WAN NURIZZATI BINTI W IDRIS MASTER OF SCIENCE 2024

THE DEVELOPMENT OF SPERM CRYOPRESERVATION PROTOCOL FOR PATIN BUAH, *Pangasius nasutus* (Bleeker, 1863)

WAN NURIZZATI BINTI W IDRIS

MASTER OF SCIENCE
UNIVERSITI MALAYSIA TERENGGANU

2024

THE DEVELOPMENT OF SPERM CRYOPRESERVATION PROTOCOL FOR PATIN BUAH, *Pangasius nasutus* (Bleeker, 1863)

WAN NURIZZATI BINTI W IDRIS

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Institute of Tropical Aquaculture and Fisheries Universiti Malaysia Terengganu

2024

DEDICATION

Dedicated this thesis to:

My soul: Mak & Ayah,

My great supervisor: Dr. Ivan Koh Chong Chu

&

My anchor throughout the storm & turbulence:

Arwah Muhammad Nursyahidin Ampandi, my family and friends

I owe you guys, big time

Thanks a lot

May Allah bless

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu in fulfillment of the requirements for the degree of Master of Science

THE DEVELOPMENT OF SPERM CRYOPRESERVATION PROTOCOL FOR PATIN BUAH, *Pangasius nasutus* (Bleeker, 1863)

WAN NURIZZATI BINTI W IDRIS

JULY 2024

Main Supervisor : Assoc. Prof. Ivan Koh Chong Chu, Ph.D

Co-Supervisor : Prof. Abol Munafi Ambok Bolong, PhD

Abduh Muhammad Yazed, PhD

School/Institute : Institute of Tropical Aquaculture and Fisheries

The development of sperm cryopreservation for Patin Buah, *Pangasius nasutus* is necessary to meet the increasing demand through artificial fertilization. Firstly, the suitable diluent for cool storage was determined by evaluating three types of diluents which were 0.9% sodium chloride (NaCl), Calcium-free Hanks' Balance salt solution (CF-HBSS) and ringer solution. 0.9% NaCl solution was the best performing diluent with motility of 8.33 ± 1.67% at 48 hours. Then, the basic protocol of sperm cryopreservation was established by identifying the optimal condition for cryodiluents, extender, freezing condition and dilution ratio. Evaluation of nine different cryodiluents, developed from three cryoprotectants (dimethyl sulfoxide, Me_2SO; Methanol, MeOH and dimethyl acetamide, DMA) at three different concentrations (5, 10 and 15%), followed by evaluation of three types of extenders (0.9% NaCl, CF-HBSS and ringer solution) were investigated. Optimal cooling rate was evaluated by investigating four different cooling rates (11.98, 10.57, 9.23 and 6.25 °C min^{-1}) obtained by manipulating the cooling height of the straw. For optimal ratios, various dilution ratios (1:9, 1:19 and 1:49) were evaluated. The optimal protocol was developed where 10% MeOH with 0.9% NaCl as extender was a suitable cryodiluent.

Cooling rates at 9.23 °C min^{-1} was optimal and dilution ratios from 1:9 to 1:49 can be used to cryopreserve the sperm. Further evaluation on the viability and applicability of the developed protocol was investigated. For long-term storage, there was no significant change in PTM and motility duration during 12 months of storage. Fertilization and deformity trials were conducted at different sperm to egg ratios. Fresh and cryopreserved sperm showed no significant differences at sperm to egg ratio of 100,000:1, except for hatching rate. Higher sperm to egg ratio for cryopreserved sperm resulted in increased fertilization rates, with hatching rates significantly higher at ratio of 300,000:1 compared to 150,000:1. There was no significant difference in deformity rates for fresh and cryopreserved at sperm to egg ratio of 300,000:1. In conclusion, this study has developed a suitable protocol for *P. nasutus* sperm cryopreservation and demonstrated that this protocol is viable for up to 12 months and greatly assist in seed production.

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PEMBANGUNAN PROTOKOL KRIOAWETAN SPERMA UNTUK PATIN BUAH, *Pangasius nasutus* (Bleeker,1863)

WAN NURIZZATI BINTI W IDRIS

JULAI 2024

Penyelia : **Prof. Madya Ivan Koh Chong Chu, Ph.D**

Penyelia Bersama : **Prof. Abol Munafi Ambok Bolong, PhD**

Abduh Muhammad Yazed, PhD

Pusat Pengajian/Institut : **Institut Akuakultur Tropikal dan Perikanan**

Pembangunan krioawetan sperma untuk Patin Buah, *Pangasius nasutus* adalah perlu bagi memenuhi permintaan yang semakin meningkat melalui persenyawaan aruhan. Pertama, pelarut yang sesuai untuk penyimpanan sejuk ditentukan dengan menilai tiga jenis pelarut iaitu 0.9% Sodium Chloride (NaCl), Calcium-free Hanks' Balance salt solution (CF-HBSS) dan larutan ringer. Larutan 0.9% NaCl adalah pencair berprestasi terbaik dengan motiliti 8.33 ± 1.67% pada 48 jam. Kemudian, protokol asas krioawetan telah ditubuhkan dengan mengenal pasti keadaan optimum untuk *cryoprotectants*, *extender*, kadar penyejukan dan nisbah pencairan. Penilaian sembilan *cryoprotectants* berbeza, dibangunkan daripada tiga kriopelindung (dimethyl sulfoxide, Me2SO; Methanol, MeOH dan dimethyl acetamide (DMA) daripada tiga kepekatan berbeza (5, 10 dan 15%), diikuti dengan penilaian tiga jenis daripada *extender* (0.9% NaCl, CF-HBSS dan larutan ringer) telah dikaji. Kadar penyejukan optimum dinilai dengan menyiasat empat kadar penyejukan berbeza (11.98, 10.57, 9.23 dan 6.25 °C min^{-1}) yang diperolehi dengan memanipulasi ketinggian penyejukan straw. Untuk nisbah optimum, pelbagai nisbah pencairan (1:9, 1:19 dan 1:49) telah dinilai. Protokol optimum telah dibangunkan di mana 10% MeOH dengan 0.9% NaCl

sebagai *extender* adalah *cryoprotectants* yang sesuai. Kadar penyejukan pada 9.23 °C min^{-1} adalah optimum dan nisbah pencairan dari 1:9 hingga 1:49 boleh digunakan untuk mengawetkan sperma. Penilaian lanjut mengenai daya saing dan kebolehgunaan protokol yang dibangunkan telah dkaji. Untuk penyimpanan jangka panjang, tiada perubahan ketara dalam PTM dan tempoh motiliti selama 12 bulan penyimpanan. Percubaan persenyawaan dan kecacatan telah dijalankan pada nisbah sperma kepada telur yang berbeza. Sperma segar dan krioawetan tidak menunjukkan perbezaan yang ketara pada nisbah sperma kepada telur 100,000:1, kecuali kadar penetasan. Nisbah sperma kepada telur yang lebih tinggi untuk krioawetan sperma menyebabkan kadar persenyawaan meningkat, dengan kadar penetasan jauh lebih tinggi pada nisbah 300,000:1 berbanding 150,000:1. Tiada perbezaan ketara dalam kadar kecacatan bagi sperma segar dan krioawetan sperma pada nisbah sperma kepada telur 300,000:1. Kesimpulannya, kajian ini telah membangunkan protokol yang sesuai untuk krioawetan sperma *P. nasutus* dan menunjukkan bahawa protokol ini berdaya saing sehingga 12 bulan dan sangat membantu dalam pengeluaran benih.

ACKNOWLEDGEMENTS

Gratitude to Allah for His boundless blessings. Through His grace, I am able to complete my study. Thanks to Him for giving me strength and a good health throughout this journey. Profound appreciation and enduring love go to my beloved parents for their unwavering moral and financial support, understanding, and courage. Their constant encouragement has been a beacon of strength. A special acknowledgement is extended to my remarkable supervisor, Dr. Ivan Koh Chong Chu, for providing insightful guidance, unwavering support, valuable advice, patience, and constructive criticism during this journey. I express countless thanks to Dr. Ivan for his understanding and belief in my ability to successfully complete my studies. This journey has not been without its challenges, but with his support, I have found the strength to give my very best. My gratitude also goes to my co-supervisors, Prof. Abol and Dr. Abduh for their wealth of knowledge and unwavering support. To Dr. Abduh, Dr. Diyana and Donald, thank you for your companionship and making my research journey more enjoyable. The wonderful memories we've created together will forever hold a special place in my heart. I also wish to acknowledge the collaborative efforts of Three Ocean Fish Pond and Trading Sdn. Bhd for their invaluable assistance in the collection of sperm and eggs. Special appreciation goes to AKUATROP and FPSM staff, particularly the hatchery staff, for tolerating my requests and providing facilities during laboratory work periods, smoothing the path to the completion of this study. To my dearest sisters, Najihah and Dalila, a warm hug for your unwavering assistance throughout this journey. Puan Maisarah Yusoff, Syarifah Norasilah, Ahmad Dzulqharnain and my close friends, I extend my deepest thanks for your consistent support during my research endeavours. To everyone mentioned, words fall short in expressing the depth of my gratitude. Thank you!

APPROVALS

I certify that an Examination Committee has met on 2nd July 2024 to conduct the final examination of Wan Nurizzati Binti W Idris, on her Master of Science thesis entitled **"The Development of Sperm Cryopreservation for Patin Buah, *Pangasius nasutus* (Bleeker,1863)** in accordance with the regulations approved by the Senate of Universiti Malaysia Terengganu. The Committee recommends that the candidate be awarded the relevant degree. The members of the Examination Committee are as follows:

Liew Hon Jung, Ph.D
Associate Professor,
Institute of Tropical Aquaculture and Fisheries,
Universiti Malaysia Terengganu.
(Chairperson)

Rasina Abdul Rasid@Awang, Ph.D
Faculty of Fisheries and Food Sciences,
Universiti Malaysia Terengganu.
(Internal Examiner)

Ching Fui Fui @ Faihanna Ching Abdullah, Ph.D
Associate Professor,
Borneo Marine Research Institute,
Universiti Malaysia Sabah.
(External Examiner)

MUHAMMAD IKHWANUDDIN ABDULLAH, Ph.D
Professor/ Director
Institute of Tropical Aquaculture and Fisheries
Universiti Malaysia Terengganu

Date:

This thesis has been accepted by the Senate of Universiti Malaysia Terengganu in fulfilment of the requirement for the degree of Master of Science.

MUHAMMAD IKHWANUDDIN ABDULLAH, Ph.D
Professor/ Director
Institute of Tropical Aquaculture and Fisheries
Universiti Malaysia Terengganu

Date:

DECRALATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UMT or other institutions.

_____

WAN NURIZZATI BINTI W IDRIS

Date:

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

LN	Liquid nitrogen
BW	Body weight
BL	Body length
NaCl	Sodium chloride
Me_2SO	Dimethyl-sulfoxide
DMA	Dimethylacetamide
PG	Propylene glycol
MeOH	Methanol
mg/L	milligram per litre
pH	potential of Hydrogen
CF-HBSS	Calcium Free Hanks' Balance Salt Solution
KCl	Potassium Chloride
$MgSO_4.7H_2O$	Magnesium Sulfate Heptahydrate
$Na_2HPO_4.7H_2O$	Sodium Hydrogen Phosphate Heptahydrate
KH_2PO_4	Potassium Dihydrogen Phosphate
$NaHCO_3$	Sodium Bicarbonate
$CaCl_2$	Calcium Carbonate
mL	Millilitre
µl	Microliter
L	Liter
S	Second
Kg	Kilogram
mM	Millimolar
Cm	Centimetre

ANOVA	One-way analysis of variance
PTM	Post-thaw motiliy
VCL	Curvilinear velocity
spp.	Species
SPSS	Statistical Package for the Social Sciences
°	Degree
<	less than
>	more than
%	Percentage
±	plus minus

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of study

Pangasius is a genus of catfish native to Southeast Asia that belongs to family Pangasiidae and order Siluriformes (Gustiano *et al*., 2018). This genus is one of the most widely cultured freshwater fish in the world. The global production of *Pangasius* for aquaculture has increased significantly in recent decades. According to the Food and Agriculture Organization, the global production of *Pangasius* for aquaculture reached 2.9 million tonnes in 2020, up from just 220,000 tonnes in 2000. In Malaysia, *Pangasius* spp. are also important freshwater aquaculture species with the two most crucial species which are *Pangasius hypophthalmus* and *P. nasutus* (Mahmud *et al*., 2019; Yusoff *et al*., 2023). *P. hypophthalmus* is the most important and commercially available *Pangasius* species in Malaysia with the aquaculture production of 20,861.92 tonnes in 2022, ranking third in freshwater fish culture production (Department of Fisheries Malaysia, 2022). However, in comparison, the production of the native *Pangasius* species, *P. nasutus* or locally known in Malaysia as 'patin buah' is still very limited even though there is a large market demand (Rafi *et al*., 2022; Zulkiflee *et al*., 2020).

P. nasutus is a potential aquaculture species in Malaysia and is an expensive food fish where the price ranges between MYR 70 to MYR 175/kg (Yusof & Nakajima, 2019). This species has whitish flesh that is more preferred over the yellow flesh of *P. hypohthalmus* (Hidayat *et al*., 2016). Moreover, the market preferences can be observed not just in Malaysia but also in other possible export markets in Asia, Europe, and North America (Rafi *et al*., 2022). Nevertheless, the aquaculture

production for this species remains challenging in order to keep a consistent supply on the market (Jaapar *et al*., 2021) because it is not commonly farmed due to its sensitivity to the environment (Zulkiflee *et al*., 2020). Therefore, it is crucial to establish methods for breeding in captivity in order to fulfil the market demand of this species.

Numerous improvements have been achieved for the development of this species in captivity. Enhancement in seed nutrition have been developed in several studies (Asdari *et al*., 2011; Azani *et al*., 2022; Samad *et al.*, 2020), as well as in seed management (Noer *et al*., 2020; Rafi *et al*., 2022) and hybridization of this species with other *P. hypophthalmus* have been done (Hassan *et al*., 2011; Iswanto & Tahapari, 2014; Yusuff *et al*., 2019). However, there is still a scarcity of available information in broodstock management, breeding technology and genetics which are necessary to increase the production of this species in commercial aquaculture. Aside from that, no specific information regarding the natural spawning of this species has been published. Previous studies on other *Pangasius* species report that the natural spawning usually occurs once a year (Arsetyo *et al*., 2012). Their eggs and sperm are not available throughout the year as this species is a seasonal spawner. Meanwhile, in captive conditions, gonad maturation of both females and males are at times asynchronous which causes difficult for hatchery production (Arsetyo *et al*., 2012; Kwantong & Barb, 2003). This makes it challenging to synchronize gamete availability for both sexes.

Cryopreservation of sperm would therefore be crucial to enhance the breeding opportunities for *P. nasutus*. Sperm cryopreservation is the process of keeping sperm alive in liquid nitrogen at low temperatures below zero, typically at -196^{o}C (Butts *et al*., 2010; Horvath & Urbanyi, 2008). The idea behind this technique is to preserve genetic material and keep cells alive at a low temperature of liquid nitrogen where cells' biological activity is momentarily inhibited and can be preserved for a very long time (Anabella *et al*., 2020). This technique would help to store male gametes for an extended period of time and the ability to use stored sperm when eggs are available, which helps synchronize gamete availability for both sexes (Gwo, 2011). It is also useful for simplified broodstock maintenance where only females need to be induced

during off-season spawning and cryopreserved sperm can be utilized to fertilize the eggs. Desired fish characteristics can be maintained by storing the genome of valuable strains of male broodstock in order to produce high quality seed (Cabrita *et al*., 2010).

1.2 Problem statement

According to Farstad (2000), fresh sperm's quality decreases quickly unless it is diluted and stored at a lower temperature. Thus, it is critical to ensure sperm samples are stored properly and sperm cell viability is maintained in a good condition to subsequently be used in the cryopreservation process. Besides that, sperm cryopreservation studies also take a long time to complete due to the many steps of the procedure; and fish farms are usually located in isolated or rural areas with limited laboratory assistance and no specific cryopreservation equipment or supplies. Therefore, the identification of a suitable diluent for cool storage would be extremely crucial to prolong the functional life of the *P. nasutus* sperm cells in order to maintain the sperm in non-motile but viable state until it is used for cryopreservation or transporting the sperm samples from the farm to another location.

Cryopreservation of sperm for *Pangasius* species has been investigated since 1995 for *P. hypophthalmus*, *P. larnaudii*, *P. gigas*, *P. bocourti* and Albino pangasius catfish (Hasanah *et al*., 2020; Kainin *et al*., 2014; Kwantong & Bart 2003; 2006; 2009; Mongkopunya *et al*., 1995; Yang *et al*., 2023), but sperm cryopreservation for *P. nasutus* has yet to be explored and no study has been established. Current protocols are not applicable for *P. nasutus* sperm due to the fact that sperm cryopreservation is species specific, and various species might require differing conditions, necessitating the establishment of a specific sperm cryopreservation protocol for each species (Muchlisin, 2005). For successful sperm cryopreservation, the optimization of several important parameters is necessary. The selection of suitable candidates for the cryoprotectant, extender, cooling rate and dilution ratios are important in order to develop the protocol for sperm cryopreservation of this species (Sansone *et al*., 2002; Yang *et al*., 2020).

Cryoprotectant are chemicals that plays an important role in cryopreservation to protect the sperm cell from cold and hot shock treatments; and also, to prevent cell dehydration (Tiersch, 2011). However, cryoprotectants are often toxic to the sperm cells and can produce non-viable cells prior to freezing (Tiersch, 2011). Thus, the choice of cryoprotectant and their suitable concentration must be balanced within protection and noxious. Meanwhile, extender is the diluent prepared from salts but sometimes it is also prepared with addition of sugar such as glucose (Chew & Zulkafli, 2012). Extender plays an important role to inhibit the energy used by sperm during cryopreservation as well as to prolong the life of sperm cells (Muchlisin, 2005). Besides that, the selection of suitable cooling rate and dilution ratios also needs to be considered where successful sperm cryopreservation is also dependent on these factors (Yang *et al*., 2022). Cooling rate should be slow enough to minimize the formation of ice crystals that form in the sperm cells and yet be fast enough to minimize the length of time cells are exposed to the solution effect (Tiersch, 2011), while the dilution ratios of sperm and freezing diluent need be to optimized for preservation efficiency (Yang *et al*., 2020).

In sperm cryopreservation, there is no strict time limit for how long sperm can be stored, as long as it remains properly sealed and submerged in liquid nitrogen to prevent exposure to air and temperature fluctuations (Park *et al*., 2022). However, it is generally accepted that the longer sperm are stored, the more potential there is for minor damage or degradation (Vishwanath & Shahnon, 1997). Therefore, in order to determine whether the developed protocol for sperm cryopreservation can be used in the aquaculture industry, it will be critical to evaluate the impact of long-term storage inside the liquid nitrogen on sperm viability. Sperm motility evaluation is the most commonly used method to investigate the quality of sperm cells after cryopreservation due to its simplicity and ease of evaluation, as well as the ability to obtain the results rapidly compared to other methods (Lang *et al*., 2003; Mongkopunya *et al*., 2000; Viveiros *et al*., 2000). Nevertheless, fertilization and hatching trials should be used to evaluate sperm quality after cryopreservation whenever possible as fertilization potential and hatchability of embryos insemination with cryopreserved sperm is the

golden standard and final indicator of sperm quality to assess its viability in captive breeding programs and applicability in seed production.

1.3 Significance of study

Dilution with suitable diluent or extender after collection is really important to avoid rapid loss of quality especially for unstudied species (Tiersch, 2011). The primary goal of the diluent is to protect the sperm by keeping it in a proper environment while also making it immotile in order to conserve energy for motility. By diluting sperm samples with diluent, volume can be optimized for efficient use and can counter any effects of contamination during the stripping process. Moreover, diluent has a vital function in preventing sperm activation and motility during sperm collection, handling, and storage. On the other hand, cool storage with addition of diluent is necessary for practical work. It is useful for transporting sperm samples from fish farming to other facilities for cryopreservation process and ability to flexibly schedule for cryopreservation so that we may do it efficiently in groups for steps that require similar preparation and ensuring everything is the same for one or more samples. Therefore, the development of cool storage for *P. nasutus* sperm by identifying the suitable diluent that can subsequently be stored at a low temperature is essential to make sure the metabolism of sperm is decreased without significant changes in sperm quality before the cryopreservation experiments are conducted.

Through this study, the established sperm cryopreservation protocols will be essential to create the breeding and seed production programs to generate the aquaculture production and enhance the quality of *P. nasutus* seed. This finding will enable farmers to implement and optimize this technique based on the availability of items and materials on their location, hence increasing efficiency and production in aquaculture operations. Moreover, the production of seed can be maintained throughout the year using cryopreservation of sperm in order to fulfill the market demand for this species through artificial fertilization. The created protocol for sperm cryopreservation can enhance broodstock management and consequently produce

juveniles through artificial fertilization (Liu *et al*., 2016). On the other side, an effective sperm cryopreservation procedure can ensure that sperm is available at all times, allowing breeders to focus only on female broodstock, which is less expensive than maintaining both sexes.

Investigating the impact of long-term storage inside the liquid nitrogen and its capacity to fertilize and hatch eggs are critical to determine whether the developed protocol can be used in the aquaculture industry. By investigating the influence of long-term storage in liquid nitrogen, we can examine the reliability and applicability of our established protocol. Besides that, fertilization and hatching success is typically regarded as the most important criteria because the ultimate goal of sperm cryopreservation is to maintain the male genetic material without significant changes in the fertilizing capability. These findings are valuable for breeding programs, as it determines the suitability and ensures a continuous supply of cryopreserved sperm for producing viable offspring and generate aquaculture production.

1.4 Objectives of study

The purpose of this study is to develop a protocol for *P. nasutus* sperm cryopreservation that will be practical for aquaculture use. Thus, the objectives of the study are:

i. To determine optimal diluent for cool storage for *P. nasutus* sperm.
ii. To develop a sperm cryopreservation protocol for *P. nasutus*
iii. To investigate the viability and applicability of *P. nasutus* cryopreserved sperm in seed production.

CHAPTER 2

LITERATURE REVIEW

2.1 *Pangasius nasutus* (Bleeker, 1863)

P. nasutus or locally known in Malaysia as ‘patin buah’ (Rafi *et al*., 2022; Zulkiflee *et al*., 2020) is as an important freshwater fish in Malaysia (Yusof & Nakajima, 2019). The market preferences of this species can be found not just in Malaysia, but also in other prospective export destinations such as Asia, Europe, and North America. The whitish flesh and fine-grained of *P. nasutus* are preferred over the yellow flesh of *P. hypohthalmus*. However, finding a continuous supply of Patin buah in the market remains difficult because it is still entirely dependent on wild captures. (Rafi *et al*., 2022). As a result, overharvesting will reduce the natural population. (Hassan *et al*., 2011). With the risk of extinction, a breeding program for *P. nasutus* must be devised to increase its stock in inland water bodies. (Chew & Zulkafli, 2012). Worse, unlike *P. hypophthalmus*, *P. nasutus* is a slow-growing fish, with little knowledge of the breeding, nursery and grow-out stages (Rafi *et al*., 2022).

2.1.1 Taxonomy

Kingdom	: Animalia
Phylum	: Chordate
Class	: Actinopterygii
Order	: Siluriformes
Family	: Pangasiidae
Genus	: *Pangasius*

Species : *Pangasius nasutus* (Bleeker, 1863)
Common name : Patin buah (Malay name)

P. nasutus (Bleeker, 1863) is a catfish species, belongs to Animalia (Kingdom), Chordate (Phylum), Actinopterygii (Class), Siluriformes (Order) and Pangasiidae (Family).

2.1.2 Morphology and characteristics

Each species of *Pangasius* species can be distinguished by their coloration on the head and upper part of the body (Figure 2.1). For *P. nasutus*, their live coloration on the head and upper part of the body is merely light gold and it is longer in fin part (Yusoff *et al*., 2018). Besides that, *P. nasutus* has been classified as a gonochoric species same as other catfish species (Rodriguez *at al*., 2019). Gonochoric species mean there is only male and female sexes for each individual organism. According to Roberts & Vidthayanon (1991), *P. nasutus* has an inferior mouth, a totally exposed tooth band in the upper jaw when the jaws are closed, and a sharply showing snout.

Figure 2.1 Patin buah, *Pangasius nasutus* (Bleeker, 1863)

2.1.3 Reproductive biology

Generally, there is only a few available information about this species (Asdari *et al*., 2011). Natural spawning for genus *pangasius* usually occurs once a year. Their eggs and sperm are not available throughout the year because most of the species are seasonal spawner. *P. nasutus* species will spawn in the natural environment during

the early rainy season (Hassan *et al*., 2011; Jaapar *et al*., 2021), usually between May and July (Chew & Zulkafli, 2012). In a captive condition, both females and males gonads are not matured at the same time in a pond or tank. Therefore, hatchery production of this species is quite difficult (Kwantong & Barb, 2003; Rahardhianto *et al*., 2012).

2.1.4 Habitat and distribution

Pangasius is a genus of fishes that native to freshwater in the South and Southeast Asian which can only be found in Malaysia, South Thailand, Sumatera and Kalimantan Indonesia (Yusof & Nakajima, 2019). In Malaysia, *P. nasutus* can be found mostly in the rivers of Pahang State (Asdari *et al*., 2011). This species has great sensitivity to the environment; thus, it is mostly found living in the wild and not farmed (Zulkiflee *et al*., 2020).

2.2 Fish sperm preservation

Sperm preservation is a key tool for fish reproduction and a topic of great interest in fish farming (Magnotti *et al*., 2018). Short-term storage and cryopreservation of sperm are two common aquaculture procedures for regular management in artificial reproduction and gene bank maintenance (Pérez-Cerezales *et al*. 2009). However, because cryopreservation is difficult to perform in some species due to poor initial quality, cold storage is the most commonly used tool because it is a viable, inexpensive, and simple procedure to perform (Bobe & Labbé 2010; Shaliutina *et al*. 2013; Trigo *et al*. 2015).

2.2.1 The importance of sperm cryopreservation in aquaculture

A few researchers in the aquaculture field have mentioned the importance of cryopreservation procedures in their publications. This technique is important to facilitate reliable, long-distance transportation of a large number of samples needed, make the sperm feasible for artificial insemination, hybridization and genetic research; and preservation of special individuals fish sperm and endangered species for future use (Kainin *et al*., 2014; Mongkonpunya *et al*., 2000). Besides that, this strategy can reduce the number of males required in the hatchery, minimize stress to the fish through less frequent stripping, facilitate knowledge in genetic domains, and lead to the production of inbred lines.

This technique would help to store male gametes for an extended period of time and the ability to use stored sperm when eggs are available, which helps synchronize gamete availability for both sexes (Gwo, 2011). It is also useful for simplified broodstock maintenance where only females can be induced during off-season spawning and cryopreserved sperm can be utilized to fertilize the eggs. Desired fish characteristics also can be maintained by storing the genome of valuable strains of male broodstock in order to produce high quality of seed (Cabrita *et al*., 2010). Besides that, cryopreservation also allows the use of the total amount of sperm collected especially for the species with a low volume of sperm. For artificial insemination, certain fish only use a particular volume of sperm. Thus, extra collected sperm can be cryopreserved and used at a later date (Cabrita *et al*., 2010).

The other advantage of this technique is sperm can be collected during high quality stages which can avoid ageing of sperm and allows for the protection of broodstock from extinction due to illness, natural disaster, or overexploitation. (Argawal, 2011). According to Vuthiphandchai *et al*., (2009), cryopreserved sperm can be a gene bank for conservation of the strain for biotechnology purposes. In addition, for species such as groupers which have large sized male broodstock, this technique will help to minimize handling stress and can simplify the broodstock maintenance by synchronizing gamete availability.

Although this method has many advantages, it also has some limitations. Cryopreserved sperm is rarely 100% applicable. This is because thawed sperm is 50% less effective than fresh sperm when used to fertilize eggs. Besides that, frozen-thawed sperm are more costly than fresh sperm (Kwantong & Barb, 2009). However, better understanding and further research in sperm cryopreservation could increase the efficiency of sperm quality and lead to commercialization of cryopreserved sperm.

2.2.2 Long-term storage/cryopreservation

Fish sperm cryopreservation is the method for storing sperm cells or genetic materials at a low temperature usually at -196°C in liquid nitrogen (LN). The principle of this method is to keep genetic materials and maintain the cells life at a low temperature of LN where the biological activity of cells is temporarily inhibited and can store for a long time (Anabella *et al*., 2020). The main objective of cell preservation is to remove as much water as possible from cells while preserving their integrity (Meryman, 2007). It is important to note that this procedure does not improve the quality of sperm cells, even the rate of motility and egg fertilization remains unchanged after the freezing process. There are numerous advantages of sperm cryopreservation that will undoubtedly benefit the aquaculture industry.

However, in all applications involving fish sperm, cryopreservation must overcome a lack of standardization of procedures and methods (Cabrita *et al*., 2010). A reliable measurement of sperm quality and the development of techniques to minimize cryoinjury are important in this method (Cabrita *et al*., 2010). In cryopreservation, a series of investigations and experiments are needed to develop practical methods to cryopreserve sperm for fish species especially for a new and unstudied species (Tiersch, 2011). Cryopreservation results also are difficult to apply from one research to another through published articles or journals because there was a lack of standardization in protocols and reporting (Tiersch *et al*., 2007; Tiersch, 2011; Yang *et al*., 2010). This is because sperm cryopreservation is species-specific, it is

difficult to standardize or optimize this procedure and apply it to all types of fish. This is due to the fact that sperm from various species require various conditions, necessitating the establishment of a specific sperm cryopreservation protocol for each species (Muchlisin, 2005).

2.2.3 Short term storage/cool storage

Cool storage or refrigerated storage, often mentioned as short-term preservation, is necessary for practical work (Tiersch, 2011). Generally, it consists of storing the sperm samples at low temperature between 0 to 4°C under oxygen atmosphere (Shaliutina *et al*., 2013) to retain sperm viability and lower sperm metabolic activity (Meyers, 2009). Most research have revealed the best conditions for sperm preservation in many species, as well as the elements that influence the quality and viability of preserved sperm. Fresh sperm loses its quality quickly unless it is diluted and stored at a lower temperature (Farstad, 2000; Lemma, 2011). Thus, sperm samples need to be diluted with suitable diluents that is isotonic to sperm cells and need to store at low temperature as mentioned above.

Cool storage permitting samples to be shipped from a location such as an operational hatchery to a laboratory with specific equipment for cryopreservation research and performance. It is also important to flexibly schedule cryopreservation for efficient processing in batches for steps that require the same preparation and standardization for one or multiple samples. Furthermore, the capability of at least 24 hours of cold storage serves as the basis for a routine quality control and assurance system for commercial scale cryopreservation (Tiersch, 2011). In order to develop the cool storage for a certain species, the sperm needs to be tested with several extenders or diluents. Various types of extenders have been evaluated, including simple ones like 0.9% NaCl solution and more complex ones that contains more than one ionic component such as KCl, CaCl, NaOH, glucose and mores, which mimics the ionic content and osmolality of fish sperm (López *et al*., 2015). On the others hand,

according to Tiersch (2011), good motility (~40-70%) at 24 hours is probably sufficient to move forward for developing the basic protocols for cryopreservation.

2.3 Development of sperm cryopreservation protocol

The protocol for sperm cryopreservation for each species of fish needs to developed in order to protect the sperm quality. Sperm cryopreservation performance can be significantly affected by a series of factors, including types and concentration of cryoprotectants and extenders, toxicity of cryoprotectant, rates of freezing and thawing, and post-thawing application (Kopeika *et al*., 2007). Therefore, all of these factors must be considered in order to develop or optimize the sperm cryopreservation protocol. The basic steps described below are usually used to develop a basic protocol for unstudied species or to improve the studied protocol.

2.3.1 Gamete collection

Generally, sperm from matured males will be obtained during the spawning seasons by either stripping or by crushing of dissected testis. Stripping of sperm involves a collection directly from the male into a sterile tube. During collection, care must be taken to avoid any contamination such as dirt, urine, feces and water (Diwan *et al*., 2010). Meanwhile, dissected testis usually involves killing the male and removal of testis using forceps and scissors. By doing this, care must be taken to avoid bacteria contamination due to cutting of the intestine. After removal, the testis must be rinsed with an extender (isotonic saline) to remove any blood or excess tissues (Diwan *et al*., 2010; Tiersch, 2011). The cleaned testis is placed in a suitable container and cut into smaller pieces to release the sperm.

2.3.2 Extenders

The term ‘extender’ refers to a solution or diluent of salts, sometimes including organic compounds such as glucose or sugar (Tiersch, 2011). Extender and dilution are correlated to each other. Dilution with extenders after sperm collection is really important to avoid rapid loss of quality especially for unstudied species. By diluting sperm samples with extenders, volume can be optimized for efficient use and can counter any effects of urine contamination. Contamination with urine usually happened when sperm samples were collected by stripping, where urine can reduce the percentage of motility by activating the sperm and decrease the storage lifetime (Diwan *et al*., 2010). Stripping samples may be contaminated with urine, which can activate the sperm and shorten its storage lifespan. Freshwater fish urine, for example, is hypotonic to the body's tissues and would activate the sperm by lowering the sample's osmotic pressure. Thus, diluting the samples with extender can mitigate the effect of urine contamination by shifting the osmotic pressure to an isotonic range. (Tiersch, 2011).

Extender or diluent is important during the freezing process as to maintain sperm viability. This solution is a balance of salt buffer of osmotic strength and specific pH. It is usually prepared from salts but sometimes it is also prepared with addition of glucose which is an organic compound (Chew & Zulkafli, 2012). Extender plays an important role to inhibit the energy used by sperm during cryopreservation (Thilak Pon Jawahar & Betsy, 2020). Therefore, extender can extend fertilizing capability and prolong the functional life of the sperm. Meanwhile, Muchlisin (2005) stated that the extender is a medium to dilute sperm and to obtain a large amount of diluted sperm for artificial breeding purposes. For some species of fish, they only produce small volumes of sperm. Thus, extender plays an important role to dilute sperm and provides a higher fertilization of cryopreserved sperm (Thilak Pon Jawahar & Betsy, 2020). However, the choice of suitable extender must be considered in order to avoid activating sperm cells during the cryopreservation process as well as to achieve highest motility of cells after freezing-thawing process (Tiersch, 2011).

Extenders are commonly being prepared to be compatible with the seminal plasma composition of the selected species in order to maintain sperm in a non-motile but viable state (Bustani & Baiee, 2021; Mongkonpunya *et al*., 2011). It has been proved that maintaining osmotic pressure at levels isotonic or hypertonic to sperm cells was the major component to maintain fertilizing capability and viability of pangasiid sperm (Mongkonpunya *et al*., 2011). Moreover, lower osmotic pressure can activate the sperm motility of *P.gigas* and *P.hypophthalmus* (Mongkonpunya *et al*., 1995). Furthermore, 0.9% Sodium chloride (NaCl) is recommended as an extender for Pangasiid sperm species because it is inexpensive and easy to prepare (Mongkopunya *et al*., 2011). Easy methods and simple ingredients as well as inexpensive extenders can be useful and more preferable for the farmers (Mongkonpunya *et al*., 2011).

2.3.3 Cryoprotectant

Cryoprotectants are chemicals used in cryopreservation to protect cells from damage caused by freezing and thawing (Tiersch, 2011). The accomplishment of the cryoprotectants depends greatly on the fish species or the combination of the type and concentration of the cryoprotectant. Thus, specific cryoprotectant must promote a partial sample of dehydration to inhibit the formation of ice crystals and to protect all components as they must permeate the cells in order to cryopreserve any tissues or biological structure (Kainin *et al*., 2014; Robles *et al*., 2009). Cryoprotectants are also an important part of a successful cryopreservation protocol. This is because cryoprotectants protect the cells during the freezing process by entering the cells and replacing the water that comes out to minimise the osmotic shock and reduce the formation of intracellular ice (Hasanah *et al*., 2020; Tiersch *et al*., 2007). However, in numerous studies, the choice of cryoprotectant and their optimal concentration must be balance between protection and toxicity where too little cryoprotectant entering the cells before cooling can reduces the effectiveness and too much can cause osmotic swelling and rupture during thawing (Tiersch *et al*., 2007).

Cryoprotectants that are commonly used for all aquatic species are dimethyl-sulfoxide (Me_2SO), dimethylacetamide (DMA), dimethyl-formamide, glycerol, propylene glycol (PG), methanol (MeOH) and sucrose (Tiersch *et al*., 2006). Over the last ten years, the most commonly employed cryoprotectants for catfish sperm cells have been MeOH, Me_2SO, and DMA ranging from 5-15% (Table 2.1). These cryoprotectants also are frequently used for sperm cryopreservation for genus *Pangasius* (Kwantong & Barb, 2003;2009; Kainin *et al*., 2014).

Table 2.1 Optimal cryoprotectant concentration that yielded the highest PTM for catfish species.

Cryoprotectant	**Species**	**References**
9% Me_2SO	*P. gigas*	Mongkopunya *et al*., 1995
10% and 15% DMA	*S. glanis*	Ogier de Baulny *et al*., 1999
10% Me_2SO	*C. gariepinus*	Urbanyi *et al*., 2000
10% MeOH	*C. gariepinus*	Viveiros *et al*., 2000, 2001
12% Me_2SO	*P. hypophthalmus*	Kwantong & Bart, 2003
10% MeOH	*M. nemurus*	Muchlisin *et al*., 2004
10% Me_2SO or 10% MeOH	*C. gariepinus*	Miskolczi *et al*., 2005
10% Me_2SO	*P. laurnadii*	Kwantong & Bart, 2006
10% MeOH	*P. fulvidraco*	Pan *et al*., 2008
12% Me_2SO	*P. hypophthalmus*	Kwantong & Bart, 2009
10% MeOH	*C. gariepinus*	Kovács et *al*., 2010

As stated by Tiersch (2011), she suggested that the practical protocols for the most majority of aquatic species can be developed using a small number of cryoprotectants such as methanol and Me_2SO and secondarily glycerol and DMA, perhaps in combination with specific sugars. These should be the starting points in a strong research pathway controlling the major variables. On the other hand, methanol has been chosen as the best cryoprotectant for cryopreservation of sperm for many freshwater species, for example common carp (Horváth *et al*., 2003), catfish (Horváth & Urbányi, 2000), salmonids (Horváth *et al*., 2015) and many more. MeOH is famous

for extreme rapidity to enter the cells and its permeability is greater than Me_2SO (Gwo *et al*., 2019; Koh *et al*., 2017;).

In addition, cryoprotectants are often toxic to the sperm cells and can produce non-viable cells prior to freezing (Tiersch, 2011). Toxicity usually depends on the time of cryoprotectant penetrate to the cells and their concentration. Hence, it is important to know their toxic effect on the sperm within a time interval during refrigerated storage by observing the motility of sperm (Tiersch, 2011). However, this method is uncommonly stated or not typically reported in previous studies on sperm cryopreservation because mostly they focused on a part of equilibrium time. Investigation on toxicity of cryoprotectants plays an important role as it is given a lot of advantages such as allowing sufficient time for handling the samples, exposing how much motility loss before cryopreservation and helps to optimize equilibrium time.

Moreover, after the addition of cryoprotectants to the sperm cells, time needed for the cryoprotectants to permeate the cell was usually set at 15 to 30 minutes. However, for cryoprotectants such as Me_2SO and MeOH, the time for them to permeate the cells is very fast as both of them are permeating cryoprotectants. Besides that, toxicity of cryoprotectants is also known as equilibrium of motility. The toxicity of cryoprotectants is the most well known as the crucial parameter to indicate the success of sperm cell cryopreservation (Tiersch *et al*., 2007). Therefore, for each experimental design for each species, the effect of cryoprotectants at various concentrations before freezing process should be investigated to identify the suitable concentrations and equilibrium times (Hasanah *et al*., 2020). Increasing in cryoprotectant concentration and increase of exposure time before freezing resulted in reduction of sperm motility and viability of pangasiid and clariid sperm cells (Kainin *et al*., 2014). Toxicity studies are critical for narrowing the list of cryoprotectants and determining the concentrations to utilize in subsequent cryopreservation trials. By doing these toxicity investigations, we can determine whether the sperm are damaged or immotile due to cryoprotectant toxicity before they are cooled (Tiersch, 2011).

On the others hand, sperm dilution ratio in the cryodiluents (cryoprotectant + extender) is a critical factor for the success of cryopreservation (Yang *et al*., 2020). Sperm to diluent ratios between 1:3 and 1:9, as reported in various publications, achieve the best results in fish sperm cryopreservation tests (Yang *et al*., 2020). Nevertheless, the diluents and dilution ratios that function to preserve sperm motility appear to differ between fish species. The evaluated sperm that is fit for further processing is diluted with different diluents in various dilution ratios to determine the most effective and appropriate ratio for fertility trials (Thilak Pon Jawahar & Betsy, 2020). The dilution is crucial because it increases the volume of semen, allowing for numerous inseminations. Therefore, in order for any protocol to be effective, this variable must be standardized.

2.3.4 Freezing/ Cooling

The choice of cooling rate has been another major focus of many sperm cryopreservation studies. The cooling rate in fish sperm cryopreservation refers to how quickly the sperm's temperature drops throughout the freezing procedure (Suquet *et a*l., 2000). This is an important parameter since the rate of cooling has a large impact on the success of cryopreservation. The goal is to establish a controlled and slow cooling process that minimizes cellular damage, notably from the creation of ice crystals (Dalal *et al*., 2018). To be considered minimal, a rate should be slow enough to minimize the formation of ice crystals that form in the sperm cells and yet be fast enough to minimize the length of time cells are exposed to the solution effect, which is the concentration or precipitation of materials that happen when solubility reach the limit during the dehydration caused by the formation of ice (Tiersch, 2011).

Generally, there are two different methods for freezing. Computer-controlled freezers provide accurate and consistent rates (Viveiros *et al*., 2000; Kwantong & Bart 2009), but are typically costly; and a simpler and cheaper method which is freezing fish sperm in nitrogen vapour on a floating tray or in the neck of a container then the straws are immersed in liquid nitrogen (Kovács *et al*., 2010; Miskolczi *et al*., 2005;

Yang *et al*., 2023). For this method, cooling rate is measured by adjusting the height of the samples above liquid nitrogen and controls the temperature as well as times taken to plug the samples into liquid nitrogen (Suquet *et al*., 2000). As stated by Tiersch (2011), the time required for the samples to change from 0°C to -80°C is usually used to calculate cooling rates and the method used to calculate the rate should be reported carefully. Moreover, the concept of optimal cooling rates is related and dependent on the choice of cryoprotectant and container. Formation of ice crystals are lethal to the sperm cells when sperm is cooling too fast during the freezing process where the level of necessary dehydration will not reach to avoid intracellular freezing (Viveiros, 2011). With this point of view, there are a lot of protocols that use different ranges of cooling rates for different species.

2.3.5 Thawing

Thawing process also is related to the cooling or freezing method that were used to cryopreserve the sperm. Too fast cooling can produce huge intracellular ice crystals to the sperm cells, whereas too slow can produce small or insignificant intracellular ice crystals. Therefore, rapid thawing is suitable for fast cooling to avoid the crystals grow in the process of recrystallization and it is not recommended for the slow cooling process. This is because the cells are probably much dehydrated and could be lethal to them caused by rapid rehydration (Leibo, 2004; Viveiros, 2011).

In general, samples are considered thawed when air bubbles within the container can easily travel throughout the liquid. Transparent containers, such as cryo-straw, will make it easier to see the samples. When thawed, the samples should be cool to the touch not warm (Tiersch, 2011). Motility after thawing should be immediately observed especially for research purposes. This is due to the fact that sperm motility can be quickly lost after thawing, and sperm might be motile inside the container (without activating solution). As a result, fertilization trials should be conducted right away after thawing.

2.4 Sperm quality assessment

According to Rurangwa et al. (2004), sperm quality is a measure of the sperm's capacity to successfully fertilize an egg. Any quantifiable physical measure that directly correlates with sperm fertilization capacity could be used to assess sperm quality. Generally, fertilization success will depend on sperm quality and focusing on males, it crucial to identify and understand the means of sperm quality. Sperm quality can be determined by assessing many criteria such as sperm motility activation, damage of plasma membrane, seminal plasma composition, DNA damage and mitochondrial function impairment, all of which have an impact on sperm fertilization capacity (Cabrita *et al*., 2014).

2.4.1 Sperm motility

Assessing the motility of fish sperm is an important step and crucial component (Yang *et al*., 2022) in determining the quality of sperm samples, particularly in the context of artificial reproduction, selective breeding programs, and cryopreservation (Kime *et al*., 2001). Sperm motility has been the main parameter that has been used in the most studies (Cabrita *et al*., 2011). Sperm motility analysis has relied on subjective estimates of motility characteristics for a long time by identifying the percentage of motile sperm cell, the duration of sperm motility or by a combination of both characteristics (Cabrita *et al*., 2010). Motility analysis can be used to compare different experimental conditions such as collecting procedures, medium of sperm dilution and conditions for sperm storage (Yang *et al*., 2017). Besides that, sperm motility has a critical influence on successful fertilization because sperm need to find and enter the micropyle in the limited period (Fauvel *et al*., 2010). Therefore, sperm that are highly motile will have the highest chance of fertilization. However, as stated by Chew & Zulkafli (2012), generally, sperm motility will decrease after the thawing and freezing process compared to the motility of fresh sperm.

There are a few methods to measure the motility of sperm such as subjective methods, semi-quantitative methods and quantitative computer assisted method (Rurangwa *et al*., 2001). Subjective method is the method in which the percentage of motile sperm are estimated by using scale criteria from 0 (immotile) to 5 (all sperm motile) (Mortimer & Mortimer, 2013). According to Viveiros et al. (2003), percent of moving sperm was evaluated in the field of view by using naked eyes as; 0, no motility observe; 1, above 25% moving forward; 2, above 50% moving forward; 3, above 75% moving forward; 4, more than 75% are moving forward. However, the values from this scale cannot be used for any statistical test as this is a nonlinear scale. Moreover, this method led to variable results between observers or laboratories and the value of scales is still in doubt due to subjectivity of the method (Rurangwa *et al*., 2004).

Meanwhile, the semi-quantitative method is the method of using video recording to observe and measure the motility of sperm. The video recordings are usually viewed by two or more independent observers (Rurangwa *et al*., 2004). For percent motility estimation, only sperm that swim actively in a forward movement will be counted as motile. Meanwhile, sperm that remains in place and only shows a vibratory movement is considered as non-motile (Tiersch, 2011). The video recordings are examined at a slow speed while a grip is secured to the screen, and the number of motile sperm in percentage is determined. This allows for individual sperm to be counted based on motility. This method offers a less subjective measure of sperm motility than most scoring methods. A stopwatch is usually used to record the time for the total duration of motility, as well as the times when 50% and 95% of the sperm stopped moving. This method can also be used to calculate velocity from the distance travelled and the frame frequency of the videotape. Although this method is quantitatively more accurate to determine the quality of sperm in terms of motility, motility duration and VCL, this method is very time-consuming.

Quantitative computer-assisted methods, also known as Computer-Aided Sperm Analysis (CASA) systems is the evolution of several photomicrography exposure and video-micrography approach for tracking the sperm cells, with use of a computer equipped with imaging software. A CASA system includes the physical

equipment that required to view sperm images, as well as the procedures used for processing and evaluating them (Gallego *et al*., 2018). Moreover, CASA systems quantify various sperm motility variables, including those that cannot be measured manually. Aside from being quick, CASA approach provides for a more in-depth investigation of the components of sperm motility. However, temperature, sperm size and concentration, as well as speed, could all have an impact on the validity of the data and must be considered during calibration. CASA-based computer tracking of sperm mobility requires expensive technology and knowledge that many laboratories and farms do not possess. However, the process for recording sperm motility is rather easy, and the equipment, which is not complicated, is readily accessible in any laboratory or farm with a qualified employee (Rurangwa *et al*., 2004).

2.4.2 Sperm motility duration

Identification of sperm duration or motility duration is important to plan the experiments by indicating the timing involved for motility estimation or to ensure optimum fertilization when mixing the gamete with eggs. In most freshwater species, sperm normally moves for less than 2 minutes and often actively moves for less than 30 seconds (Kime *et al*., 2001). According to Rurangwa *et al*. (2004), the motility duration for freshwater teleost is thought to be shorter than that of marine fish species. The sperm motility duration of *Pangasius* species typically ranges from 30 seconds to a few minutes; the motility duration for *P. hypophthalmus* and *P. bocourti*, is only around 1 to 2 minutes (Kwantong & Barb, 2009). However, for marine fish species, the duration can vary widely but often lasts several minutes to hours (Cosson *et al*., 2008).

Identification of sperm motility will give benefits in handling and makes the assessment of motility easier and more accurate. Besides that, the motility duration might be different for sperm at the time collection, after cool storage, after diluting with a cryoprotectant and after thawing process (Tiersch, 2011). Moreover, longer motility duration is better, which can prolong the movement of sperm to swim forward

in order to reach the micropyle of eggs. Motility and duration are correlated to each other. Higher motility usually has a higher duration. The duration of sperm motility is critical in artificial fertilization. It establishes the window of time during which insemination should take place in order to enhance the chances of fertilization.

2.4.3 Fertilization and hatching

Basic damage may occur in sperm cells during the cryopreservation process which can lead to decrease the fertilization capacity. The most important uses of fertilization trials are to check the validity of other measures of sperm quality, where sperm motility or DNA damage and a fertilization capacity of either fresh or post-thawed sperm are correlated (Rurangwa *et al*., 2004). Therefore, in any cryopreservation protocol, fertilization and hatching trials have been concluded as the most crucial test. Depending on how fertilization is assessed either by early cell division or hatching rates, one may discuss probable causes for fertility unsuccessful. Thus, fertilization trials should be used as the method to identify sperm quality after post-thaw whenever possible.

Besides that, artificial spawning involves collecting unfertilized eggs from females and combining them with sperm (Tiersch, 2011). Usually in cryopreservation, after thawing, sperm samples are added to the eggs and thoroughly mixed, then the sperm will be activated by using appropriate solution. This technique is called a dry fertilization method and can be applied to a variety of crosses such as the use of sperm samples from the same male to fertilize the eggs from a few females, or for the eggs from a single female with sperms from several males. This can lead to a breeding matrix in which a select group of males can mate with a select group of females to develop a population with unique or desired traits (Tiersch, 2011).

Moreover, there are a few factors that are related to fertilization success such as the number of sperm per egg, the duration of contact between eggs and sperm, and fertilization protocol employed (Rurangwa *et al*., 2004). Thus, optimal sperm to egg

ratio has been recommended in commercial production, while in research work, fertilization success is commonly used as an endpoint of sperm quality. Sperm motility evaluation is the most used to investigate the viability of sperm cells after thawing. However, previous studies showed that too little motility after post-thaw still can produce successful fertilization (Mongkopunya *et al*., 2000; Lang *et al*., 2003; Viveiros *et al*., 2000). Pérez-Cerezales *et al*. (2010) examined multiple procedures for sperm cryopreservation in rainbow trout and found a high rate of abortion throughout embryo development, registering a 63-77% fertility rate (during early cell division) but only a 15-37% hatching rate. Cabrita *et al*. (2009) discovered the same thing with grouper sperm.

CHAPTER 3

METHODOLOGY

3.1 Introduction

The research was conducted at two locations, i) Three Ocean Fish Pond Sdn. Bhd. a private fish farm located in Rawang, Selangor and ii) Biosecurity Lab at Institute of Aquaculture Tropical and Fisheries (AKUATROP) in Universiti Malaysia Terengganu (UMT), Malaysia. Sperm and eggs were collected from broodstock of *P. nasutus* reared at the Three Ocean Fish Pond Sdn. Bhd., a private fish farm located in Rawang, Selangor. Fertilization and hatching trials were conducted in May and August 2023 using 9 months and 1-year cryopreserved sperm. This study was divided into three main experiments to answer the objectives, which were:

Experiment 1: Determination of optimal diluent for cool storage of *P. nasutus* sperm.
Experiment 2: Development of sperm cryopreservation protocol for *P. nasutus.*
Experiment 3: Investigation on the viability and applicability of *P. nasutus* cryopreserved sperm in seed production.

3.2 Broodfish management

Broodstock of *P. nasutus* were cultured and maintained outdoors inside net cages at the rate of 60 fish per cage in earthen pond at a private fish farm, Three Ocean Fish Pond Sdn. Bhd., Rawang, Selangor (Figure 3.1). Rainwater was the primary supply of freshwater, and it was cycled using a flow-through system. Broodstocks were given fish commercial pellets containing 32% protein and 4% lipid (Star feed,

TP 2) two times a day until they were satiated. The water parameters in the earthen pond were measured using a YSI meter. The measured values for water quality parameters were 28-30°C, 5.11 mg/L and 7.6-8 for temperature, dissolved oxygen and pH, respectively.

Figure 3.1 Net cages in earthen pond at Three Ocean Fish Pond Sdn. Bhd. where broodstocks of *Pangasius nasutus* were maintained.

3.3 Sperm collection

Matured broodstocks were selected according to sexual maturity criteria where matured males expressed sperm with simple pressure on the abdomen. The sperm was produced naturally without any induction of hormones. The selected broodstocks were anesthetized by immersing them in an anesthetic bath containing an adequate amount of clove oil (80 mg/L) for no longer than 10 minutes. Then, males were properly dried with a clean towel and placed on a flat surface area with the ventral region facing up. Then, sperm was collected by stripping (gentle abdominal massage) below the pelvic fin to the anus and dispensed into separate 2.0 mL tubes for each respective male with care to avoid any contamination of freshwater, blood or urine (Figure 3.2). Collected sperm was immediately placed on crushed ice in an icebox for further analysis. Assessment of initial sperm motility and motility duration was determined within 30

minutes after sperm collection. This study received ethical approval from the Institutional Animal Care and Use Committee (IACUC), Research Management and Innovation Centre (CRIM), Universiti Malaysia Terengganu (UMT), (CRIM_IACUC UMT V1/2020). The guidelines for the care and use of animals were followed.

Figure 3.2 Sperm collection by using stripping technique.

3.4 Sperm concentration

Upon sperm collection, sperm concentration for three males was determined by using a hemocytometer chamber. Diluted sperm was prepared at 10,000 times dilution with 0.9% NaCl. Two centrifuge tubes were prepared and labelled A and B respectively. 1 µl of fresh sperm was added into 99 µL of 0.9% NaCl in the centrifuge tube A and immediately mixed the diluent by using a vortex. Then, 1 µL from centrifuge tube A was transferred into centrifuge tube B that already contained 99 µL of 0.9% NaCl and mixed again by using vortex. After that, 15 µL of the diluted sperm was placed under the cover slip on each side of the hemocytometer and carefully placed the hemocytometer on the biological microscope (CX43; Olympus; 40× magnification). The sperm cells were counted in 4 squares on each side of the hemocytometer and the average was used for calculation. The equation below was used to convert the counts in 4 squares to concentration/mL (Vanderwall, 2008):

Concentration/mL = (Dilution Factor) (Count in 4 squares) (10^4)

3.5 Experiment 1: Determination of optimal diluent for cool storage of *Pangasius nasutus* sperm

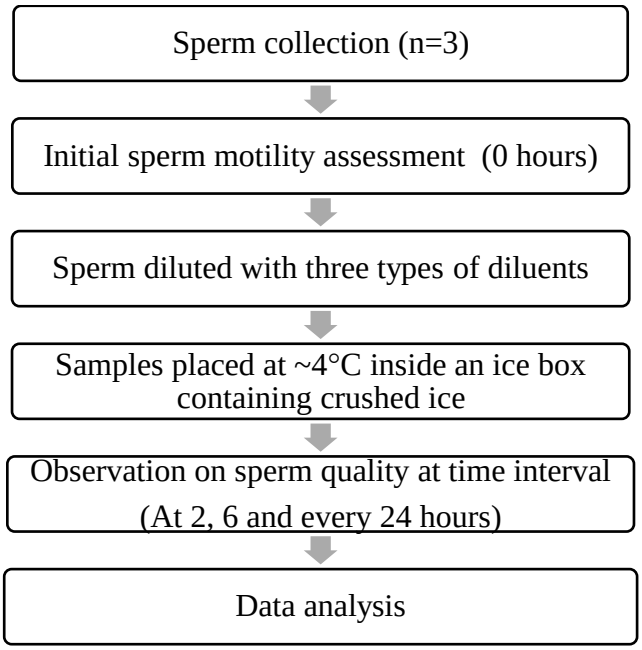


Figure 3.3 An outline of major steps to determine the optimal diluent for cool storage of *Pangasius nasutus* sperm

3.5.1 Sperm collection

Sperm was collected from three males weighing between 0.8 to 1.0 kg without hormone induction. Milt was collected through gentle abdominal massage as described in Section 3.3. The volume of milt collected per male was around 2 to 2.5 mL. Initial motility was observed at the sampling sites by using hemocytometer chamber and dilution of samples with three types of diluents were conducted immediately within 10 minutes after collection. Only samples with motility over 40% were used for storage trials.

3.5.2 Experimental design

Storage trials were done by diluting 500 µL of milt with 500 µL of diluent in a 2.0 mL microtube. Diluents were freshly made in the laboratory the day before. Diluents were cooled on ice before being diluted with milt. For this experiment, four conditions were investigated which was undiluted and diluted sperm samples. Each condition was tested in three replicates where each individual male represent one replicate (n=3). Sperm samples were diluted in three different types of diluents, which were i) 0.9% sodium chloride (NaCl), ii) Calcium Free Hanks' Balance Salt Solution (C-F HBSS) (Kainin *et al*., 2014) and iii) ringer solution (Muchlisin *et al*., 2004) containing varying ion components: 0.9% NaCl (NaCl 154 mM), CF-HBSS (NaCl 152 mM, KCl 6mM, $MgSO_4.7H_2O$ 0.9 mM, $Na_2HPO_4.7H_2O$ 0.5 mM, KH_2PO_4 0.5 mM, $NaHCO_3$ 4.6 mM, Glucose 6.2 mM), Ringer (NaCl 128.34 mM, KCl 2.7 mM, $NaHCO_3$ 2.4 mM, Glucose 25.3 mM, $CaCl_2$ 1.4 mM). All samples were placed at 2~4°C inside an ice box containing crushed ice. The samples were then transported by car from the sampling site and stored in a refrigerator in the laboratory following a 6 hour journey for further observations. In order to maintain the temperature and facilitate transport, the samples were placed vertically inside the crushed ice, which made it possible to maintain the average temperature of ~4°C. The temperature was monitored using a thermometer that was fixed inside the icebox. The sperm motility was investigated at 0, 2 and 6 hours after initial dilution and storage at the sampling site, and subsequently every 24 hours in the Biosecurity Lab of AKUATROP until no motility was detected.

3.5.3 Sperm quality observation

Percentage of sperm motility was recorded by using Dino eye video camera and software (DinoCapture 2.0, version 1.5.45) where a video camera combined with the optical lens of a biological microscope (CX43; Olympus; 10× magnification). The motility of diluted sperm was observed at the sampling sites (Figure 3.4) and in the laboratory at UMT. The motility of undiluted and diluted sperm was observed after

rapid mixing with freshwater as an activating solution at a sperm to solution ratio of 1:999 and 1:499, respectively in a 1.5 mL microtube. The video timer was recorded starting from sperm sample activation.

After mixing, 8 µL of the mixture was taken and immediately put onto 21 well coated glass slide (Yusoff *et al*., 2018). Sperm motility was recorded from 5 s until all sperm stopped moving. However, hemocytometer chamber was utilized to replace coated glass slide at the sampling sites, in order to avoid any sources of wind during motility observation. For hemocytometer, after the sperm sample was mixed with an activating solution, 10 µL of the mixture was taken and immediately placed under the cover slip on one side of the hemocytometer. Sperm motility was recorded from 5 s until all sperm stopped moving.

Figure 3.4 Equipment setup for sperm motility evaluation at the sampling site.

Sperm motility (%) was examined by reviewing the video recordings. From the video images, sperm cells were randomly chosen and the motile sperm were counted. Only sperm that swam vigorously in a forward direction were counted as motile and sperm that remained stationary or only vibrated were regarded as non-motile. Sperm motility was presented in percentage (%) which was calculated as below (Ohta & Izawa, 1996). Measurements were repeated in duplicate for each replicate and the average was used in the data analyses (Koh *et al*., 2017; Yusoff *et al*., 2018).

% Motility = (Motile sperm / Total sperm counted) × 100

Duration of sperm motility was assessed by reviewing the video recording with the sperm motility duration beginning from the mixing of sperm into freshwater until all the sperm were non motile. For each dilution, measurements were repeated in duplicate and the averages were used in data analyses.

The agglutination of undiluted and diluted sperm was evaluated after rapid mixing with freshwater as an activating solution. Before mixing with freshwater, all sperm samples in the microtubes were homogenously mixed using a vortex to prevent sperm sedimentation. Then, 1 µL from each sample was taken and mixed rapidly with freshwater at a sperm to solution ratio of 1:999 (undiluted sperm) and 1:499 (diluted sperm), respectively in a 1.5 mL microtube. The sperm agglutination was characterized by visual examination inside the microtube, where agglutinated sperm can be seen clumped together (Dong *et al.*, 2007). Meanwhile, sperm samples without any clumps inside the microtube were characterized as homogenous suspension. The observation was conducted once for each replicate at each time interval, in conjunction with sperm motility measures.

3.6 Experiment 2: Development of sperm cryopreservation protocol for *Pangasius nasutus*

3.6.1 Sperm collection

Sperm was obtained from three male broodstocks of *P. nasutus* by using the method described in Section 3.3. The broodstock's body weight (BW) and total length (TL) were between 0.75-1.10 kg and 45.4-48.0 cm, respectively. The volume of milt collected per male was around 2 to 2.5 mL. Collected sperm was immediately mixed with 0.9% NaCl (except for the experiment on optimal extender) at a 1:1 ratio to avoid drastic decrease in initial motility, then immediately placed on crushed ice in an

icebox. For initial sperm motility and motility duration, the measurement was done immediately within 30 minutes after sperm collection while cryopreservation process was conducted within ~1 hour.

3.6.2 Standard sperm cryopreservation protocol

Diluted sperm (fresh sperm to 0.9% NaCl ratio of 1:1, except for the experiment on optimal extender) was mixed thoroughly with cryodiluent (cryoprotectant + extender) in a 2 mL microtube at dilution ratio of 1:9 (final sperm to cryodiluent ratio 1:19) and equilibrated for 5 minutes at room temperature (except for the experiment on optimal dilution ratio). After equilibration, 250 µL of the dilution was loaded into 0.25 mL straw (I.M.V., France) and the open ends of straws were sealed with straw powder (Fujihara Kogyo Co., Tokyo, Japan).

The straws were frozen with liquid nitrogen (LN) vapor in a cylindrical stainless-steel jar which filled with LN to a depth 5-8 cm (Koh *et al*., 2017) (Figure 3.5). Straws were placed horizontally on an angular floating frame made of styrofoam block at a height of 14 cm from the surface of LN (except for the experiment on optimal freezing rates) to expose the straws to LN vapor for 7 minutes. After 7 minutes, the final temperature reached -60 °C (cooling rates of 9.23°C min^{-1}) and straws were then immediately immersed in the LN. All cryopreserved samples were held in the LN storage tank for at least 24 hours before sperm quality assessment.

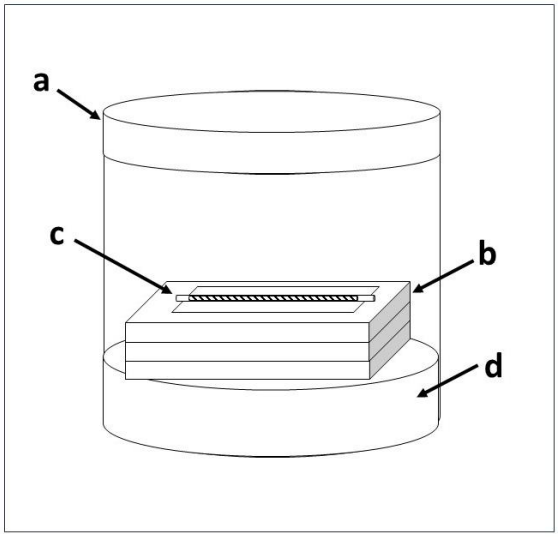


Figure 3.5 Freezing setup for cryopreservation process. a) cylindrical stainless steel jar with a lid; b) hollow angular floating frame made of styrofoam block in which height can be adjusted; c) 0.25 mL straw (I.M.V., France) was placed horizontally; d) liquid nitrogen was filled to a depth 5-8 cm.

3.6.3 Thawing protocol

Thawing was done by removing straws from the LN storage tank with tweezers and placed right away in a water bath filled with water at room temperature (25 °C) for 15 s (Koh *et al*., 2017). Sperm was expelled into 1.5 mL centrifuge tubes after cutting them using a straw cutter with care to ensure no water was added. Thawed sperm were immediately used within 1 minute for motility observations.

3.6.4 Sperm quality evaluation

Percentage of sperm motility was recorded by using Dino eye video camera and software (DinoCapture 2.0, version 1.5.45) where a video camera combined with the optical lens of a biological microscope (CX43; Olympus; 10x magnification). The motility of diluted sperm was observed at the sampling sites and post-thaw motility samples were measured in the laboratory at UMT. The initial motility of diluted and

post-thaw motility of sperm were observed after rapid mixing with freshwater as an activating solution at a sperm to solution ratio of 1:499 and 10:490, respectively in a 1.5 mL microtube. The video timer was recorded starting from sperm sample activation.

After mixing, 8 µL of the mixture was taken and immediately put onto 21 well coated glass slide (Yusoff *et al*., 2018). Sperm motility was recorded from 5 s until all sperm stopped moving. However, hemocytometer chamber was utilized to replace coated glass slide at the sampling sites, in order to avoid any sources of wind during motility observation. For hemocytometer, after the sperm sample was mixed with an activating solution, 10 µL of the mixture was taken and immediately placed under the cover slip on one side of the hemocytometer. Sperm motility was recorded from 5 s until all sperm stopped moving.

Sperm motility (%) was examined by reviewing the video recordings. From the video images, sperm cells were randomly chosen and the motile sperm were counted. Only sperm that swam vigorously in a forward direction were counted as motile and sperm that remained stationary or only vibrated were regarded as non-motile. Sperm motility was presented in percentage (%) which was calculated as below (Ohta & Izawa, 1996). Measurements were repeated in duplicate for each replicate and the average was used in the data analyses. Only fresh sperm with initial motility over 40% were used for the following experiments (Koh *et al*., 2017; Yusoff *et al*., 2018).

% Motility = (Motile sperm / Total sperm counted) × 100

Duration of sperm motility was assessed by reviewing the video recording with the sperm motility duration beginning from the mixing of sperm into freshwater until all the sperm were non motile.

Spermatozoa velocity according to the actual path (curvilinear velocity, VCL; µm s^{-1}) was evaluated by using a semi-subjective method. The video recording of

sperm motility was reviewed and the movement of sperm was observed until 10 s. The actual length of sperm movement was measured using a stage micrometer. In each condition, at least 50 sperm cells were examined and the average results were used for data analysis. This assessment was limited to the determination of optimal cryodiluent.

Adopting the standard cryopreservation protocol, thawing method and sperm quality evaluation above, the effects of changes in the following parameters were examined in order to develop the sperm cryopreservation protocol for *P. nasutus* species. For each parameter, the initial motility of diluted sperm was investigated before the experiments were conducted to make sure the sperm quality was high (maintained motility of 30-40%).

3.6.5 Determination of optimal cryodiluent

Three different concentrations **(**5, 10 and 15%) of dimethyl sulfoxide (Me_2SO), dimethylacetamide (DMA) and methanol (MeOH) were evaluated. Based on previous studies on sperm cryopreservation of other *Pangasius* species, 0.9% NaCl was chosen as an extender in this experiment. Briefly, diluted sperm from each male (n=3) were mixed with 9 types of cryodiluents at a ratio 1:9 (diluted sperm:cryodiluent) where final dilution ratio was 1:19, and transferred into 0.25 mL straws. Each condition was tested in three replicates where each individual male represents one replicate (n=3). After samples equilibrated for 5 minutes at room temperature, freezing conditions and thawing were conducted as previously mentioned above at 3.6.2 and 3.6.3. Sperm motility percentage, duration and VCL after post-thaw was analyzed using the same method and technique in 3.6.4. Two straws for each replicate were thawed and measurements on sperm motility and motility duration were repeated in duplicate for each and the average was used in the data analyses. The control for this experiment was 10% MeOH with 0.9% NaCl as a cryodiluent.

In order to assess the toxicity of cryoprotectants, the sperm motility percentage and duration was examined both before being mixed with cryodiluent and at intervals of 10, 25, and 50 minutes thereafter, using the procedure outlined in Section 3.6.4.

3.6.6 Determination of optimal extender

Using the best results from Section 3.6.5, the trial for various extenders was carried out. Three types of extenders that were successfully being used and tested in other *Pangasius* species and freshwater fish were chosen. 0.9% Sodium Chloride, NaCl, Calcium-free Hanks' Balance salt solution, CF-HBSS (152 mM NaCl, 6 mM KCl, 0.9 mM MgSO4.7H2O, 0.5 mM Na2HPO4.7H2O, 0.5 mM KH2PO4, 4.6 mM NaHCO3, 6.2 mM Glucose) and freshwater fish ringer solution (128.34 mM NaCl, 2.7 mM KCl, 1.4 mM CaCl, 2.4 mM NaHCO3, 25.3 mM Glucose) were investigated in this study. Each extender was tested in three replicates where each individual male represents one replicate (n=3). Upon sperm collection, fresh sperm was diluted with three types of extenders at ratio 1:1. Then, diluted sperm was mixed with the best cryodiluent (10% MeOH + 90% extender) at a ratio 1:9, to achieve final dilution ratio 1:19. The samples were immersed in LN after being exposed to LN vapour for 7 minutes at a height of 14 cm. Samples were kept for at least 24 h before sperm quality assessment. Sperm motility percentage and duration after post-thaw was analyzed using the same method and technique in 3.6.4. Two straws for each condition were thawed and measurements on sperm motility and motility duration were repeated in duplicate for each and the average was used in the data analyses.

3.6.7 Determination of optimum cooling rates

Based on results from Section 3.6.6, the best cryodiluent was used for this experiment. Diluted sperm was suspended with 10% MeOH + 90% 0.9% NaCl as extender at final dilution ratio of 1:19 and was dispensed into a 250 µL straw. Then, straws were put on the hollow square floating frame at different heights and cooled.

Each cooling rate was tested in three replicates where each individual male represents one replicate (n=3). The cooling rates were controlled by adjusting the height of straws to 10, 12 ,14 and 16 cm on the floating frame (actual cooling rates of 11.98, 10.57, 9.23 and 6.25°C min^{-1}, respectively) and cooling for 7 minutes (corresponding to immersion temperature of -80, -70, -60 and -40°C, respectively) before immediately immersing the straws in LN. Cooling rates were computed based on the time taken for the temperature to change from 0°C to final temperature. Samples were kept for at least 24 h before sperm quality assessment. For each freezing height, the procedure was carried out in duplicate. Sperm motility percentage and duration after post-thaw was analyzed using the same method and technique in 3.6.4. Two straws for each condition were thawed and measurements on sperm motility and motility duration were repeated in duplicate for each and the average was used in the data analyses.

3.6.8 Determination of optimum dilution ratio

To determine the optimum ratio, diluted sperm was mixed with the best result at Section 3.6.7, 10% MeOH + 90% 0.9% NaCl at three dilution ratios (1:4, 1:9 and 1:24), where final solution contained of 1:9, 1:19 and 1:49 of sperm to cryodiluent. Each dilution ratio was tested in three replicates where each individual male represents one replicate (n=3). The dilutions were exposed to LN vapor for 7 minutes at 14 cm height before immersed to LN. Samples were kept for at least 24 h before motility assessment. Sperm motility percentage and duration after post-thaw was analyzed using the same method and technique in 3.6.4. The post-thaw motility of sperm was observed after mixing with freshwater as an activating solution at a sperm to solution ratio of 5:245, 10:490 and 10:390, respectively in a 1.5 mL microtube for final dilution of 1:999. Two straws for each condition were thawed and measurements on sperm motility and motility duration were repeated in duplicate for each and the average was used in the data analyses.

3.7 Experiment 3: Evaluation of *Pangasius nasutus* cryopreserved sperm quality and its applicability in seed production

3.7.1 Evaluation on the effect of long-term storage inside liquid nitrogen

In this study, sperm was obtained from three males (n=3) of *P. nasutus* cultured at a private fish farm, Three Oceans Fish Pond located in Rawang, Selangor, Malaysia in August 2022 using the method described at Section 3.3. The sperm was produced naturally without any induction of hormones. The broodstock's BW and TL were between 1.5-2.0 kg and 48.0-52.3 cm respectively. Sperm was collected by stripping (gentle abdominal massage) below the pelvic fin to the anus and dispensed into separate 2.0 mL tubes for each respective male and placed on crushed ice in an icebox. Initial sperm motility and duration were determined and dilution of samples with 0.9% NaCl at ratio 1:1 conducted within 30 mins of collection. Sperm was then cryopreserved by using developed protocol and the motility and duration of post-thaw sperm was evaluated at 3, 6, 9 and 12 months of storage.

3.7.1.1 Cryopreservation protocol

From the results of Experiment 2, the following protocol for long term preservation was adopted. Diluted sperm (fresh sperm to 0.9% NaCl ratio of 1:1) was mixed thoroughly with 10% methanol (MeOH) + 90% 0.9% NaCl at a dilution ratio of 1:9 (final sperm to cryodiluent ratio 1:19) and equilibrated for 5 minutes at room temperature. After equilibration, 250 µL of the dilution was loaded into 0.25 mL straw (I.M.V., France) and the open ends of straws were sealed with straw powder (Fujihara Kogyo Co., Tokyo, Japan).

The straws were frozen with liquid nitrogen (LN) vapor in a cylindrical stainless-steel jar which filled with LN to a depth 5-8 cm (Koh *et al*., 2017). Straws were placed horizontally on an angular floating frame made of styrofoam block at a height of 14 cm from the surface of LN for 7 minutes, then immediately immersed in

the LN. All cryopreserved samples were stored in a LN storage tank for 1 year at the Biosecurity Lab of AKUATROP.

For thawing, straws were removed from the LN storage tank with tweezers and placed right away in a water bath filled with water at room temperature (25 °C) for 15 seconds. After that, sperm was expelled into 1.5 mL centrifuge tubes after cutting them using a straw cutter with care to ensure no water was added. Thawed sperm were immediately used for sperm quality observations.

3.7.1.2 Sperm quality evaluation

The motility and duration of post-thaw sperm was evaluated at 3, 6, 9 and 12 months of storage using the same method described in 3.6.4. Percentage of sperm motility was recorded by using Dino eye video camera and software (DinoCapture 2.0, version 1.5.45) where a video camera combined with the optical lens of a biological microscope (CX43; Olympus; 10× magnification). The post-thaw motility of sperm was observed after rapid mixing with freshwater as an activating solution at a sperm to solution ratio of 10:490 in a 1.5 mL microtube.

After mixing, 8 µL of the mixture was taken and immediately put onto 21 well coated glass slide (Yusoff *et al*., 2018). The video timer was recorded starting from sperm sample activation. Sperm motility was recorded from 5 s until all sperm stopped moving. Sperm motility (%) was examined by reviewing the video recordings. For each duration, two straws were thawed and measurements were repeated in duplicate for each and the average was used in the data analyses. Duration of sperm motility was assessed by reviewing the video recording with the sperm motility duration beginning from the mixing of sperm into freshwater until all the sperm were non motile.

3.7.2 Trial 1: Fertilization and hatching trials

Fertilization and hatching trials were done using long-term cryopreserved sperm (9-months) at the same sperm to egg ratio for fresh and cryopreserved sperm. Fertilization and hatching were done at a private fish farm, Three Ocean Fish Pond Sdn. Bhd. Rawang, Selangor, Malaysia**,** in May 2023.

3.7.2.1 Gamete collection

Sperm samples from three males (n=3) were collected in August 2022 and cryopreserved using developed protocol as previously mentioned in Section 3.7.1.1. The broodstock's BW and TL were between 1.5-2.0 kg and 48.0-52.3 cm respectively. After 9 months of storage, cryopreserved sperm was thawed in a water bath at room temperature (25°C) for 15 s and transferred into 2.0 mL centrifuge tubes. PTM was then evaluated immediately after thawing. Fresh sperm was also extracted from three males (n=3) before fertilization occurred. The broodstock's BW and TL were between 0.75-0.95 kg and 45.4-46.2 cm respectively. Sperm was collected by stripping (gentle abdominal massage) below the pelvic fin to the anus as mentioned in Section 3.3, dispensed into separate 2.0 ml tubes for each respective male and placed on crushed ice in an icebox. Initial sperm motility was determined within 30 mins of collection. The motility of fresh and cryopreserved sperm was evaluated using the method described in 3.7.1.2.

For egg collection, a matured female broodstock of *P. nasutus* was selected from the earthen pond and maintained inside the rectangular tank for hormone injection. Determination of fish sexual maturity was conducted through abdominal massage because *P. nasutus* females showed no obvious external signs of sexual maturity. The broodstock's body weight and total length were 0.85 kg and 45 cm, respectively. The female was given a hormone injection with the farmer's assistance. Eggs were then collected via stripping technique, a gentle pressure at the abdomen after 24 hours injection of hormone. Female broodstock was anesthetized by

immersing them in an anesthetic bath containing clove oil (80 mg/L) for no longer than 10 minutes before egg collection. Then, the female was covered with a plastic bag except their abdomen to reduce the stress and the eggs were gently squeezed out into a bowl with care to avoid any contamination of freshwater, blood or urine (Figure 3.6). Eggs were yellowish in colour and total weight of the collected eggs was ~140 g (Figure 3.6).

Figure 3.6 The head and tail of the fish was covered with the plastic bag and eggs were collected using stripping technique.

3.7.2.2 Fertilization and activation

The amounts of collected eggs were transferred into 2 bowls with the same amount (~7.5g) for each treatment. The eggs were them fertilized in triplicates for each treatment within 30 minutes. All sperm samples (fresh and cryopreserved sperm) were diluted using 0.9% NaCl to 10 mL (same final volume for fresh and cryopreserved sperm) in the 15 mL tubes at the same sperm amount of 7.94×10^8. The final sperm to egg ratio was ~100,000 to 1. Diluted pooled sperm were added into the eggs and gently mixed manually by twirling (spin lightly and quickly round) until the sperm homogeneously spreads in the egg mass. After that, 100 mL of freshwater was added directly into the bowl after addition of sperm samples for activation and fertilization. Freshwater was added until it covered all the number of eggs. Mixing was done by rolling or twirling the petri dish for 1 minute in order to achieve the maximum fertilization.

3.7.2.3 Incubation and hatching

The eggs were immediately transfer into a triplicate floating net at the same amount (1.5 g; ~1500 eggs) for each treatment for incubation and further observation. The floating nets were placed inside a rectangular tank (30 cm × 73 cm × 106 cm) filled with 180 L of freshwater. A flow-through system with a gentle aeration was used for incubation and the temperature was maintained at 29~31˚C by using heater (Figure 3.7 and 3.8).

Figure 3.7 Flow-through system used for eggs incubation. Red arrow shows the water outlet and the yellow arrow for the water inlet.

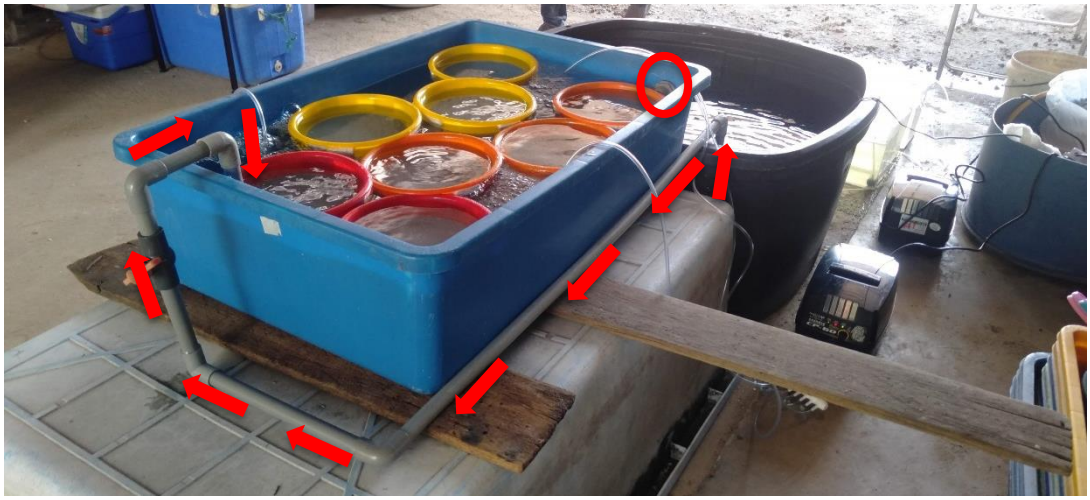


Figure 3.8 Flow-through system used for eggs incubation. Red arrow shows the flow of water from the storage tank into the incubation tank. Red circle shows the water outlet.

After 3 hours of insemination, the fertilization rate was evaluated. Around 100 eggs in each replicate for both treatments were randomly collected from the floating

net and placed on a petri dish for observation under compound microscope (Figure 3.9).

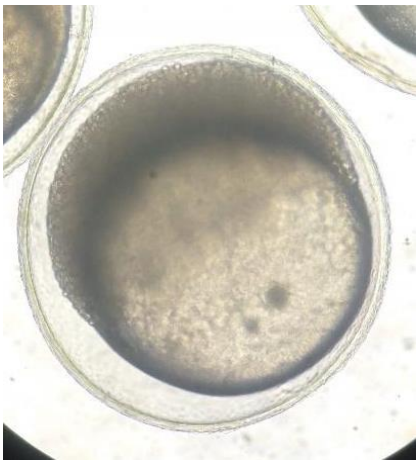


Figure 3.9 Fertilized eggs at gastrula stage after 3 hours insemination. Image was observed under a compound microscope at magnification 40×.

The eggs started hatching at 25 hours after insemination but fully hatched only at 5 h after initial observation of hatching. Therefore, the hatching rate was evaluated at 30 h after insemination. Hatching rate was calculated as hatched larvae in relation to the total number of eggs inside the floating net. For deformities, fifty larvae for each replicate were randomly sampled and preserved in 0.1% formalin for deformity observation. Larvae were classified as malformed if their spines were curved and distorted. The following formulas were used to calculate the rates of fertilization (Hassan *et al*., 2011; Haque *et al*., 2021), hatching (Gallego *et al*., 2013) and deformities (Linhart *et al*, 2023).

Fertilization rate (%) = (no. of fertilized eggs / no. of total eggs) × 100

Hatching rate (%) = (no. of hatched larvae / no. of total eggs) × 100

Larvae deformity rate (%) = (no. of deformed larvae / no. of total larvae observed) × 100

3.7.3 Trial 2: Fertilization and hatching at different sperm to egg ratios

Increased sperm to egg ratios were used in trial 2 to compare between 1-year cryopreserved and fresh sperm. The goal of the second trial was to identify the effect of increasing concentration of sperm with fertilization, hatching and deformity rates. Fertilization and hatching were done at a private fish farm, Three Ocean Fish Pond Sdn. Bhd. Rawang, Selangor, Malaysia**,** in August 2023.

3.7.3.1 Gamete collection

Sperm samples from three males (n=3) were collected in August 2022 and cryopreserved using developed protocol as previously mentioned in Section 3.7.1.1. The broodstock's BW and TL were between 1.5-2.0 kg and 48.0-52.3 cm, respectively. After 1 year of storage, cryopreserved sperm was thawed in a water bath at room temperature (25°C) for 15 seconds and transferred into 2.0 mL centrifuge tubes. Thawed sperm were then procced for motility evaluation and immediately used for fertilization trial. Fresh sperm was also extracted from three males (n=3) before fertilization occurred. The broodstock's BW and TL were between 0.68–0.8 kg and 42.5-43.4 cm respectively. Sperm was collected by stripping (gentle abdominal massage) below the pelvic fin to the anus as mentioned in Section 3.3, dispensed into separate 2.0 ml tubes for each respective male and placed on crushed ice in an icebox. Initial sperm motility was determined within 30 mins of collection. The motility of fresh and cryopreserved sperm was evaluated using the method described in 3.7.1.2.

For eggs collection, a matured female broodstock of *P. nasutus* was selected from the earthen pond and maintained inside the rectangular tank for hormone injection. Determination of fish sexual maturity was conducted through abdominal massage because *P. nasutus* females showed no obvious external signs of sexual maturity. The broodstock's body weight and total length were 0.75 kg and 44.2 cm respectively. The female was given a hormone injection at a specific dosage with the

farmer's assistance. Eggs were collected using the same methods as described in Section 3.7.2.1 and dispensed into a bowl.

3.7.3.2 Fertilization and activation

Collected eggs were transferred by spoon to 60 ml sample bottles. Each spoon contained ~1000 eggs with the actual number of eggs for each condition counted later. The eggs were fertilized with 3 types of sperm treatments: i) fresh sperm at sperm to egg ratio of 1.5×10^5; ii) cryopreserved sperm at sperm to egg ratio of 1.5×10^5 (C1); iii) cryopreserved sperm at sperm to egg ratio of 3.0×10^5 (C2); in triplicates within 30 minutes after eggs were collected. The motility of fresh and cryopreserved sperm was evaluated simultaneously using the method described in 3.7.1.2. while fertilization was on-going.

Each sperm samples (n=3) for fresh and cryopreserved sperm were diluted using 0.9% NaCl to 1 mL at sperm amounts of fresh sperm, 1.5×10^8 sperm; C1, 1.5×10^8 sperm and C2, 3.0×10^8 sperm, respectively and added into a sample bottle. The final sperm to eggs ratios were 150,000 to 1, 150,00 to 1 and 300,000 to 1 for fresh, C1 and C2, respectively. 10 ml of freshwater was added directly after addition of sperm samples for activation and fertilization. Mixing was done by gently rolling the sample bottles for 1 minute to achieve maximum fertilization.

3.7.3.3 Incubation and hatching

The eggs were immediately transferred into a triplicate floating net (n=3) for each treatment (~200 to 300 eggs) for incubation and further observation. The floating nets were placed inside a rectangular tank (30cm × 73cm × 106cm) filled with 180 liters of freshwater. Using the same method outlined in Section 3.7.2.2, a flow-through

system with a gentle aeration was used for incubation with the temperature maintained at 29~31˚C by using a heater.

After 3 hours of insemination, fertilization rate was evaluated. Around 50 eggs from each replicate for both treatments were randomly collected from the floating net and put into a petri dish to observe under compound microscope. Hatching was counted 5 hours after initial hatching was observed. Hatching rate was calculated as hatched larvae in relation to the total number of eggs inside the floating net. For deformities, fifty larvae from each replicate were randomly sampled and preserved in 0.1% formalin for deformity observation. The same formulas at Section 3.7.2.2 were used to compute the rates of fertilization, hatching, and deformities.

3.8 Statistical data analysis

All values were expressed as mean ± SE. Prior to analysis, percent motilities were transformed using arcsine square root transformation. All data in Experiment 1 (except for observation on sperm agglutination) and observation on toxicity of cryodiluents in Experiment 2 were analysed using repeated measure analysis of variance (ANOVA) (Bozkurt *et al*., 2009; Gonzalez-lopez *et al*., 2020; Liu *et al*., 2022). One-way ANOVA was used to determine the best condition for sperm quality in Experiment 2 and 3 (Koh *et al*., 2017;2010; Yang *et al*., 2023; Yusoff *et al*., 2018). Fertilization, hatching and deformity rate in Trial 2 was also analysed using one-way ANOVA. Experiments which had significant differences were then evaluated using a post-hoc (Tukey) test to determine significance between treatments. Pearson's correlation was conducted to determine the relationship between PTM vs motility duration and PTM vs VCL in various cryodiluents in Experiment 2. Fertilization, hatching and deformity values (Trial 1) were compared by using an independent Sample t-test. The significance level was set at ($P < 0.05$) for all statistical analysis. All statistical analyses were performed using SPSS 27.0 software (SPSS, Chicago, IL USA).

CHAPTER 4

RESULTS

4.1 Experiment 1: Determination of suitable diluent for cool storage

This experiment was aimed to investigate the suitable diluent for short term preservation of *P. nasutus* sperm. Sperm samples were stored in four different conditions (undiluted, 0.9% NaCl, CF-HBSS and Ringer solution) and demonstrated different degrees of sperm motility throughout 120 hours of storage (Figure 4.1). The control which is the undiluted fresh sperm can only last for a short time period; at 2 hours of storage at 4°C, the motility was completely zero. Percent motility showed a gradual decrease in all diluents, however, 0.9% NaCl showed the highest motility between 0 until 24 hours which was not significantly different compared to initial. CF-HBSS also performed well, highest motility was observed from 0 to 6 hours; at 24 hours onward motility was significantly lower. Ringer solution demonstrated the worst results, with motility significantly decreasing as early at 6 hours. At 48 hours, motility in 0.9% NaCl significantly decreased to 8.33 ± 1.67% and was not significantly different compared to the other diluents. At 72, 96 and 120 h, motility mostly was very low (<1%) in all diluents.

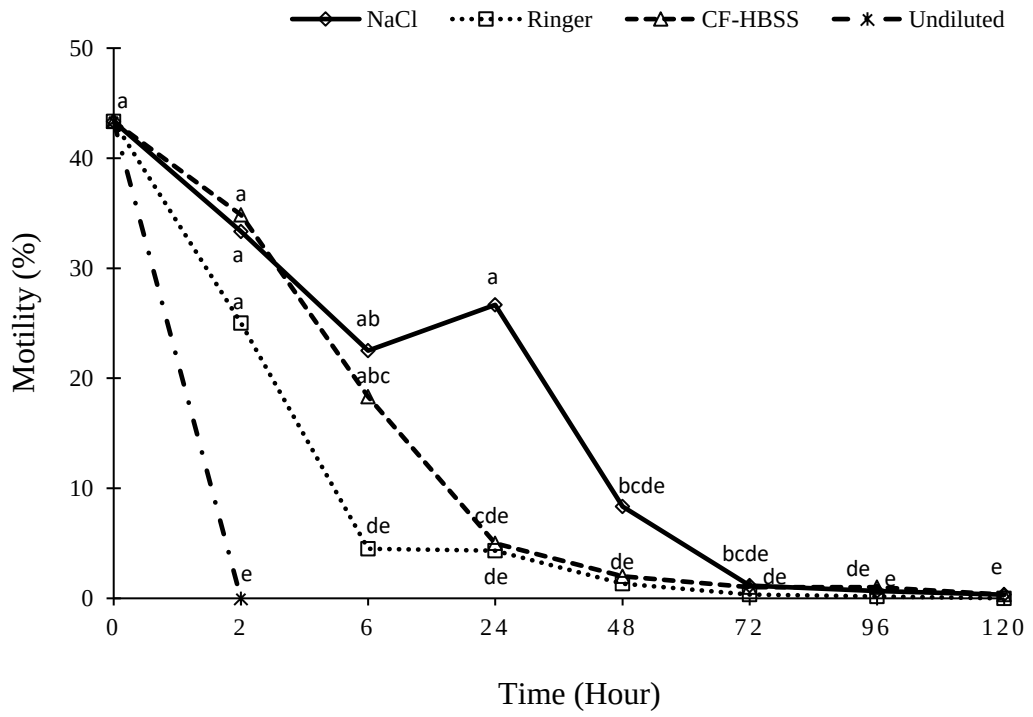


Figure 4.1 Effects of various diluents during 120 hours of storage at ~4°C on the motility of *Pangasius nasutus* sperm (n=3). 0.9% NaCl, 0.9% Sodium chloride; Ringer; CF-HBSS, Calcium free hanks' balance salt solution. Means followed by different superscripts are different ($P < 0.05$).

Motility duration of sperm samples before and after storage in different diluents showed a gradual decrease except for 0.9% NaCl from 0 to 24 hours (Figure 4.2). The initial duration for sperm samples showed the highest duration of 32.67 ± 0.88s. Motility duration after storage was significantly longest in 0.9% NaCl up to 24 h with motility duration of over 30 s. Motility duration significantly decreased in 0.9% NaCl from 48 h onwards, but it still had the longest motility duration when compared to all other diluents up to 96 h. CF-HBSS was the second-best diluent in terms of motility duration. Nevertheless, motility duration of CF-HBSS was only not significantly lower that initial duration until 2 hours of storage. The lowest motility duration was observed in Ringer solution at 96 hours of storage which were not significantly different with 0.9% NaCl at 120 hours and CF-HBSS at 72 and 96 hours of storage trials. At 120 hours of storage, no motility was detected for Ringer.

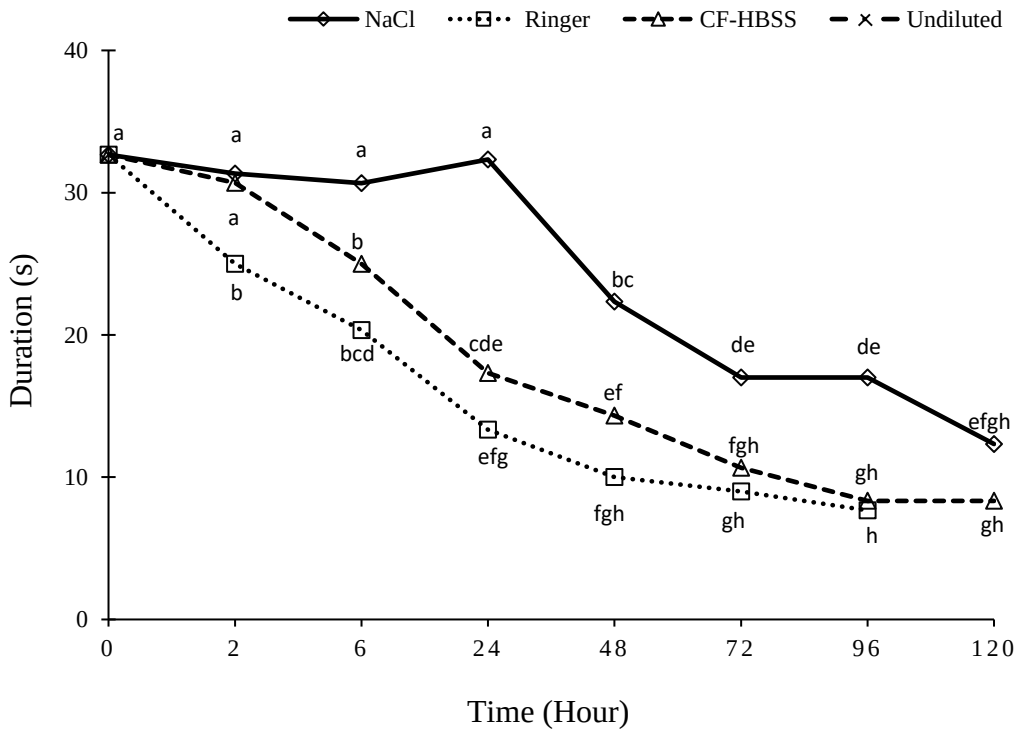


Figure 4.2 Effects of various diluents during 120 hours of storage at ~4°C on the motility duration of *Pangasius nasutus* sperm (n=3). 0.9% NaCl, 0.9% Sodium chloride; Ringer; CF-HBSS, Calcium free hanks' balance salt solution. Means followed by different superscripts are different ($P < 0.05$).

Sperm samples in various diluents exhibited varying periods of agglutination when we mixed it with the activating solution (Table 4.1). According to our observations, agglutination occurred in undiluted sperm samples within 2 hours of storage. Sperm samples in 0.9% NaCl showed agglutination after 96 hours of storage. However, with ringer and CF-HBSS, sperm agglutination was seen after 48 hours.

Table 4.1 Observation on agglutination of *Pangasius nasutus* sperm at various diluents during 120 hours of storage at ~4°C.

Dilution/ Hour	0	2	6	24	48	72	96	120
Undiluted	0	x	nd	nd	nd	nd	nd	nd
0.9% NaCl	0	0	0	0	0	0	0	x
Ringer	0	0	0	0	x	x	x	x

CF-HBSS	0	0	0	0	x	x	x	X

0, Homogenous suspension; x, agglutination; nd, shows no data.

4.2 Experiment 2: Development of sperm cryopreservation protocol

4.2.1 Optimal cryodiluent

The results on the effect of three types of cryoprotectants (Me_2SO, MeOH and DMA) at various concentrations (5, 10 and 15%) on post-thaw motility are shown in Figure 4.3. Initial sperm motility, duration and velocity were 51.7 ± 2.4%; 31.3 ± 0.33 s and 58.50 ± 1.97µm/s, respectively. The 10% MeOH + 90% of 0.9% NaCl treatment resulted in post thaw motility (PTM) of 26.3 ± 0.9% which was higher than all other conditions ($P < 0.05$). The PTM values of 15% MeOH (17.7 ± 3.7%) was the second highest followed by 10% Me_2SO (16.7% ± 2.4%), 5% Me_2SO (15.0 ± 3.4%), 10% DMA (12.0 ± 2.6%), 5% DMA (11.0 ± 2.5%) as well as 5% MeOH (4.7 ± 2.7%). Both 15% Me_2SO and DMA recorded the lowest PTM values at 2.2 ± 0.7% and 2.7 ± 0.35, which was not significantly different ($P > 0.05$) compared to 5% MeOH.

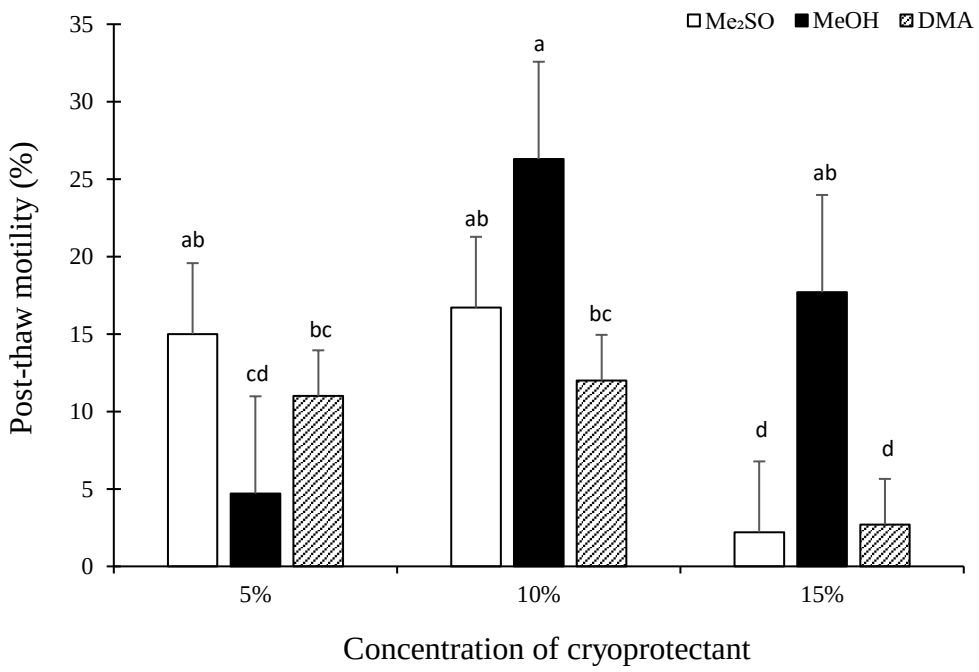


Figure 4.3 Post-thaw motility of *Pangasius nasutus* sperm with various cryoprotectants (Me_2SO, MeOH, DMA) at 3 different concentrations (5, 10 and 15%). Me_2SO, dimethyl sulfoxide; MeOH, methanol; DMA, dimethyl acetamide; NaCl, sodium chloride. All values are mean ± SE of sperm from three replicates (n=3). Values with different letters are significantly different ($P < 0.05$).

The concentration of 15% MeOH (38.2 ± 0.4s) showed the highest motility duration after post-thaw followed by 10% MeOH (35.2 ± 1.0s) and 10% DMA (31.2 ± 3.2s). There were no significant differences ($P > 0.05$) between the top 3 treatments; 15% MeOH was however significantly different with all other treatments (Figure 4.4). At 5% concentration, all cryoprotectants had a low motility duration of 25 to 26 s. The shortest motility duration was detected in 15% Me_2SO (16.2 ± 1.7s) which was not significantly different with 15% Me_2SO (16.2 ± 1.7s) and 10% Me_2SO (22.0 ± 0.8s).

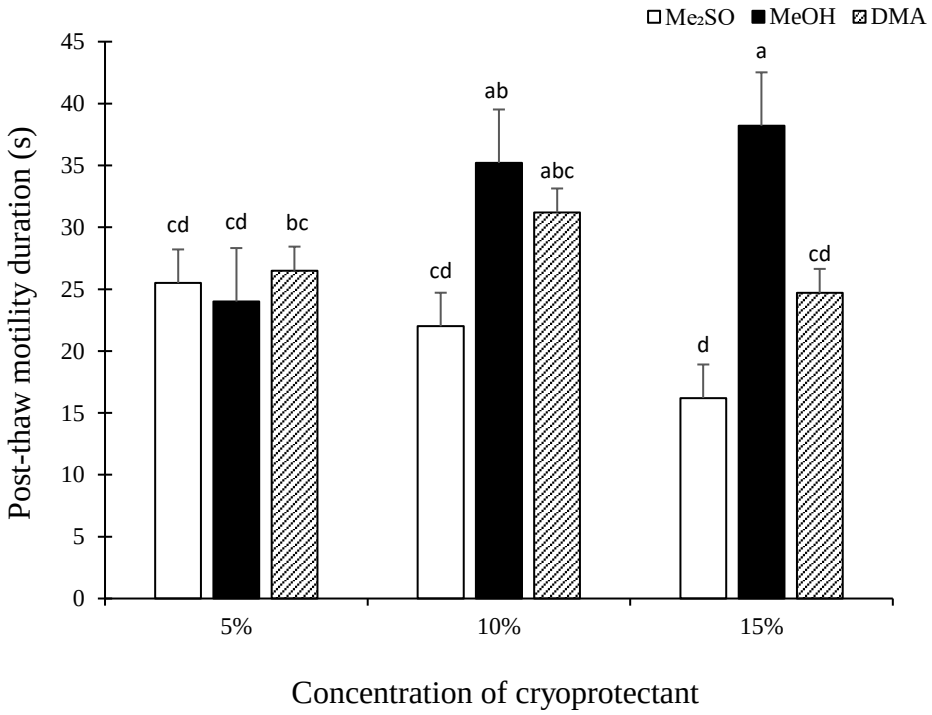


Figure 4.4 Post-thaw motility duration of *Pangasius nasutus* sperm with various cryoprotectants (Me_2SO, MeOH, DMA) at 3 different concentrations (5, 10 and 15%). Me_2SO, dimethyl sulfoxide; MeOH, methanol; DMA, dimethyl acetamide; NaCl, sodium chloride. All values are mean ± SE of sperm from three replicates (n=3). Values with different letters are significantly different ($P < 0.05$).

Velocity curvilinear (VCL) of 10% MeOH (58.04 ± 2.45 µm/s) was significantly higher ($P > 0.05$) compared to other cryoprotectant conditions tested here (Figure 4.5). The second highest VCL was 15% MeOH (46.60 ± 3.10µm/s), followed by 5% (39.80 ± 1.8µm/s) and 10% DMA (40.30 ± 1.50µm/s). VCLs for 15% DMA, 5% Me_2SO and 5% MeOH were between 28 to 37 µm/s. The lowest VCL was observed in 15% Me_2SO (18.8 ± 0.8 µm/s) which was not significantly different to 10% (25.0 ± 1.1 µm/s) and 5% Me_2SO (28.3 ± 1.5 µm/s).

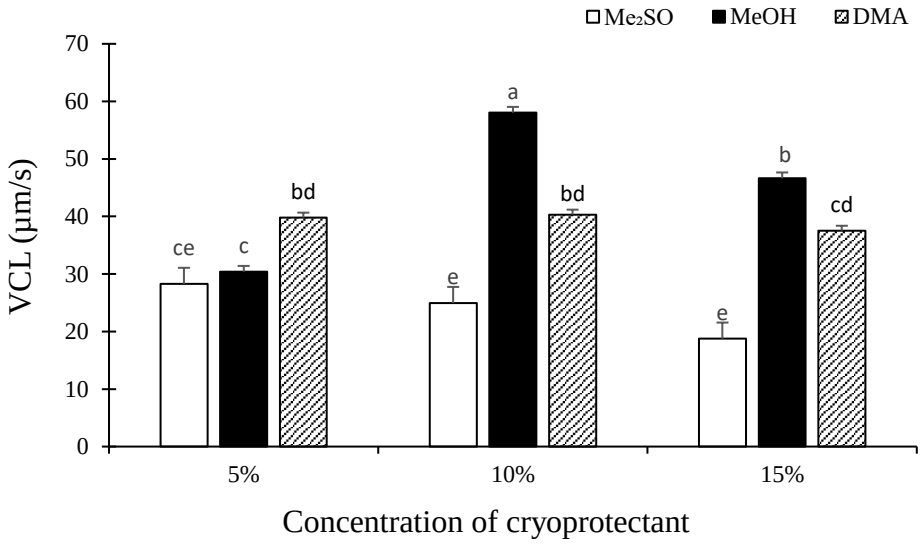


Figure 4.5 VCL of *Pangasius nasutus* cryopreserved sperm with various cryoprotectants (Me_2SO, MeOH, DMA) at 3 different concentrations (5, 10 and 15%). Me_2SO, dimethyl sulfoxide; MeOH, methanol; DMA, dimethyl acetamide; NaCl, sodium chloride. All values are mean ± SE of sperm from three replicates (n=3). Values with different letters are significantly different ($P < 0.05$).

Pearson product-moment correlation was conducted to determine the relationship between PTM, motility duration and VCL in various cryodiluents (Figure 4.6 and 4.7). There was a strong, positive correlation between PTM and motility duration, which was statistically significant ($r= 0.679$, $n =27$, $p < 0.001$). Meanwhile, moderate positive correlation was found between PTM and VCL, which was statistically significant ($r= 0.600$, $n =27$, $p < 0.001$). From these results, we observed that the highest PTM showed higher motility duration and VCL, while the lowest PTM showed lower motility duration and VCL.

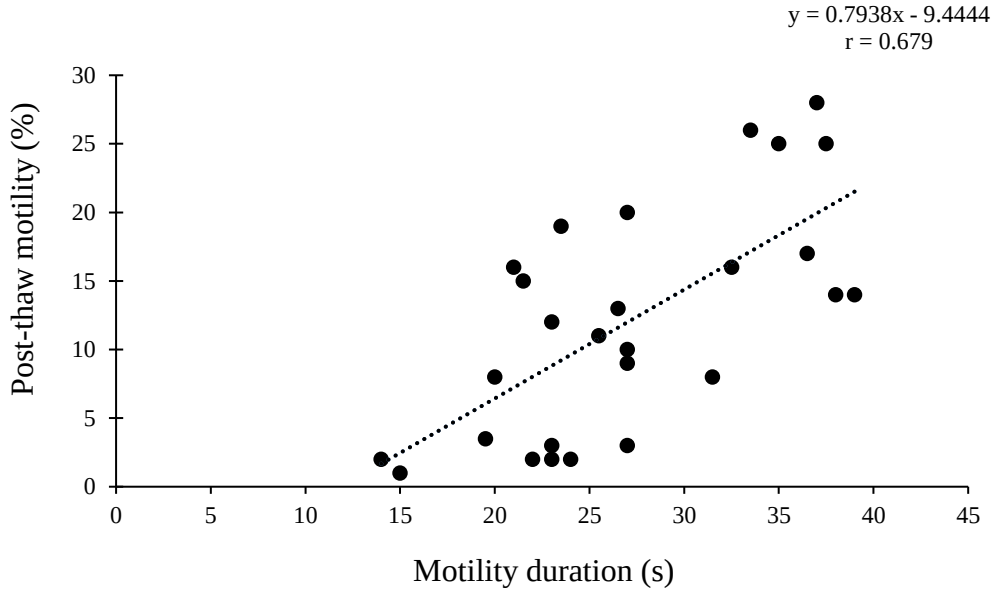


Figure 4.6 Scatter graph of Pearson correlation for post-thaw motility (PTM) and velocity curvilinear (VCL) in different types of cryodiluents at various concentrations of *Pangasius nasutus* sperm.

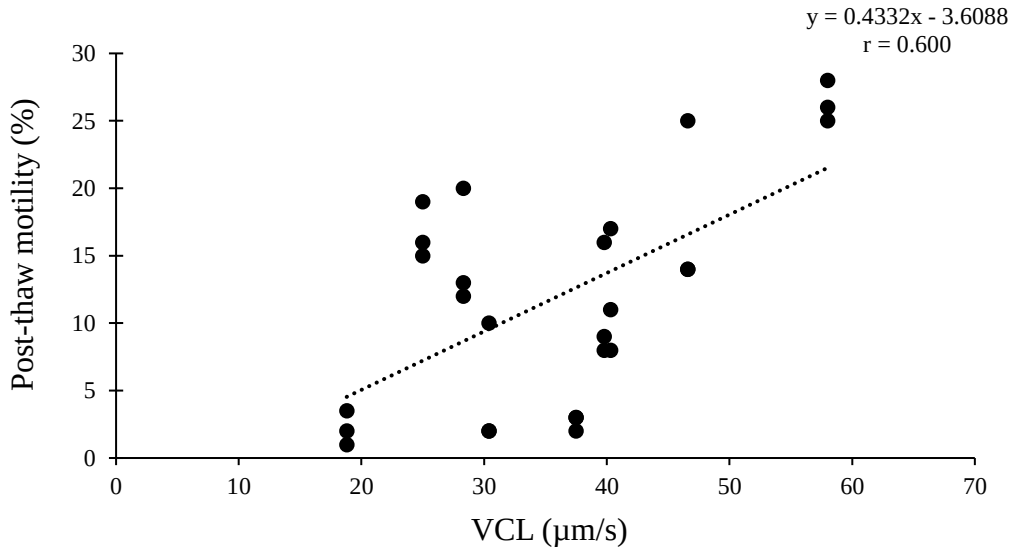


Figure 4.7 Scatter graph of Pearson correlation for post-thaw motility (PTM) duration (s) and velocity curvilinear (VCL) in different types of cryodiluents at various concentrations of *Pangasius nasutus* sperm.

Toxicity of cryoprotectants was conducted to identify the toxic effect of cryoprotectants on the sperm motility within a time interval before the freezing process (Table 4.2). The initial sperm motility and duration were 17 ± 1.3% and 23 ± 1.1 s, respectively. MeOH at 5 and 10% as well as 5% Me_2SO were least toxic at all intervals compared to other conditions. However, there were no significant differences ($P > 0.05$) detected for 10% Me_2SO up to 5 minutes and 5% DMA up to 25 minutes exposure. All cryoprotectants at 15% were considerably more toxic compared to other cryoprotectant concentrations (Table 4.2). However, the values for motility duration were not significantly different at various cryoprotectants tested here (Table 4.3).

Table 4.2 Toxicity of *Pangasius nasutus* sperm suspended with various concentrations of cryoprotectants and 0.9% NaCl as an extender at a final dilution ratio 1:19. Me_2SO, dimethyl sulfoxide; MeOH, methanol; DMA, dimethyl acetamide. Significant differences are represented by different letters ($P < 0.05$).

Motility (%)	**Times (minutes)**		
	10	25	50
Me_2SO			
5%	14.33 ± 1.2 ^{ab}	9.67 ± 0.33 ^{abcd}	12.0 ± 1.15 ^{abcd}
10%	7.33 ± 1.33 ^{abcd}	5.67 ± 0.88 ^{cde}	6.67 ± 1.76 ^{bcde}
15%	5.33 ± 0.88 ^{cde}	0.67 ± 0.67 ^{gh}	0.0 ± 0.0 ^{h}
MeOH			
5%	15.33 ± 1.76 ^{a}	12.67 ± 0.67 ^{abc}	11.67 ± 1.45 ^{abcd}
10%	15.33 ± 1.2 ^{a}	14.33 ± 1.45 ^{ab}	14.33 ± 2.19 ^{ab}
15%	8.67 ± 0.67 ^{abcd}	4.67 ± 0.33 ^{def}	2.0 ± 0.58 ^{efg}
DMA			
5%	10.67 ± 1.76 ^{abcd}	9.67 ± 2.0 ^{abcd}	4.67 ± 0.33 ^{def}
10%	5.0 ± 1.73 ^{def}	1.33 ± 0.67 ^{efg}	0.0 ± 0.0 ^{h}
15%	2.0 ± 0.58 ^{efg}	0.0 ± 0.0 ^{h}	0.0 ± 0.0 ^{h}

Table 4.3 Sperm motility duration of *Pangasius nasutus* sperm suspended with various concentrations of cryoprotectants and 0.9% NaCl as an extender at a final dilution ratio 1:19. Me_2SO, dimethyl sulfoxide; MeOH, methanol; DMA, dimethyl acetamide; nd, shows no data. Values are means ± SE of sperm from three replicates.

Duration (s)	**Times (minutes)**		
	10	25	50
Me_2SO			
5%	18.33 ± 0.89	17.0 ± 0.58	20.0 ± 2.65
10%	17.67 ± 1.85	16.33 ± 2.73	22.0 ± 0.33

15%	11.0 ± 1.0	14.0 ± 1.33	n.d
MeOH			
5%	23.83 ± 1.67	21.00 ± 2.30	21.67 ± 3.84
10%	19.67 ± 2.60	19.50 ± 2.75	21.67 ± 3.17
15%	18.67 ± 3.18	18.0 ± 0.0	17.0 ± 0.0
DMA			
5%	21.00 ± 4.93	21.50 ± 1.80	13.83 ± 7.67
10%	15.67 ± 2.96	15.33 ± 1.45	n.d
15%	11.67 ± 1.67	n.d	n.d

4.2.2 Optimum extender

The effects of different extenders in the diluents on post-thaw motility and motility duration are shown in Figure 4.8. The initial sperm motility and duration for this experiment were 35.2 ± 2.7% and 22.9 ± 0.1 s, respectively. There were no significant differences ($P > 0.05$) for post-thaw sperm motility (0.9% NaCl, 23.3 ± 4.6%; ringer, 14.5 ± 0.5%, CF-HBSS, 24.2 ± 5.1%) and duration (0.9% NaCl, 55.3 ± 3.5 s; ringer, 48.7 ± 5.1 s; CF-HBSS, 46.8 ± 2.7s) for all types of extenders observed in this study.

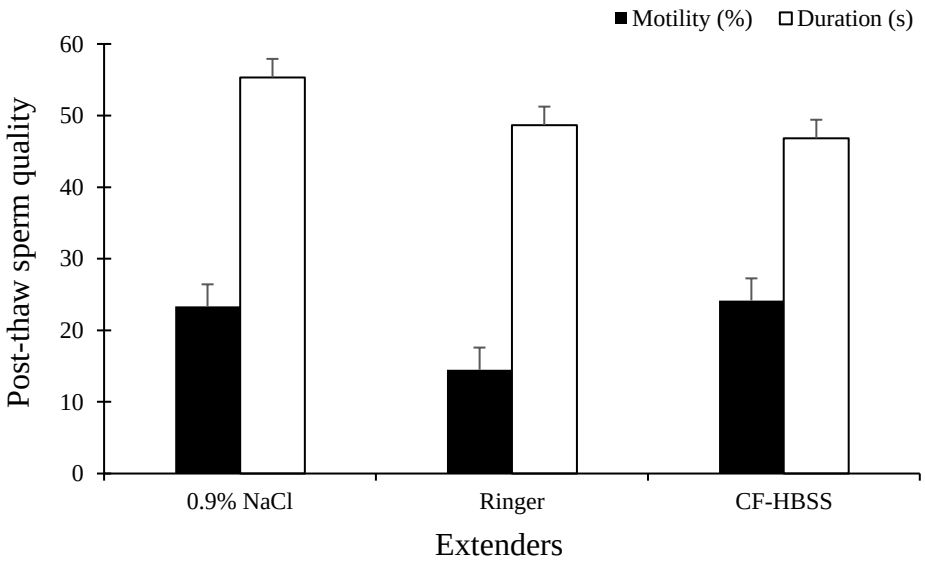


Figure 4.8 Post-thaw motility and duration of *Pangasius nasutus* sperm cooled at 14 cm height above the surface of liquid nitrogen for 7 minutes in 10% MeOH and various extenders. MeOH, methanol; 0.9% NaCl, 0.9% Sodium chloride; Ringer; CF-HBSS, Calcium free hanks' balance salt solution. All values are mean ± SE of sperm from

three replicates (n=3). There were no significant differences between values ($P > 0.05$).

4.2.3 Optimum cooling rate

The effects of cooling rates on post-thaw motility were investigated by adjusting the height of straw above LN vapor (10-16 cm), and the time exposed to LN vapor was set to 7 minutes before plunged into LN. Initial for sperm motility and duration were 46.7 ± 2.4% and 22.7 ± 0.9 s, respectively. The highest PTM result (17 ± 0.6%) was observed at cooling rate of 9.23°C min^{-1}, at 14 cm height (corresponding to immersion temperature -60°C) which was significantly higher ($P < 0.05$) than others (Figure 4.9). When cooling rates were below or above 9.23°C min^{-1}, PTM dropped drastically. The values for motility duration were not significantly different ($P > 0.05$) at various heights above the LN surface, except for cooling at 6.25°C min^{-1}, at 16 cm of height which showed no data.

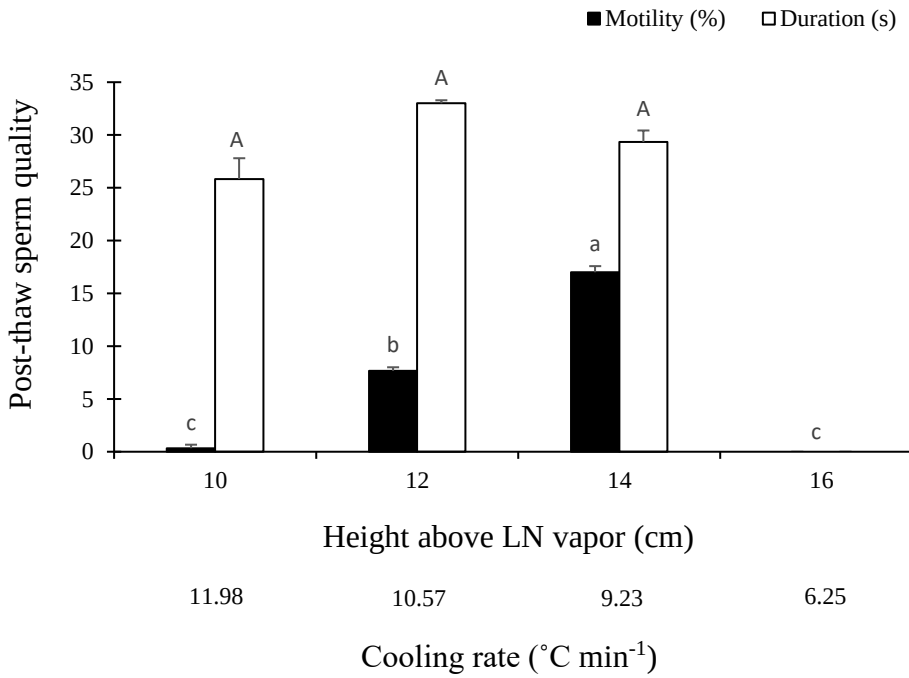


Figure 4.9 Post-thaw motility and duration of *Pangasius nasutus* sperm at different cooling rate and heights of straw above the surface of liquid nitrogen. All values are mean ± SE of sperm from three replicates (n=3). Values with different letters are significantly different ($P < 0.05$).

4.2.4 Optimum dilution ratio

The results of different dilutions ratios on the post-thaw motility and duration of *P. nasutus* sperm at 14 cm height of straw above the surface of liquid nitrogen for 7 minutes are seen in Figure 4.10. Initial values for sperm motility and duration were 45.7 ± 1.9% and 23.7 ± 1.5 s, respectively. There were no significant differences ($P > 0.05$) for post-thaw motility and duration at all dilution ratios (1:9, 1:19 and 1:49) investigated in this study.

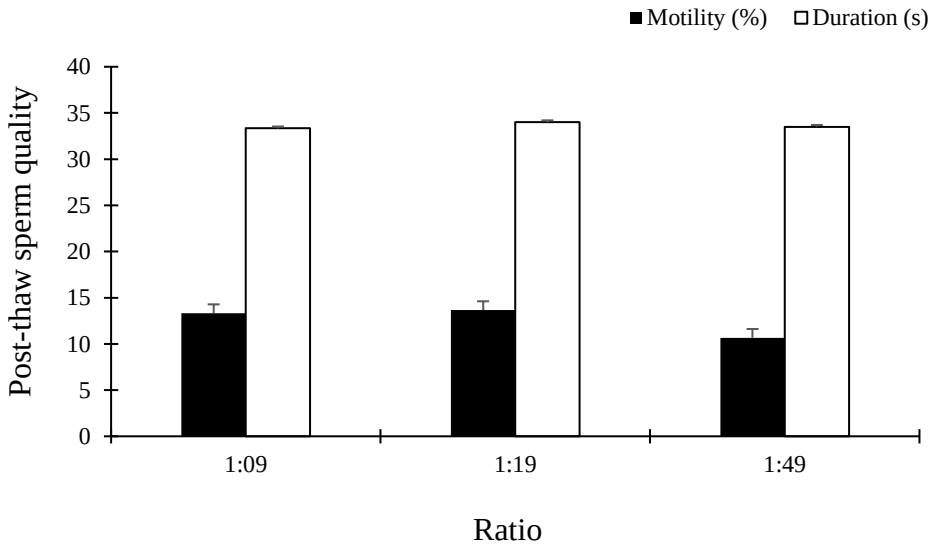


Figure 4.10 Post-thaw motility and duration of *Pangasius nasutus* sperm at 14 cm height of straw above the surface of liquid nitrogen for 7 minutes at different dilution ratios. Values are means ± SE of sperm from three replicates (n=3). There were no significant differences between values ($P < 0.05$).

4.3 Experiment 3: Investigating *Pangasius nasutus* cryopreserved sperm quality and its applicability in seed production.

4.3.1 Effect of long-term storage of cryopreserved sperm

The effect of cryopreservation period on sperm motility and duration of *P. nasutus* cryopreserved sperm is shown in Figure 4.11. Post-thaw motility of cryopreserved sperm within storage period was significantly lower ($P < 0.05$) compared to the initial motility of fresh sperm (51.67 ± 2.4%). However, there was no significant change of PTM ($P > 0.05$) during 12 months of storage (3 months, 33.3 ± 2.33%; 6 months, 32.0 ± 2.52%; 9 months, 32.67 ± 1.76%; 12 months, 33.33 ± 1.67%).

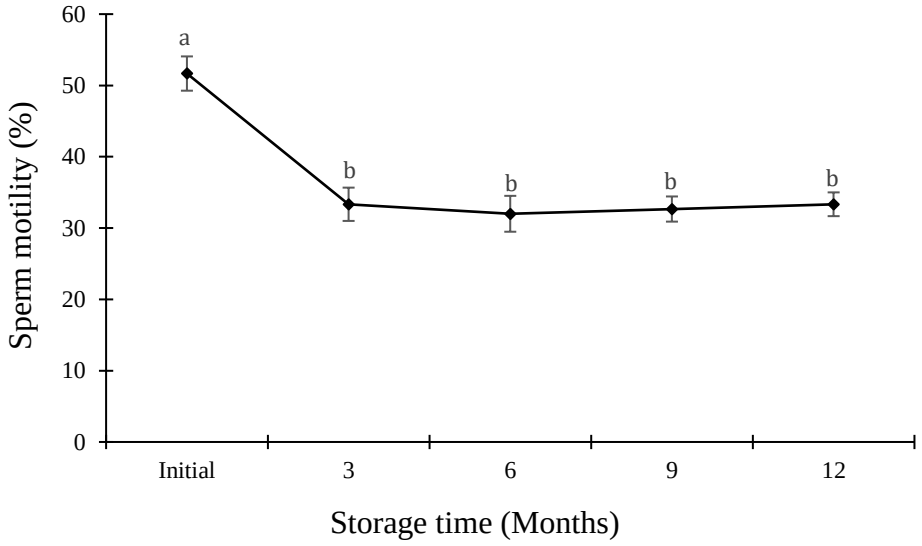


Figure 4.11 Sperm motility percentage of *Pangasius nasutus* cryopreserved sperm for 12 months. Initial, fresh sperm. Means with different letters are significantly different ($P < 0.05$).

In addition, the motility duration was unaffected by storage (Figure 4.12) as there was no significant difference ($P > 0.05$) to that fresh (Fresh, 31.67 ± 0.33 s; 3 months, 35.33 ± 1.76 s; 6 months, 38.33 ± 0.88 s; 9 months, 33.33 ± 0.88 s; 12 months, 38.33 ± 0.88 s).

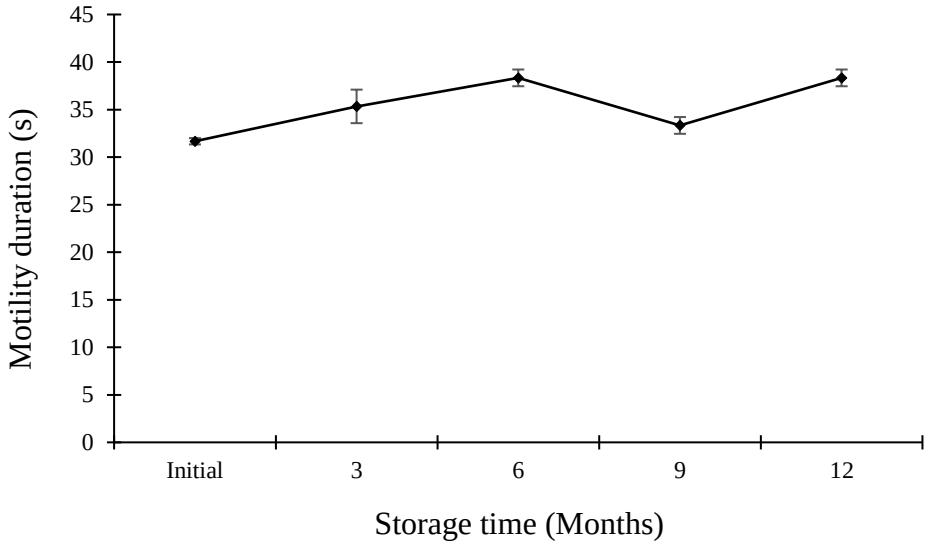


Figure 4.12 Motility duration of *Pangasius nasutus* sperm cryopreserved for 12 months. Initial, fresh sperm. There were no significant differences between values ($P < 0.05$).

4.3.2 Trial 1: Fertilization and hatching trials

There were no significant differences ($P > 0.05$) in motility, fertilization and deformity rates for fresh and 9-month cryopreserved sperm (Figure 4.13). Fertilization rates were all greater than 90% for both treatments. However, the hatching rate was significantly lower ($P < 0.05$) in cryopreserved sperm ($14.29 \pm 1.08\%$) compared to that fresh ($36.24 \pm 3.0\%$). The deformity rates of newly hatched larvae were less than 7% for both treatments. Hatched larvae of *P. nasutus* were categorized as deformed or abnormal if their spine at the abdominal or caudal area were curved and distorted. This was the only deformed feature observed in both treatments (Figure 4.14).

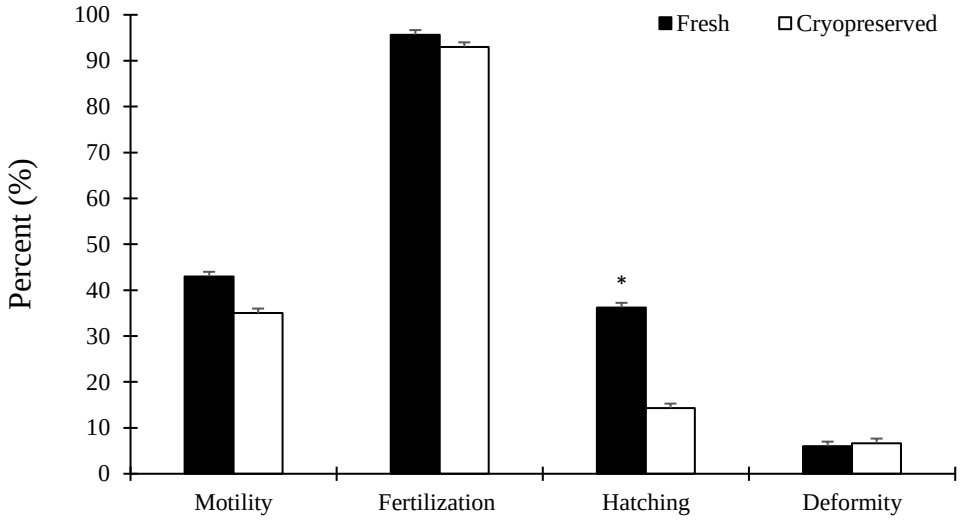


Figure 4.13 Comparison of motility, fertilization, hatching and deformity rates of fresh and cryopreserved sperm of *Pangasius nasutus*. Cryopreserved samples were diluted with 10% MeOH and 90% of 0.9% NaCl. Fresh, freshly obtained spermatozoa; MeOH, methanol; NaCl, sodium chloride. Values are means ± SE of sperm from three replicates (n=3). Means with * are significantly different ($P < 0.05$).

Figure 4.14 A) Normal hatched larvae, B) Abnormal C-shaped hatched larvae where the spine was curved and distorted, C) Hatched larvae with abnormal tail.

4.3.3 Trial 2: Fertilization and hatching trials at different sperm to egg ratios

There were no significant differences ($P > 0.05$) for fertilization rates in all sperm to egg ratios for fresh and 1-year cryopreserved sperm (Figure 4.14). Fertilization rates were all greater than 80% for all conditions. Cryopreserved sperm, C2 obtained higher hatching rates at a ratio 300,000:1 (9.24 ± 4.68%) compared to C1 at ratio 150,000:1 (3.44 ± 1.7%.), which was not significantly different to that fresh. The deformity rates of newly hatched larvae were less than 15% for all conditions. There was no significant difference ($P > 0.05$) in deformity rates for fresh and C2 at

sperm to egg ratio of 300,000:1. However, deformity rates at ratio C1 for cryopreserved sperm was significantly lower compared to fresh and C2. Hatched larvae of *P. nasutus* were categorized as deformed or abnormal if their spine at the abdominal or caudal area as well as tail were curved and distorted (Figure 4.15). This was the only deformed feature observed in all conditions.

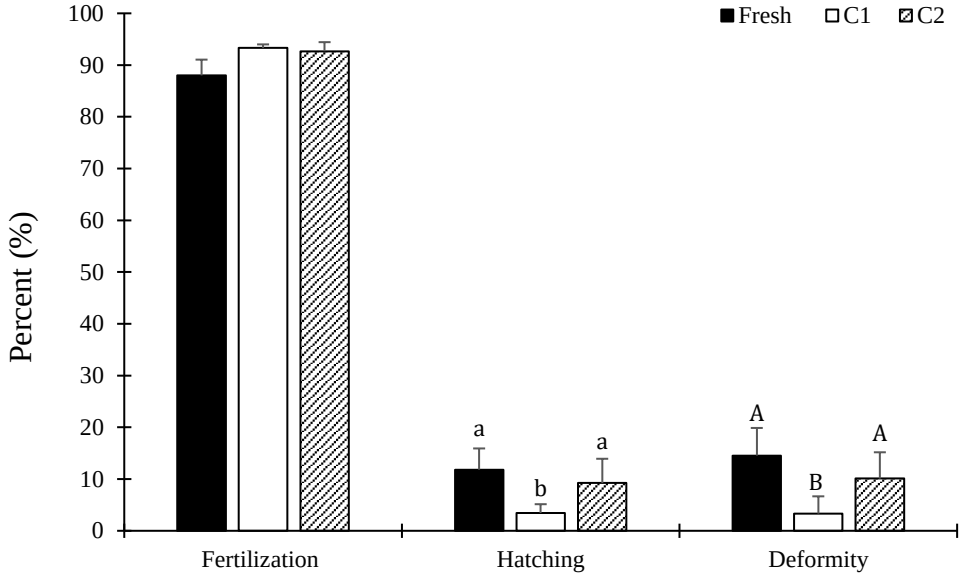


Figure 4.15 Comparison of fertilization, hatching and deformity rates of fresh and 1-year cryopreserved sperm of *Pangasius nasutus* at different sperm:egg ratios (mean ± SE, n=3). Fresh, freshly obtained spermatozoa at amount of sperm to 1 oocyte of $1.5x10^5$; C1, cryopreserved sperm at amount of sperm to 1 oocyte of $1.5x10^5$; C2, cryopreserved sperm at amount of sperm to 1 oocyte of $3.0x10^5$. Significant differences at different sperm to egg ratios are noted by different letters ($P < 0.05$).

CHAPTER 5

DISCUSSION

5.1 Optimum extender for short term preservation of *Pangasius nasutus* sperm

Based on our results, undiluted fresh sperm can only last for a short time period. At 2 hours of storage at 4°C, no motility was observed and agglutination also occurred when sperm was mixed with the activating solution. These issues were clearly influencing the quality of fresh sperm. Thus, the poor initial quality of sperm will make it difficult to perform the cryopreservation process as sperm quality is required to be maintained in a good condition. Short-term preservation, also known as cool storage, is an alternative method frequently used for gamete preservation that allows sperm to be stored at a low temperature for a short period (Contreras *et al*., 2020). It basically stores a thin layer of sperm in the absence of light, at room temperature between 0°C to 4°C in an oxygenated atmosphere (Shaliutina *et al*., 2013). While it is the most commonly utilized instrument since it is a practical, economical, and straightforward procedure to perform, it has its limitations as storage periods are much shorter compared to cryopreservation (Contreras *et al*., 2019; Shaliutina *et al*., 2013; Trigo *et al*., 2015). However, this makes it a good intermediatory before the cryopreservation process to help maintain sperm quality. Several experiments have been conducted to determine whether cool storage of fish sperm can be followed by cryopreservation process without impairing post-thaw sperm quality (Lahnsteiner *et al*., 1996; Tiersch *et al*., 2004; Viveiros *et al*., 2017; Yang *et al*., 2018; Yang *et al*., 2020). These findings demonstrated that cool storage is useful for practical procedures, particularly when there is a delay between semen collection and cryopreservation process.

Agglutination was observed in undiluted fresh sperm after 2 hours of storage. Agglutination is a phenomenon where individual sperm cells clump together. This agglutination might be caused by contamination during stripping or bacterial growth within storage periods. According to Contreras *et al*. (2020), the reduction of sperm motility is unavoidable due to factors such as cell metabolism, oxygen consumption and potential bacteria growth during storage periods. These problems can produce non-motile or dead sperm inside the sperm samples which can lead to the agglutination, in which dead sperm clump together. Moreover, sperm samples were collected using stripping procedures in this study, which had a higher risk of contamination with urine. Thus, to avoid agglutination and to lengthen the shelf life of sperm, the sperm must be mixed with appropriate diluent and stored at a low temperature (~4°C) (Viveiros *et al*., 2014). The present results clearly demonstrated that all tested diluents can avoid the formation of sperm agglutination up until 48 hours. As stated by Tiersch (2011), mixing sperm samples with diluent can counter any effects of urine contamination. Freshwater fish urine is hypotonic to the body's tissues and would activate the sperm by lowering the sample's osmotic pressure. Thus, diluting the samples with diluent can mitigate the effect of urine contamination by shifting the osmotic pressure to an isotonic range (Tiersch, 2011).

Cool storage with an appropriate diluent can aid to slow down metabolic processes and sustain cellular viability of sperm cells, ensuring that sperm remain alive and functional until ready for cryopreservation (Contreras *et al*., 2019). Therefore, the identification of cool storage with suitable diluent were required prior to the cryopreservation process. The addition of diluents is proposed to allow the sperm's survival and viability to be further extended (Trigo *et al*., 2015). There have been numerous studies on the use of diluents to enhance the storage period of various fish sperm species (Glenn *et al*., 2011). Our study also showed the same in which diluents helped to enhance the storage duration. In this particular study, this flexibility was useful in instances where immediate cryopreservation was not possible, and it allowed sperm samples to be transferred from sampling sites to a suitable location for the freezing process.

On the other hand, diluent efficacy can vary greatly between species due to differences in the biochemical composition of their seminal plasma (Beirão *et al*., 2019). Fish sperm is diluted in isotonic saline solutions or diluents that are similar in chemical composition to the physiological environment in which sperm are found in the testes, and they are also known as an 'extender' (Contreras *et al*., 2020). In this study, three types of extenders were prepared which were 0.9% NaCl, Calcium Free Hank's Balance Salt Solution (CF-HBSS) and ringer solution. The main role of extender is to produce an ideal environment for sperm storage by keeping them immotile in which protecting sperm motility and maintaining metabolism to retain viability and energetic resources for sperm activation (Wayman & Tiersch, 2011). However, it appears that *P. nasutus* sperm in diluted form performed better during cool storage. This could be explained by the fact that extenders can aid in the preservation of sperm quality by providing a sufficient aerobic environment and preventing agglutination (Contreras *et al*., 2020). Nevertheless, sperm quality reduction is unavoidable during refrigerated storage due to factors such as cell metabolism, oxygen consumption, and potential bacterial development (Contreras *et al*., 2020).

Regarding *P. nasutus*, the sperm samples stored in three different diluents demonstrated different degrees of motility throughout 120 hours of storage. In this study, all diluents (0.9% NaCl, CF-HBSS and ringer solution) could store sperm at 4°C for up to 24 hours. It has been found that there is a considerable loss in sperm quality during in vitro storage of fish sperm, affecting the motility (Trigo *et al*., 2015; Contreras *et al*., 2017). Decreased in sperm motility could be caused by oxidative stress which can lead to accelerate cellular ageing during cool storage (Contreras *et al*., 2020). Oxidative stress is a physiological state that develops when there is an imbalance between the ability to neutralize or repair the damage produced by reactive oxygen species (Storz & Imlayt, 1999). This oxidative event which occurs during the storage causes damage in mitochondria resulting in a decrease in sperm motility (Sanocka & Kurpisz, 2004).

Damage in mitochondria also might be associated with the trend of decreasing motility duration from 0 to 120 hours of storage trials for all diluents. Mitochondria

in the midpiece of the sperm cells generate Adenosine Triphosphate (ATP), the primary energy of cells (Park & Pang, 2021). ATP is essential for sustaining prolonged periods of motility which allows sperm to swim over long distances. This ATP provides the energy needed for the flagellum of the sperm to beat and propel the sperm forward (Chen *et al*., 2010). It is a critical element in sperm motility duration, and mitochondrial injury results in a drop in ATP levels resulting in lowered sperm motility and motility durations which was observed in our study. This was consistent with reports of ATP depletion over the duration of cool storage in basa catfish, *Pangasius bocourti* (Yang *et al*., 2020), Persian sturgeon, *Acipenser persicus* (Aramli *et al*., 2014), Baltic salmon, *Salmo salar L.* (Dziewulska *et al*., 2010), and Patagonian blennies, *Eleginops maclovinus* (Contreras *et al*., 2017), which resulted in decreased sperm motility.

In our study, 0.9% NaCl showed the highest motility between 0 until 24 hours which was not significantly different compared to initial. This diluent is a simple ionic compound composed of sodium (Na+) ions and chloride (Cl-) ions. These ions are important for the tonicity of the solution (Argawal, 2011). The tonicity of the solution is related to its effect on the volume of the cell in which solutions that do not change the volume of cells are said to be isotonic (Koeppen, 2013). Therefore, when cells are diluted with 0.9% NaCl, it maintains their normal volume and provides isotonic conditions to sperm cells. A solute must not permeate a membrane in order to apply an osmotic pressure across it. Because the sperm cell membrane is impermeable to sodium (Na+) ions and chloride (Cl-) ions, it imposes an osmotic pressure equal to and opposite to that produced by the sperm cell contents. Moreover, use of 0.9% NaCl is recommended as a diluent for refrigerated storage because it is easy to prepare and compatible with physiochemical composition of the seminal plasma of most *Pangasius* species.

In the present study, 0.9% NaCl was found to prevent agglutination in samples up to 96 hours of cold storage. Unfortunately, sperm samples in ringer and CFHBSS exhibited obvious clumps of agglutination after 48 hours of storage. According to Bernath *et al*. (2016), the component of glucose in the ringer and CFHBSS is

responsible for causing sperm agglutination to occur more quickly compared to 0.9% NaCl. However, the presence of this component is important to provides the energy source for sperm. This observation gives direct proof that a suitable diluent is required to avoid agglutination during the storage period. Results presented in this experiment suggested that sperm samples should be immediately diluted with 0.9% NaCl after sperm collection and stored at 4°C to retain sperm quality.

Sperm motility decreased more rapidly in Ringer and CF-HBSS compared to 0.9% NaCl. These diluents are more complex than 0.9% NaCl since they contain several numbers of ions in different amounts (mM). Sodium (Na+), Chloride (Cl), Potassium chloride (KCI), Sodium bicarbonate ($NaHCO_3$) and glucose are included in both diluents. Na+ maintains the osmotic pressure and CI regulate electrolyte balance of the cell's fluid (Perez *et al*., 2022). Potassium (K+) is reported to have the same function as Cl (Perez *et al*., 2022). Meanwhile, $NaHCO_3$ control the pH of the diluent (Tanaka et al., 2002) and glucose provides the energy source for sperm cells (Perez *et al*., 2022). Calcium (Ca^{2+}) in Ringer solution maintain cell membrane permeability while Magnesium (Mg^{2+}) in CF-HBSS protects the cells from aging (Alavi & Cosson, 2006).

The presence of these ions is thought to cause nonmotile sperm and prolong the functional life of the sperm (Muchlisin e*t al*., 2004). However, Morisawa *et al*. (1983) and Muchlisin *et al*. (2004) found that increasing the concentration of Ca^{2+} ions and decreasing the concentration of K+ ions in the diluent resulted in sperm activation, which resulted in decreased motility. Thus, we hypothesize that the Ca^{2+} and K+ ions in ringer solution, as well as K+ in CF-HBSS, activated the sperm cells, resulting in reduced motility during the storage period. Since there were dead sperm inside the samples, agglutination also occurred quickly at 48 hours of storage for both diluents. However, in our investigation, ringer solution showed the worst results with motility significantly decreasing as early at 6 hours. This can be explained by the lower concentration of K+ ions in ringer (2.7 mM) compared to CF-HBSS (6 mM), as well as the presence of Ca^{2+} which caused the initiation of sperm motility to occur more quickly in ringer.

5.2 Effect of cryoprotectant on *Pangasius nasutus* sperm

The most common parameters used to assess the quality of the sperm after cryopreservation is motility, which typically has a positive correlation with fertility (Cabrita *et al*., 2010; Kime *et al*., 2001). In the present study, motility or post-thaw motility of sperm had been used as the main parameter to evaluate quality of sperm. However, there is no information about sperm motility and its characteristics for *P. nasutus*. In this study, the initial motility of fresh sperm for *P. nasutus* was lower than 60% which was 51.67 ± 2.4% and sperm became rapidly immotile within one hour after stripping. For this study, milt was obtained male broodstocks belonging to a private fish farm. As milt was obtained during the commercial production phase of the farm, hormonal induction was not considered to increase male *P. nasutus* sperm quality following the protocols of the farm. However, the sperm quality of *P. nasutus* sperm obtained was similar to that our previous trials in which we obtained milt from wild caught fish (unpublished data).

This was the first study to report on the cryopreservation of *P. nasutus* sperm. For a new species, standardization of each step of procedure or the protocol is needed and involves the identification of numerous factors including initial sperm motility, cryoprotectants, dilution ratios and cooling rates (Suquet *et al*., 2000). The selection of an appropriate cryoprotectant at optimum concentration is very important for developing sperm cryopreservation protocol (Yang *et al*., 2020). In the present study, we evaluated three different cryoprotectants; dimethyl sulfoxide (Me_2SO), dimethylacetamide (DMA) and methanol (MeOH) which had previously been used to cryopreserve fish sperm at different concentrations, varying from 5 to 15%.

The most successful cryoprotectant with the highest post-thaw motility of 26.3 ± 0.9% was 10% MeOH with 0.9% NaCl when cooled at height of 14 cm from LN surface for 7 minutes. MeOH has been successfully used to cryopreserved sperm for many freshwater fish such as catfish (Kovács *et al*., 2010; Viveiros *et al*., 2000), common carp (Horváth *et al*., 2003), salmonids (Lahnsteiner *et al*., 1997; Horváth *et*

al., 2015) and eels (Koh *et al*., 2017; Müller *et al*., 2004; Szabó *et al*., 2005). This chemical is known as an intracellular cryoprotectant with a low molecular weight that diffuses into the cells more quickly than other cryoprotectants (Kainin *et al*., 2014). When applied as a cryoprotectant, MeOH enhances cell survival throughout the cryopreservation process by penetrating quickly into the sperm and enables sperm to achieve osmotic equilibrium readily under freezing (Chong *et al*., 2016) without suffering any osmotic shock-related toxicity (Szabó *et al*., 2005). Moreover, corresponding to our toxicity results, motility of sperm suspended in 10% MeOH was not affected up to 50 minutes, indicating that this cryoprotectant has a good permeability and does not cause osmotic stress in *P. nasutus* sperm. This may be explained by the fact that MeOH penetrates into the sperm cells at a rate comparable to the rate of water transport, therefore preventing osmotic stress. (Zhang *et al*., 2005).

Our results also revealed that there were differences with motility duration and VCL when cryopreserved using the different concentration of cryoprotectants. 10% MeOH resulted in highest motility duration (35.2 ± 1.0 s) and VCL (58.04 ± 2.45 μm/s) compared to other conditions. Motility duration and velocity are closely related to the energy production and propulsion mechanisms within the sperm cell (Inaba & Shiba, 2018). This is primarily influenced by the presence of ATP (adenosine triphosphate), the role of mitochondria, and the structure and function of the flagella. Mitochondria produces ATP, which serves as the energy source for the flagellum's movement (Guthrie & Welch, 2012). The efficient production of ATP in the mitochondria is crucial for maintaining the energy levels required for the sperm's rapid and sustained movement (Piomboni *et al*., 2011). Hence, this indicates that 10% MeOH can avoid depletion of ATP levels or damage to mitochondria in sperm cells during the freezing and thawing process.

Consequently, it is important to maintain the motility, duration and velocity of the sperm after the freezing process in order to successfully reach the oocytes and fertilize the eggs. There was a strong positive correlation between PTM and motility duration as well as PTM and VCL for *P. nasutus*. This suggests that good quality sperm does not only have high PTM but also high motility duration and fast velocity,

whereas poor quality sperm is characterised by having low scores in all 3 categories. As a result, utilizing cryopreserved sperm with the highest PTM while additionally exhibiting a high motility duration and VCL will increase the probability of fertilization success.

Based on previous studies of sperm cryopreservation in *Pangasius* species, Me_2SO is the best cryoprotectant where 10% Me_2SO is the best for *P. bocourti* sperm cells (Kainin *et al*., 2014) as well as for *P. larnaudii* (Kwantong & Barb, 2006), 9% Me_2SO for *P. gigas* (Mongkopunya *et al*., 1995) and 12% Me_2SO for *P. hypophthalmus* (Kwantong & Barb., 2003). Me_2SO is a permeating cryoprotectant (Denniston *et al*., 2011) that is widely used as a cryoprotectant for a variety of aquatic species (Suquet *et al*., 2000; Yang *et al*., 2022). This substance is distinguished by its tiny molecular size, rapid penetration ability, and interaction with plasma membrane phospholipids. (Suquet *et al*., 2000). However, Me_2SO has been reported to be toxic and has higher osmolality compared to MeOH (Marco-Jiménez *et al*., 2006). From our toxicity result, a significant reduction in sperm motility was observed in 10% and 15% concentrations of Me_2SO compared to MeOH. Moreover, motility duration and velocity of post-thaw sperm for all concentrations of Me_2SO was significantly lower than 10% MeOH. This suggest that Me_2SO may be unsuitable as cryoprotectant for *P. nasutus* sperm.

DMA was less successful in providing protection than MeOH and Me_2SO for *P. nasutus* sperm cells. DMA is also a permeating cryoprotectant which is similar to Me_2SO and MeOH. According to Argawal (2011), DMA is toxic to sperm when used in high concentration. Similar results were obtained in others *Pangasius* species in which DMA was the second highest for black ear catfish, *P. laurnadii* and striped catfish, *P. hypophthalmus* in terms of sperm motility (Kwantong & Bart, 2003; 2006), as well as Basa catfish, *P. bocourti* where this cryoprotectant resulted in lower motility, viability and fertilization (Kainin *et al*. 2014). However, DMA as a cryoprotectant has been successfully used for other catfish species such as African catfish, *Clarias gariepinus* (Urbanyi *et al*., 2000) and European catfish, *Silurus glanis* (Ogier de Baulny *et al*, 1999). According to Yang *et al*., (2022), it is important to note that the

efficacy of cryoprotectants varies greatly per species due to differences in cryoprotective capacity and sperm toxicity. Based on our results, DMA was not a suitable cryoprotectant as motility at all tested concentrations was poor; it was not able to protects the *P. nasutus* sperm cells from cryoinjuries during freezing process.

5.3 Optimum extender

The selection of an appropriate extender is critical for the successful cryopreservation of fish sperm. Cryoprotectants are hazardous to sperm cells at high concentrations, thus they are usually diluted with extenders (Koh *et al*., 2010). In the present study, three types of extenders or diluents were investigated. All extenders (0.9% NaCl, CF-HBSS and freshwater ringer) used in the study produced good results which did not produce significantly different motility values. Of all the solutions tested, 0.9% NaCl is potentially the best for farm or commercial use as it is the cheapest and easiest to prepare as the mixing of only one ingredient is required. Previous studies in sperm cryopreservation of other *Pangasius* species also reported good results when using 0.9% NaCl as an extender (Kwantong & Bart 2003; 2006; 2009; Mongkopunya *et al*., 1995). This solution seems compatible with the physiochemical composition of the seminal plasma which is necessary in order to maintain the sperm in a non-motile but viable state until it is used (Mongkonpunya *et al*., 2011). Therefore, our results suggest that 0.9% NaCl is the most suitable extender for *P. nasutus*.

In our study, Ringer and CF-HBSS also showed similar to 0.9% NaCl. Ringer have been successfully used in others catfish species such as bagrid catfish, *Mystus nemurus* (Muchlisin, 2004) and yellow catfish, *Pelteobagrus fulvidraco* (Pan *et al*., 2008). Meanwhile CF-HBSS have been reported to be an effective extender for albino pangasius catfish (Hasanah *et al*., 2020) and Mekong giant catfish, *Pangasius gigas* (Mongkonpunya *et al*., 2000). However, one of weakness of these extender is required many components to prepare, including NaCl, KCl, $CaCl_2$, $NaHCO_3$, glucose, $Na_2HPO_4.7H_2O$ and others. It is more expensive and complicated to prepare than 0.9% NaCl, particularly if the farm is located in a rural area. Nevertheless, both extenders

are suitable substitute to 0.9% NaCl as an extender for *P. nasutus* species.

5.4 Optimum cooling rate

Cooling rate is an important factor affecting the post-thaw motility of sperm, as the optimal cooling rates vary with different species. The optimum cooling rate in our study was 9.23 °C min^{-1}, at 14 cm height above LN vapor (corresponding to final temperature -60°C; calculated from 0 to -60°C) which was similar to the optimal cooling rates in other *Pangasius* sp. where samples were cryopreserved using a computer-controlled freezer at cooling rates of 10 °C min^{-1} (Mongkopunya *et al*., 1995; Kwantong & Barb, 2003; 2006; 2009; Kainin *et al*., 2014). This indicated that slower cooling rates were more suitable for freezing of *Pangasius* sp. sperm and *P. nasutus* shared the same traits.

Additionally, it should be noted that the type of cryoprotectant and effective cooling rates are connected (Denniston *et al*., 2011). MeOH is believed to be more effective at slower cooling rates as a cryoprotectant because of the reduced slow cooling damage especially that of intracellular ice (Dennniston *et al*., 2011). However, dehydration and long-term exposure to high solute concentration might kill cells if the cooling rate is too slow (Hagedorn *et al*., 2004). This was also noted in our study as freezing at a slower cooling rate of 6.25 °C min^{-1} (at height of 16 cm above LN vapor) resulted in total loss of motility in post thaw sperm. Meanwhile, cooling rates of 11.98 °C min^{-1} and 10.57 °C min^{-1} are too fast for *P. nasutus* sperm cells and would result in cell damage by the formation of intracellular ice crystals (Tiersch, 2011). Fast cooling rates do not allow sufficient egress of intracellular water to maintain equilibrium and the supercooled water in the cells undergoes intracellular ice formation which causes damage to the cell by disruption of cellular structure and function (Tiersch, 2011). Moreover, the formation of intracellular ice is followed by recrystallization during thawing if the thawing rate is slow, which is also harmful to cells (Mazur *et al*., 1972). According to Gao & Critser (2000), it is recommended that the thawing rate should be fast enough to prevent intracellular ice from recrystallizing. Nevertheless, in our

study, fast thawing rates were used and thawing rates probably did not affect the results.

The present results clearly indicate that *P. nasutus* sperm has a narrow range of optimal cooling rate. Some freshwater fish species have a narrow range or lower freezing tolerance for sperm cryopreservation. Similar results were observed in stripped catfish, *P, hypophthalmus* (Kwantong & Bart, 2003) and major carp, *Lebeo calbasu* (Nahiduzzaman *et al*., 2012), where the cooling rate was only optimal at 10°C min^{-1}. If the rate is somewhat lower or higher than the optimal value, the quality of PTM suffers significantly. Meanwhile, many marine species exhibit optimal cooling rates that have a wide range. Examples are, in brown-marbled grouper, *Epinephelus fuscoguttatus*, optimal cooling rates of 64.9-5.7°C min^{-1} (Yusoff *et al*., 2018) and 33.2-141.2°C min^{-1} (Yang *et al*., 2020); in silver barb, *Barbodes gonionotus* (Vithiphandchai *et al*., 2015) cooling rates of 5-8°C min^{-1} were optimal; in seven-band grouper, *Epinephelus septemfasciatus* (Koh *et al*., 2010) optimal cooling rates were 27.1-94.3°C min^{-1} and striped bass, *Morone saxatilis* (Thirumala *et al*., 2006) reported cooling rates of 14-20°C min^{-1}. The PTM showed no significant differences over a wide range of cooling rates, indicating a wide range of suitable cooling rate as well as high freezing tolerance for these species.

The cooling protocol in our study involved the manipulation height of the straws from the LN surface using the vapor to cryopreserve sperm samples. This method of adjusting the height and floating of the styrofoam frame on the surface of LN has previously been used successfully for sperm cryopreservation of *P. bocourti* (Yang *et al*., 2023), *C. gariepinus* (Kovác *et al*., 2010; Miskolczi *et al*., 2005), *I. furcatus* (Bart *et al*., 1998), *P. fulvidraco* (Pan *et al*., 2008), *E. septemfasciatus* (Koh *et al*.,2010), *E. fuscoguttatus* (Yusoff *et al*., 2018) and *Anguilla japonica* (Koh *et al*., 2017). While usage of the program freezers allows for specific and adjustable cooling rates which the equipment is costly and requires an investment that breeders and farmers are reluctant to commit. The cooling protocol used here is therefore an acceptable alternative to using the program freezer, as it is a convenient and reliable method for field operations or in rural areas where there is no advanced or costly

equipment.

5.5 Optimum dilution ratio

In the current study, the application of dilution ratio 1:9 to 1:49 did not show any significant differences among them. Therefore, any ratio between these ranges can be used because the cryodiluent is enough to protect and cover the amount of cells during the cryopreservation process. It has been proven that the concentration of protective chemicals (e.g., protein, glucose, and ions) that are naturally present in seminal fluid (Marco-Jiménez *et al*., 2006) is sufficient to protect the sperm membrane at higher dilution ratios. From an application standpoint, due to the greater volume of spermatozoa in cryodiluent, ratios of 1:9 to 1:19 are often adequate for the breeding programs. Meanwhile, a minimal amount of sperm is required for research activities, and dilution ratios of 1:19 to 1:49 can be utilized for a variety of experimental setups. The small amount of milt collected which was around 2.5 ml from male *P. nasutus* makes it difficult to compare at minor dilution ratios, hence the dilution ratio of 1:1 to 1:3 was not tested in this study.

5.6 Effect of long-term storage on motility percent and duration

In the present study, we evaluated the effect of long storage periods inside liquid nitrogen up to 1 year. Post-thaw motility (PTM) and duration were unaffected by up to 1 year of storage, but the PTM was significantly lower compared to fresh sperm. Similar result was reported in sea bream, *Sparus Aurata L.* where the motility of sperm before freezing was significantly higher than 1-months and 5 years of cryopreserved sperm (Fabbrocini *et al*., 2014). Lahnsteiner *et al*., (2024) also reported the motility rate of cryopreserved sperm was significantly lower than that of fresh sperm for brown trout, *Salmo trutta*; charr, *Salvelinus umbla* and rainbow trout, *Oncorhynchus mykiss*. As stated by Chew & Zulkafli (2012), generally, sperm motility will decrease after the thawing and freezing process compared to the motility

of fresh sperm.

However, the negative impact of sperm cryopreservation on *P. nasutus* appears to be minimal, as our results revealed no alterations in sperm motility and duration throughout 12 months, which would allow better flexibility in timing of artificial fertilization. These results demonstrated that ATP levels of the cells remained stable throughout the storage period. ATP is a key element in sperm motility and motility duration, and mitochondrial injury results in an unavoidable reduction in ATP levels (Chen *et al*., 2010). The consistent results of motility and duration of cryopreserved sperm showed that biological activity of sperm ceased at -196°C during storage periods. Similar results were obtained in others studies in which long-term storage of cryopreserved sperm did not cause a significant decrease in PTM. Giant grouper, *Epinephelus lanceolatus*, (Park *et al*., 2022) can maintain good quality of PTM for 4 years of storage, while brown-marbled grouper, *Epinephelus fuscoguttatus* (Chen *et al*., 2023) maintained high PTM throughout 3 years of storage. Moreover, in sea bream, *Sparus aurata L.* (Fabrrocini *et al*., 2015) storage of 1 months to 5 years did not affect the parameters of sperm on activation, as well as in silver barb, *Barbodes gonionotus* (Vuthiphandchai *et al*., 2014) where PTM was not affected during 9 months of storage.

As long-term cryo-storage data for *P. nasutus* become accessible, it provides a number of benefits. This finding is valuable for breeding programs, as it ensures a continuous supply of cryopreserved sperm for up to 12-months to generate aquaculture production. It is useful during off-season spawning where only females can be induced and cryopreserved sperm can be used to fertilize the eggs. This is critical and beneficial for this species as it has specific breeding seasons and allows the use of stored sperm when needed regardless of natural reproductive cycles. Moreover, this technique improves selective breeding programs by allowing certain individuals with desirable features to be cryopreserved and used for controlled breeding later (Cabrita *et al*., 2010). Fish sperm cryopreservation also allows for efficient utilization of sperm in which the total volume of available sperm from selected individuals can be stored for a certain period and used to achieve the breeding goals (Suquet, 2000).

Nevertheless, successful implementation requires resolving the uncertainty and risk aspects. The cost of cryopreservation equipment such as cryotanks and liquid nitrogen supplies can be quite expensive. However, these costs can be offset by increased productivity and reduced need for frequent breeding. Training is also required to handle cryopreservation techniques, as mistakes in handling freezing and thawing procedures might affect the quality of sperm.

5.7 Viability of long-term cryopreservation protocol for *Pangasius nasutus* for seed production

Sperm cryopreservation causes unavoidable cell damage as a result of cryoinjuries during the freezing and thawing processes, resulting in a reduction in sperm quality and fertility potential (Figueroa *et al*., 2016; Yang *et al*., 2023). The most important purpose of sperm cryopreservation is to preserve the ability of sperm cells to fertilize the eggs, hence the changes in motility percent that result from cryopreservation should be taken into account alongside the sperm's ability to fertilize. In this study, there were no significant differences in motility and fertilization rates for fresh and 9 months cryopreserved sperm. Motility typically has a positive correlation with fertility. Thus, the fertilization rate of our cryopreserved sperm was not significantly different to that fresh. We found that cryopreserved sperm at the sperm to egg ratio of ~100,000 to 1 had the same fertilization capacity as fresh sperm (same amount of sperm to egg ratio) where fertilization rates for both treatments were all greater than 90%. Our results indicated that cryopreserved sperm using our developed protocol had an adequate amount of motile sperm to fertilize the eggs as the same as fresh sperm.

However, the hatching rate was significantly lower in cryopreserved sperm (14.29±1.08%) compared to fresh sperm (36.24±3.0%). Injuries might occur to the chromatin or plasma membranes of sperm cells during freezing or thawing process, affecting embryo development and causing low egg hatchability. It has been suggested that sperm chromatin has a greater impact on early embryonic development than it

does on fertilization (Speyer *et al*., 2010). Sperm chromatin contains the genetic information that combines with the egg's genetic material during fertilization, which then undergoes cell divisions to develop into embryo (Middelkamp *et al*., 2020). If the damaged sperm chromatin does successfully fertilize the egg, it can introduce genetic mutations or abnormalities into the developing embryo (D'Occhio *et al*., 2007). These mutations can result in genetic disorders or developmental abnormalities. Moreover, damage to sperm chromatin can disrupt the normal progression of cell divisions in the early embryo (Mohammad *et al*., 2005). Thus, this can lead to embryo arrest, where the development of the embryo is halted, preventing it from reaching the blastocyst and preventing the successful hatching of the eggs.

Studies on *Pangasius* species mostly demonstrated the outcome of fertilization rate without hatching rate in order to determine the ideal condition for sperm cryopreservation and its applicability of the developed protocol (Kainin *et al*., 2014; Kwantong & Barb, 2003; 2006 Mongkopunya *et al*., 1995). Moreover, the latest study on cryopreserved sperm of *Pangasius* species, which is *P. bocourti* (Yang *et al*, 2023) presented only the fertilization result by using sperm to egg ratio of 200,000:1 to indicate that their developed protocol can be used in artificial insemination. At present, the impact of sperm cryopreservation on production of *Pangasius* species remains unknown as many studies do not proceed the evaluations until hatching. It is critical to prove that the eggs can developed until hatching when fertilized with cryopreserved sperm, so that farmers can be certain that cryopreserved sperm can be used for fertility. Therefore, in addition to fertilization rate, evaluations such as hatching and deformity rate were performed in this study to determine the applicability of cryopreserved sperm. According to Rurangwa *et al*. (2001), unfertilized eggs can reach the 12 h stage before dying, hence the hatching rate at 24 h onwards is crucial for expressing the fertilizing potential of sperm. The appearance of malformed larvae is also important for determining the developmental potential of fertilized eggs.

There was no significant difference in the deformity rates between both treatments indicating that cryopreserved sperm of *P. nasutus* has an equal ability to produce normal hatched larvae as fresh sperm. The deformity rates of newly hatched

larvae in this study were less than 7% for both treatments. In contrast, percent of deformed larvae for African catfish was more than 30% in cryopreserved sperm while fresh sperm was 28.3% (Miskolczi *et al*., 2005) with no significant differences between both treatments. Meanwhile in *P. hypophthalmus*, the lowest percent abnormality in cryopreserved sperm was 8.25% (Fanni *et al*., 2018) and 5.25% in rainbow trout (Young *et al*., 2009) with no significant differences with fresh sperm. Based on these findings, it appears that our developed protocol produces the minimal percent of deformities.

Although the hatching rate was lower, we successfully developed the protocol and produced normal larvae by using our approach. According to Chew & Zulkafli (2012), cryopreservation process for a species is regarded successful if the preserved sperm successfully fertilizes eggs and produces progeny. In fact, cryopreservation damage was present during the hatching stage, however hatching eggs produced mainly normal larvae. In this study, there were several phases involved in causing damage due to freezing. First, disruption to sperm motility resulted in an unfertilized egg. Second, sperm cell damage prevented eggs from hatching effectively, and third, eggs matured until they hatched but produced deformed larvae. Nevertheless, result presented in this study demonstrated that our cryopreserved sperm were viable to produce normal larvae but was not yet optimized for hatchery seed production due to lowered hatching rates.

Thus, the second fertilization trial aimed to optimize the sperm to egg ratio to enhance the hatching rates by increasing sperm to egg ratio for both fresh (150,000:1) and cryopreserved sperm (300,000:1). The results showed that at higher sperm to egg ratios, hatching rates were similar to that of fresh sperm. It is generally known that a greater number of cryopreserved sperm is usually necessary to compensate and obtain the same results as fresh sperm (Gwo, 2011). Thus, the increase in sperm concentration for cryopreserved sperm increased the amount of viable sperm, which have a normal function for eggs to develop until hatching. According to Kwantong & Barb (2009), over 3 times more cryopreserved sperm of *P. hypophthalmus* was required to fertilize the same number of eggs as fresh sperm. Similar results were

reported in Atlantic salmon, *Salmo salar L*. in which 5 times more cryopreserved sperm was needed to reach an equivalent reproductive success compared to fresh sperm (Erraud *et al*., 2022).

Additionally, hatching rates in our study were below 15% for both fresh and cryopreserved sperm which indicated the eggs were not of good quality. According to Hassan *et al*. (2011), the hatching rate for *P. nasutus* was 68.26%. However, in our study, the hatching rate for fresh sperm was much lower than that. The percentage of hatching and survival appears to be determined by egg quality rather than sperm (Kwantong & Bart, 2009; Subagja *et al* 1999). This could explain why our hatching rates were below 12% for both fresh and cryopreserved sperm with no significant differences between them. The factor of egg quality is a major hurdle in conducting a fertilization and hatching trial. Egg quality is dependant on many factors, such as female broodstock condition, age, size, maturity and etc (Rurangwa *et al*., 2001). In our study, egg was obtained by a female cultured at the commercial farm and all broodstock related management, feeding, culture systems and conditions and handling was conducted by the farm staff and workers. We were therefore unable to directly influence and improve the female broodstock condition and subsequently the egg quality.

Moreover, seed production at the farm generally did not focus on increasing individual female and male broodstock quality and focused on cost effective measures as evident by the lack of hormonal injection for males resulting in relatively low sperm motility (~40%). Instead, the farm emphasized on large volume production using increased numbers of fish to achieve the seed production targets. For sperm and eggs collection, we were hampered by the need to follow the commercial breeding protocols and production schedules of the farmer involved as sperm and eggs samples were only obtained during the commercial production phase. For sperm collection, we were able to select 3 males that have sufficient initial sperm quality (motility of >40%) and quantity (volume required for the experiments). However, we were unable to choose high-quality eggs because mostly females produced the same quality of eggs and we also received the eggs directly from the farmer.

An interesting phenomenon was found in our results in which the fertilization rate among sperm to egg ratios (~150,000 and 300,000:1) of cryopreserved sperm showed no differences but the hatching rate was significantly lower at ratio 150,000:1. We hypothesize that this is related to the velocity according to the actual path (VCL) of cryopreserved sperm. While we were unable to evaluate the VCL of the sperm during the second fertilization trials, VCL results of 10% MeOH from Experiment 2, showed that there were 2 distinct sperm groups according to velocity among the motile sperm. This suggests that when increased sperm to egg ratios were used, the amount of high velocity cryopreserved sperm also increased. This increase in sperm concentration of high velocity sperm increases the chances in which the less damaged sperm fertilized the eggs, unlike at lower concentrations where low velocity sperm had a higher chance to fertilize the eggs. As higher sperm VCL denotes better sperm quality, we hypothesize that this group consists of sperm with less cryoinjuries and damage compared to the low VCL group. This might have led to the higher hatching rates in high VCL group, thus presenting in our study the higher hatching rates in higher sperm to egg ratio. This positive corelation between VCL of sperm and hatching rate was also observed by Rurangwa *et al*. (2001) in African catfish, *Clarias gariepinus*.

While sperm with a lower velocity can fertilize the eggs, the sperm cells might have already been damaged in the form of their structure, which was plasma membrane, mitochondria or chromatin, which can result in embryos not developing as previously mentioned. Moreover, motility and velocity of sperm cells are affected by several factors, including mitochondrial state, ATP generation, flagellar structure, and plasma membrane (Cabrita *et al*., 2010). ATP produces energy to the sperm cells and sperm require energy to power their movement (Misro & Ramya, 2012). High ATP levels are associated with better sperm motility and fast velocity. Sperm with adequate ATP reserves are more likely to exhibit vigorous and sustained motility, contributing to their ability to reach and fertilize the egg. Therefore, as sperm concentration increases, the fast velocity of cryopreserved sperm also increases and competes to reach the egg's micropyle. In fact, frozen-thawed sperm contain both immotile and

motile sperm cells (Rurangwa *et al*., 2001). Thus, the minimum concentration of good-quality cryopreserved sperm in terms of motility and velocity are required for fertilization to guarantees that these sperm have the best chance of reaching the micropyle and developing the eggs. Sperm with a high velocity are identified as faster sperm in which they reach the micropyle of eggs first, limiting the entry of slower sperm, thereby contributing to reproductive success (Rudolfsen *et al*., 2008).

According to the findings, it appears reasonable to utilize a sperm to egg ratio of 150,000:1 for fresh sperm. However, in order to achieve the same reproductive success as fresh sperm, cryopreserved sperm would have to be two times more concentrated. Therefore, for *P. nasutus* with a concentration of 2.09×10^{10}, for fertilization of up to 100,000 (~100g) it is necessary to cryopreserved 1.4 ml (2.09×10^{7}) of fresh milt. When it is considered that *P. nasutus* female have a high fecundity of 140,000 eggs per kg of body weight, cryopreservation of 1.96 mL of fresh sperm is required to adequately fertilize the total amount of eggs from a female. This volume is easily obtained, as we were able to obtain at least 2.5 mL from males of *P. nasutus* in our study. The refining of the sperm-to-egg ratio is useful for artificial insemination and propagation in *P. nasutus* as it helps avoid the wasting of cryopreserved sperm in aquaculture production. The optimal sperm to egg ratio also can contribute to successful embryonic development.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

To summarise, a practical and effective sperm cryopreservation protocol for *P. nasutus* sperm was developed. Based on our results, we can conclude that sperm samples diluted with 0.9% sodium chloride (NaCl) showed the highest percentage of motility and duration throughout 24 hours of storage trials. Following this outcome, the basic sperm cryopreservation protocol for *P. nasutus* was established by identifying the optimal conditions for cryoprotectant, extenders, freezing condition and dilution ratio. 10% methanol with 0.9% NaCl as an extender is a suitable cryodiluent and cryopreservation process should be conducted within 10 minutes after dilution with cryodiluent. Cooling rates at 9.23 °C min^{-1} obtained by freezing at height of 14 cm above LN vapor for 7 minutes (corresponding to final temperature -60°C) was the optimal and dilution ratios from 1:9 to 1:49 can be used to cryopreserve the sperm. These results obtained here will be important for further studies on the optimization of cryopreservation procedures and application of artificial fertilization for *P. nasutus*.

Moreover, our findings showed that *P. nasutus* sperm could be efficiently cryopreserved for 1 year using 10% MeOH and 0.9% NaCl as an extender. Despite the fact that storage time may decrease the quality of cryopreserved sperm, it produced good fertility that was comparable to fresh sperm in all sperm to egg ratios. However, increasing the sperm concentration (sperm to egg ratio: 300,000:1) in cryopreserved sperm can improve the hatching rate for this species. Moreover, our cryopreserved perm has an equal ability to produce normal hatched larvae as fresh sperm and the damage caused by our protocol was minimal. These data are important for assisting reproduction in this species by artificial fertilization. This demonstrated that our

cryopreserved sperm were reusable but not at optimum range for hatchery production. This advantage also can be used to facilitate the transport of cryopreserved sperm from one location to another and can be stored for a long-term to synchronize the gamete availability for breeding purposes.

However, further studies on optimization of freezing and thawing processes need to be done to enhance the hatching rates. Other evaluation methods such as sperm viability and DNA integrity should also be considered to evaluate sperm quality. Additional investigation on varieties of packaging such as different volumes of straws or cryotube and cryovials and low dilution ratios (1:1 to 1:3) are needed to improve the efficiency of sperm cryopreservation protocol for this species.

REFERENCES

Abdul Halim, S. A. A., Esa, Y., Gan, H. M., Zainudin, A. A., & Mohd Nor, S. A. (2023). The complete mitochondrial genomes of *Pangasius nasutus* and *P. conchophilus (Siluriformes: Pangasiidae)*. *Mitochondrial DNA Part B: Resources*, 8(1).

Alavi, S. M. H., & Cosson, J. (2006). Sperm motility in fishes (II) Effects of ions and osmolality: a review. *Cell biology international*, *30*(1), 1-14.

Anabella, N. A., Abinawanto, Subagja, J., Arifin, O. Z., Muhiardi, I., & Arief, M. Z. (2020). The motility of Tor soro fish (Valenciennes, 1842) using post cryopreservation sperm: the effect of grape juice (*Vitis vinifera*) as a natural antioxidant. *IOP Conference Series*.

Agarwal, N. K. (2011). Cryopreservation of fish semen. Himalayan Aquatic Biodiversity Conservation and New Tool in Biotechnology. *Transmedia Publication*. (pp. 104-127).

Aramli, M. S., & Nazari, R. M. (2014). Motility and fertility of cryopreserved semen in Persian sturgeon, *Acipenser persicus*, stored for 30–60 min after thawing. *Cryobiology*, *69*(3), 500-502.

Arsetyo, R., Abdulgani, N., & Trisyani, N. (2012). Pengaruh Konsentrasi Larutan Madu Dalam Nacl Fisiologis Terhadap Viabilitas Dan Motilitas Spermatozoa Ikan Patin (*Pangasius Pangasius*) Selama Masa Penyimpanan. *Jurnal Sains Dan Seni ITS*, *1*(1), 15850.

Asdari, R., Aliyu-Paiko, M., & Hashim, R. (2011). Effects of different dietary lipid sources in the diet for *Pangasius nasutus* (Bleeker, 1863) juveniles on growth performance, feed efficiency, body indices and muscle and liver fatty acid compositions. *Aquaculture Nutrition*, *17*(4), 883–891.

Azani, N., Abol-Munafi, A. B., Liew, H. J., Kamaruddin, S., Arshad, A., Hassan, M. M., Mustafa Kamal, A. H., & W. Rasdi. (2022). Different dietary effects on growth and reproduction of freshwater zooplankton *Ceriodaphnia cornuta* (Sars, 1885) and its potential use in *Pangasius nasutus* larval rearing.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J. K., Peixoto, S., Robeck, T. R., & Cabrita, E. (2019). Sperm handling in aquatic animals for artificial reproduction. *Theriogenology*, *133*, 161-178.

Bernáth, G., Żarski, D., Kasa, E., Staszny, Á., Varkonyi, L., Kollár, T., & Horváth, Á. (2016). Improvement of common carp (*Cyprinus carpio*) sperm cryopreservation using a programable freezer. *General and Comparative Endocrinology*, *237*, 78-88.

Butts, I., Litvak, M., Kaspar, V., & Trippel, E. (2010). Cryopreservation of Atlantic cod *Gadus morhua L*. spermatozoa: Effects of extender composition and freezing rate on sperm motility, velocity and morphology. *Cryobiology*, *61*(2), 174–181.

Bustani, G. S., & Baiee, F. H. (2021). Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. *Veterinary World*, *14*(5), 1220.

Bobe, J., & Labbé, C. (2010). Egg and sperm quality in fish. *General and comparative endocrinology*, *165*(3), 535-548.

Bozkurt, Y., Öğretmen, F., & Seçer, F. S. (2009). Effect of different extenders and storage periods on motility and fertilization success of grass carp (*Ctenopharyngodon idella*) sperm during spawning season.

Dalal, J., Kumar, A., Dutt, R., Singh, G., & Chandolia, R. K. (2018). Different cooling rate for cryopreservation of semen in various livestock species: a review. *International Journal of Current Microbiology and Applied Sciences*, *7*(8), 1903-1911.

Dziewulska, K., Rzemieniecki, A., & Domagała, J. (2010). Motility and energetic status of Atlantic salmon (*Salmo salar L*.) sperm after refrigerated storage. *Journal of Applied Ichthyology*, *26*(5), 668-673.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., & Herráez, M. (2010). Cryopreservation of fish sperm: applications and perspectives. *Journal of Applied Ichthyology*, *26*(5), 623–635.

Cabrita, E., Engrola, S., Conceição, L. E., Pousão-Ferreira, P., & Dinis, M. T. (2009). Successful cryopreservation of sperm from sex-reversed dusky grouper, *Epinephelus marginatus*. *Aquaculture*, *287*(1-2), 152-157.

Chairak, N. (1996). Toxicity test of cryoprotectants on *Clarias macrocephalus* Gunther sperm.

Chereguini, O., De La Banda, I. G., Rasines, I., & Fernandez, A. (1999). Artificial fertilization in turbot, *Scophthalmus maximus* (L.): different methods and determination of the optimal sperm–egg ratio. *Aquaculture Research*, *30*(5), 319-324.

Chew, P., & Zulkafli, R. (2012). Sperm Cryopreservation of Some Freshwater Fish Species in Malaysia. *Current Frontiers in Cryopreservation*. *Current Frontiers in Cryopreservation*.

Chen, Y. K., Liu, Q. H., Li, J., Xiao, Z. Z., Xu, S. H., Shi, X. H., & Ma, D. Y. (2010). Effect of long-term cryopreservation on physiological characteristics, antioxidant activities and lipid peroxidation of red seabream (Pagrus major) sperm. *Cryobiology*, *61*(2), 189-193.

Chen, X., Ma, H., Wu, S., Fu, Q., Lin, Z., & Yang, S. (2023). Evaluation of long-term cryostorage on sperm function and fertility of brown-marbled grouper (*Epinephelus fuscoguttatus*). *Aquaculture Reports*, *30*, 101620.

Contreras, P., Dumorné, K., Ulloa-Rodríguez, P., Merino, O., Figueroa, E., Farias, J. G., & Risopatrón, J. (2020). Effects of short-term storage on sperm function in fish semen: a review. *Reviews in Aquaculture*, *12*(3), 1373-1389.

Contreras, P., Ulloa, P., Merino, O., Valdebenito, I., Figueroa, E., Farías, J., & Risopatrón, J. (2017). Effect of short-term storage on sperm function in Patagonian blenny (*Eleginops maclovinus*) sperm. *Aquaculture*, *481*, 58-63.

Chong, G., Tsai, S., Wang, L., Huang, C., & Lin, C. (2016). Cryopreservation of the gorgonian endosymbiont Symbiodinium. *Scientific Reports*, 6(1).

Denniston, R. S., Michelet, S., Bandioli, K., R. Godke, R., A., T.R. Tiersch, C.C. Green (Eds.), Cryopreservation in Aquatic Species (second ed.), World Aquaculture Society, Baton Rouge, Louisiana (2011), pp. 274-290.

Diwan, A. D., Ayyappan, S., Lal, K. K., & Lakra, W. S. (2010). Cryopreservation of fish gametes and embryos. *The Indian Journal of Animal Sciences*, *80*(4), 109-124.

Dong, Q., Huang, C., & Tiersch, T. R. (2007). Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters. *Cryobiology*, *54*(1), 87-98.

D'occhio, M. J., Hengstberger, K. J., & Johnston, S. D. (2007). Biology of sperm chromatin structure and relationship to male fertility and embryonic survival. *Animal reproduction science*, *101*(1-2), 1-17.

Erraud, A., Cornet, V., Baekelandt, S., Neus, Y., Antipine, S., Lambert, J., & Kestemont, P. (2022). Optimal sperm–egg ratios for successful fertilization

using fresh and cryopreserved sperm in wild anadromous Atlantic salmon (*Salmo salar* L. 1758). *Aquaculture*, *549*, 737758.

Farstad, W. (2000). Assisted reproductive technology in canid species. *Theriogenology*, *53*(1), 175-186.

Fabbrocini, A., D'Adamo, R., Pelosi, S., Oliveira, L. F. J., Del Prete, F., Silvestri, F., & Sansone, G. (2015). Sperm motility evaluation following long-term storage (5 years) of cryopreserved sea bream (*Sparus aurata L*., 1758) semen. *Journal of Applied Ichthyology*, *31*, 104-107.

Fanni, N. A., Santanumurti, M. B., Suprayogi, T. W., & Bendryman, S. S. (2018). Quality enhancement of cryopreserved spermatozoa of Sutchi Catfish (*Pangasianodon hypophthalmus*) with honey addition. 231-238.

Fauvel, C., Suquet, M., Dreanno, C., Zonno, V., & Menu, B. (1998). Cryopreservation of sea bass (*Dicentrarchus labrax*) spermatozoa in experimental and production simulatin conditions. *Aquatic Living Resources*, *11*(6), 387-394.

Félix, F., Antunes, R., Vera, L. M., Oliveira, C. C., & Cabrita, E. (2023). The protective effect of endogenous melatonin on gilthead seabream sperm during cryopreservation. *Aquaculture*, *577*, 739997.

Figueroa, E., Lee-Estevez, M., Valdebenito, I., Watanabe, I., Oliveira, R. P. D. S., Romero, J., & Farías, J. G. (2019). Effects of cryopreservation on mitochondrial function and sperm quality in fish. *Aquaculture*, *511*, 634190.

Gallego, V., Pérez, L., Asturiano, J. F., & Yoshida, M. (2013). Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). *Aquaculture*, *416*, 238-243.

Gallego, V., Herranz-Jusdado, J. G., Rozenfeld, C., Pérez, L., & Asturiano, J. F. (2018). Subjective and objective assessment of fish sperm motility: when the technique and technicians' matter. *Fish physiology and biochemistry*, *44*, 1457-1467.

Gao, D., & Critser, J. K. (2000). Mechanisms of cryoinjury in living cells. *ILAR journal*, *41*(4), 187-196.

Glenn, T., Tsyusko, O., Yi, Y., Joice, G., Jones, K., Aizawa, K., and Hinton, T. (2011). Differential genetic responses to ionizing irradiation in individual families of Japanese medaka, *Oryzias latipes*. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 718(1-2), 18-23.

González-López, W. Á., Ramos-Júdez, S., Giménez, I., & Duncan, N. J. (2020). Sperm contamination by urine in Senegalese sole (*Solea senegalensis*) and the use of extender solutions for short-term chilled storage. *Aquaculture*, *516*, 734649.

Guthrie, H., & Welch, G. (2012). Effects of reactive oxygen species on sperm function. *Theriogenology*, *78*(8), 1700–1708.

Gustiano, R., Prakoso, V. A., & Ath-thar, M. H. F. (2018). Asian catfish genus Pangasius: Diagnosis and distribution. *Indonesian Fisheries Research Journal*, *24*(2), 99-115.

Gwo J.C., T.R. Tiersch, C.C. Green (Eds.), Cryopreservation in Aquatic Species (second ed.), World Aquaculture Society, Baton Rouge, Louisiana (2011), pp. 459-481 *78*(8), 1700–1708.

Gwo, J. C., Jamieson, B. G. M., & Leung, L. P. (2019). Live preservation of fish gametes. In *Reproductive Biology and Phylogeny of Fishes, Vol 8B: Part B: Sperm Competion Hormones* (pp. 395-484). CRC Press.

Hagedorn, M., Peterson, A. T., Mazur, P., & Kleinhans, F. W. (2004). High ice nucleation temperature of zebrafish embryos: slow-freezing is not an option. *Cryobiology*, *49*(2), 181–189.

Hasanah, U., Abinawanto, A., Alimuddin, A., Boediono, A., & Kusrini, E. (2020). Short Communication: Effect of cryopreservation on ultrastructure and mitochondrial function of albino *Pangasius* catfish spermatozoa. *Biodiversitas Journal of Biological Diversity*, *21*(10).

Hassan, A., Ambak, M. A., & A. Samad, A. P. (2011). Crossbreeding of *Pangasianodon hypophthalmus* (Sauvage, 1878) and *Pangasius nasutus* (Bleeker, 1863) and their larval development. *Journal of Sustainability Science and Management*, *6*(1), 28–35.

Haque, M. A., Hossain, M. I., Aftabuddin, S., Habib, A., & Siddique, M. A. M. (2021). First onboard fertilization of Asian seabass, *Lates calcarifer*: Effects of egg stocking density on the fertilization, hatching and survival rate. *Scientific African*, *12*, e0084.

Hidayat, K., W., Supriyono, E., Setiyanto, D., D., & Widiyati, A. (2016). Effect of three simple design micro-pore aeration on growth and survival of hybrid catfish

Horváth, Á., Labbé, C., Jesenšek, D., Hoitsy, G., Bernáth, G., Kaczkó, D., & Urbányi, B. (2015). Post-thaw storage of sperm from various salmonid species. *Journal of Applied Ichthyology*, *31*, 119-124.

Horváth, Á., Miskolczi, E., & Urbányi, B. (2003). Cryopreservation of common carp sperm. *Aquatic Living Resources*, *16*(5), 457-460.

Inaba, K., & Shiba, K. (2018). Microscopic analysis of sperm movement: links to mechanisms and protein components. *Microscopy*, 67(3), 144–155.

Iswanto, B., & Tahapari, E. (2014). Keragaan pemijahan buatan antara ikan aptin siam (*Pangasianodon hypophthalmus*) betina dan ikan patin jambal (*Pangasius djambal*) Jantan dan ikan patin nasutus (*Pangasius nasutus*) jantan.

Jaapar, M. Z., Yusof, M. Z., Yusof, H. M., Ramli, N. A., Mohamad, S. A., & Jamari, Z. (2021). Effect of different salinity concentrations on hatching rate and larval development of Patin buah, *Pangasius nasutus* (Bleeker, 1863). *Journal of Applied Aquaculture*, *34*(3), 693–701.

Kainin, S., Ponchunchoovong, S., Imsilp, U., & Singsee, S. (2014). Cryopreservation of Mekong catfish, *Pangasius bocourti* Sauvage, 1880 spermatozoa.

Kime, D. E., Van Look, K. J. W., McAllister, B. G., Huyskens, G., Rurangwa, E., & Ollevier, F. (2001). Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *130*(4), 425-433.

Klaiwattana, P., Srisook, K., Srisook, E., Vuthiphandchai, & Neumvonk, J. (2016). Effect of cryopreservation on lipid composition and antioxidant enzyme activity of seabass (*Lates calcarifer*) sperm. *Iranian Journal of Fisheries Sciences*, *15*(1), 157–169.

Koeppen, B. M. (2013). Physiology of body fluids. In *Elsevier eBooks* (pp. 1–14).

Kopeika, E., Kopeika, J., & Zhang, T. (2007). Cryopreservation of fish sperm. *Cryopreservation and freeze-drying protocols*, 203-217.

Koh, I. C. C., Yokoi, K. I., Tsuji, M., Tsuchihashi, Y., & Ohta, H. (2010). Cryopreservation of sperm from seven-band grouper, *Epinephelus septemfasciatus*. *Cryobiology*, *61*(3), 263–267.

Koh, I. C. C., Hamada, D., Tsuji, Y. A., Okuda, D., Nomura, K., Tanaka, H., & Ohta, H. (2017). Sperm cryopreservation of Japanese eel, *Anguilla japonica*. *Aquaculture*, *473*, 487–492.

Kovács, Müller, T., Márián, T., Krasznai, Z., Urbányi, B., & Horváth. (2010). Quality of cryopreserved African catfish sperm following post-thaw storage. *Journal of Applied Ichthyology*, *26*(5), 737–741.

Kwantong, S., & Bart, A. N. (2003). Effect of cryoprotectants, extenders and freezing rates on the fertilization rate of frozen striped catfish, *Pangasius hypophthalmus* (Sauvage), sperm. *Aquaculture Research, 34*(10), 887–893.

Kwantong, S., & Bart, A. N. (2006). Cryopreservation of black ear catfish, *Pangasius larnaudii*, (Bocourt) sperm. *Aquaculture Research, 37*(9), 955–957.

Kwantong, S., & Bart, A. N. (2009). Fertilization efficiency of cryopreserved sperm from striped catfish, *Pangasius hypophthalmus* (Sauvage). *Aquaculture Research, 40*(3), 292–297.

Lang, P. R., Riley, K. L., Chandler, J. E., & Tiersch, T. R. (2003). The use of dairy protocols for sperm cryopreservation of blue catfish *Ictalurus furcatus*. *Journal of the World Aquaculture Society*, *34*(1), 66-75.

Lahnsteiner, F., Weismann, T., & Patzner, R. A. (1997). Methanol as cryoprotectant and the suitability of 1.2 ml and 5 ml straws for cryopreservation of semen from salmonid fishes. *Aquaculture Research, 28*(6), 471-479.

Lahnsteiner, F. (2000). Semen cryopreservation in the Salmonidae and in the Northern pike. *Aquaculture Research, 31*(3), 245–258.

Lahnsteiner, F., Berger, B., Mansour, N., & Kunz, F. (2024). Quality of cryopreserved salmonid semen is not affected by a 25 years storage period. *Aquaculture*, *578*, 740100.

Lee, Y., Park, J. T., Lee, I. O., Zidni, I., & Lim, H. S. (2021). Effects of cryoprotective agents and treatment methods on sperm cryopreservation of stone flounder, *Kareius bicoloratus*. *Aquaculture*, *531*, 735969.

Leibo, S. P. (2004). The early history of gamete cryobiology. In *Life in the frozen state* (pp. 373-396). CRC Press.

Lemma, A. (2011). Effect of cryopreservation on sperm quality and fertility. *Artificial insemination in farm animals*, *12*, 191-216.

Liu, S., Su, Y., Yi, H., Liu, X., Chen, X., Lai, H., & Li, G. (2022). Effect of short-term storage on sperm functional parameters in sex-reversed female mandarin fish (*Siniperca chuatsi*). *Aquaculture*, *547*, 737410.

Liu, Q. H., Xiao, Z. Z., Wang, X. Y., Xu, S. H., Guan, S. G., Xu, C. A., & Li, J. (2016). Sperm cryopreservation in different grouper subspecies and application in interspecific hybridization. *Theriogenology*, *85*(8), 1399-1407.

Linhart, O., Cheng, Y., Rodina, M., Gela, D., Tučková, V., Shelton, W. L., ... & Xin, M. (2020). AQUA_2020_1080: Sperm management of European catfish (*Silurus glanis L.*) for effective reproduction and genetic conservation. *Aquaculture*, *529*, 735620.

López, D. I., Leal, M. C., & Viveiros, A. T. M. (2015). Extender composition and osmolality affects post-thaw motility and velocities of piracanjuba *Brycon orbignyanus* (Valenciennes, 1850) (Characiformes) sperm. *Journal of Applied Ichthyology*, *31*, 114-118.

Mahmud, N. S., Abdullah, S. Z., Jalal, K. C. A., Rimatulhana, R., & Amal, M. N. (2019). Assessment of Bacteria and Water Quality Parameters in Cage Cultured *Pangasius hypophthalmus* in Temerloh, Pahang River, Malaysia. *Nature Environment & Pollution Technology*, *18*(5).

Magnotti, C., Cerqueira, V., Lee-Estevez, M., Farias, J. G., Valdebenito, I., & Figueroa, E. (2018). Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. *Reviews in Aquaculture*, *10*(1), 15-25.

Marco-Jiménez, F., Garzón, D., Peñaranda, D. S., Pérez, L., Viudes-De-Castro, M. P., Vicente, J. S., Jover, M., & Asturiano, J. F. (2006). Cryopreservation of European eel (*Anguilla anguilla*) spermatozoa: Effect of dilution ratio, foetal bovine serum supplementation, and cryoprotectants. *Cryobiology*, *53*(1), 51–57.

Mazur, P., Leibo, S. P., & Chu, E. H. (1972). A two-factor hypothesis of freezing injury: evidence from Chinese hamster tissue-culture cells. *Experimental cell research*, *71*(2), 345-355.

Meyers, S. A. (2009). Sperm physiology. *Equine breeding management and artificial insemination*, 47-55.

Meryman, H. T. (2007). Cryopreservation of living cells: principles and practice. *Transfusion*, *47*(5), 935-945.

Miskolczi, E., Mihalffy, S., Várkonyi, E. P., Urbányi, B., & Horváth, Á. (2005). Examination of larval malformations in African catfish, *Clarias gariepinus* following fertilization with cryopreserved sperm. *Aquaculture*, 241 (1-4), 119-125.

Misro, M. M., & Ramya, T. (2012). Fuel/energy sources of spermatozoa. *Male Infertility: Contemporary Clinical Approaches, Andrology, ART & Antioxidants*, 209-223.

Middelkamp, S., van Tol, H. T., Spierings, D. C., Boymans, S., Guryev, V., Roelen, B. A., & Kuijk, E. W. (2020). Sperm DNA damage causes genomic instability in early embryonic development. *Science advances*, *6*(16), eaaz7602.

Mohammad, H. N. E., Mohammad, S., Shahnaz, R., Maryam, A., Shahla, R., Fariba, M., & Mohammad, M. (2005). Effect of sperm DNA damage and sperm protamine deficiency on fertilization and embryo development post-ICSI. *Reproductive biomedicine online*, *11*(2), 198-205.

Mongkonpunya, K., Pupipat, T., Chairak, N., & Tiersch, T. R. (1995). Cryopreservation of Mekong giant catfish sperm. *Asian Fisheries Science*, *8*, 211-222.

Morisawa, M., Suzuki, K., & Morisawa, S. (1983). Effects of potassium and osmolality on spermatozoan motility of salmonid fishes. *Journal of Experimental Biology*, *107*(1), 105-113.

Mortimer, D., & Mortimer, S. T. (2013). Density gradient separation of sperm for artificial insemination. *Spermatogenesis: methods and protocols*, 217-226.

Muchlisin, Z. A. (2005). REVIEW: Current Status of Extenders and Cryoprotectants on Fish Spermatozoa Cryopreservation. *Biodiversitas Journal of Biological Diversity*, *6*(1).

Müller, T., Urbányi, B., Váradi, B., Binder, T., Horn, P. A., Bercsényi, M., & Horváth, Á. (2004). Cryopreservation of Sperm of Farmed European Eel, *Anguilla anguilla*. *Journal of the World Aquaculture Society*, *35*(2), 225–231.

Nahiduzzaman, M., Hassan, M. M., Roy, P. K., Hossain, M. A., Hossain, M. A. R., & Tiersch, T. R. (2012). Sperm cryopreservation of the Indian major carp, *Labeo calbasu*: Effects of cryoprotectants, cooling rates and thawing rates on egg fertilization. *Animal Reproduction Science*, *136*(1-2), 133-138.

Noer, I., Satriawan, R., Iskandar, Bangkit, B. S. I., & Rosidah. (2020). Effectiveness of Different Bubble Size of Aeration to Survival and Growth Rate of Pangas Catfish Larvae, *Pangasius nasutus*. *Asian Journal of Fisheries and Aquatic Research*, 34–41.

Ogier de Baulny, B., C. Labbé and G. Maisse. 1999. Membrane integrity, mitochondrial activity, ATP content and motility of the European catfish *Silurus glanis* testicular spermatozoa after freezing with different cryoprotectants. Cryobiology 39:177-184

Paul L. R., Riley, K. L., Chandler, J. E., & Tiersch, T. R. (2003). The use of dairy protocols for sperm cryopreservation of blue catfish *Ictalurus furcatus*. *Journal of the World Aquaculture Society*, *34*(1), 66-75.

Pan, J., Ding, S., Ge, J., Yan, W., Hao, C., Chen, J., & Huang, Y. (2008). Development of cryopreservation for maintaining yellow catfish *Pelteobagrus fulvidraco* sperm. *Aquaculture*, *279*(1–4), 173–176.

Park, J. Y., Zidni, I., Lee, Y. H., Lee, H. B., & Lim, H. K. (2022). Effect of long-term storage on the quality of cryopreserved sperm of the giant grouper, *Epinephelus lanceolatus*. *Aquaculture*, *555*, 738154.

Park, Y. J., & Pang, M. G. (2021). Mitochondrial functionality in male fertility: from spermatogenesis to fertilization. *Antioxidants*, *10*(1), 98.

Pérez, L., Asturiano, J. F., Yoshida, M., & Gallego, V. (2022). Ionic control of sperm motility and trials for the improvement of pufferfish (*Takifugu alboplumbeus*) sperm extenders. *Aquaculture*, *554*, 738146.

Pérez-Cerezales, S., Martínez-Páramo, S., Cabrita, E., Martínez-Pastor, F., De Paz, P., & Herráez, M. P. (2009). Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. *Theriogenology*, *71*(4), 605-613.

Piomboni, P., Focarelli, R., Stendardi, A., Ferramosca, A., & Zara, V. (2011). The role of mitochondria in energy production for human sperm motility. *International Journal of Andrology*, 35(2), 109–124.

Rafi, A. I. A., M.Yusof, H., Othman, J. M. Z., & Baharuddin, H. (2022). Potential usage of SirehmaxTM, a Piper betle extract, for controlling fish pathogen prevalence in domesticated shark catfish fingerlings, *Pangasius nasutus* (Bleeker, 1863). *Malaysian Fisheries Journal*, *31*, 32–36.

Rahardhianto, A., Abdulgani, N., & Trisyani, N. (2012). The effects of honey concentration in the physiological NaCl on viability and motility of striped catfish (*Pangasius pangasius*) spermatozoa during storage period. *Journal Sains dan Seni ITS*, 1(1), E58-E63.

Roberts, T. R., & Vidthayanon, C. (1991). Systematic revision of the Asian catfish family Pangasiidae, with biological observations and descriptions of three new species. *Proceedings of the Academy of Natural Sciences of Philadelphia*, 97-143.

Robles, V., Cabrita, E., & Paz Herráez, M. (2009). Germplasm cryobanking in zebrafish and other aquarium model species. *Zebrafish*, *6*(3), 281-293.

Rudolfsen, G., Figenschou, L., Folstad, I., & Kleven, O. (2008). Sperm velocity influence paternity in the Atlantic cod (*Gadus morhua L.*). *Aquaculture Research*, *39*(2), 212-216.

Rurangwa, E., Kime, D. E., Ollevier, F., & Nash, J. P. (2004). The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture*, *234*(1-4), 1-28.

Rurangwa, E., Volckaert, F., Huyskens, G., Kime, D., & Ollevier, F. (2001). Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilization in African catfish (*Clarias gariepinus*).

Samad, A. P., Isma, M. F., Humairani, R., & Akmal, Y. (2020). Histological studies on digestive system development and early feeding activity of pangasius hybrid larvae. *Journal of Aquaculture and Fish Health*.

Sanocka, D., & Kurpisz, M. (2004). Reactive oxygen species and sperm cells. *Reproductive biology and endocrinology*, *2*, 1-7.

Sansone, G., Fabbrocini, A., Ieropoli, S., Langellotti, A., Occidente, M., & Matassino, D. (2002). Effects of extender composition, cooling rate, and freezing on the motility of sea bass (*Dicentrarchus labrax, L.*) spermatozoa after thawing. *Cryobiology*, *44*(3), 229–239.

Shaliutina, A., Hulak, M., Gazo, I., Linhartova, P., & Linhart, O. (2013). Effect of short-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeon sperm. *Animal reproduction science*, *139*(1-4), 127-135.

Subagja, J., Slembrouck, J., & Legendre, M. (1999). Elevage larvaire d'un poisson-chat asiatique *Pangasius hypophthalmus* (Siluroidei, Pangasiidae): analyses des mortalites precoces et propositions de traitements appropries. *Aquatic Living Resources*, *1*(12), 37-44.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., & Billard, R. (2000). Cryopreservation of sperm in marine fish. *Aquaculture Research*, *31*(3), 231–243.

Storz, G., & Imlayt, J. A. (1999). Oxidative stress. *Current opinion in microbiology*, *2*(2), 188-194.

Speyer, B. E., Pizzey, A. R., Ranieri, M., Joshi, R., Delhanty, J. D. A., & Serhal, P. (2010). Fall in implantation rates following ICSI with sperm with high DNA fragmentation. *Human Reproduction, 25*(7), 1609-1618.

Szabó, G., Müller, T., Bercsényi, M., Urbányi, B., Kucska, B., & Horváth, Á. (2005). Cryopreservation of European eel *(Anguilla anguilla)* sperm using different extenders and cryoprotectants. *Acta Biologica Hungarica*, *56*(1–2), 173–175.

Tanaka, S., Zhang, H., Horie, N., Yamada, Y., Okamura, A., Utoh, T., & Kurokura, H. (2002). Long-term cryopreservation of sperm of Japanese eel. *Journal of Fish Biology*, *60*(1), 139-146.

Thilak Pon Jawahar, K., & Betsy, J. (2020). Cryopreservation of fish gametes: An overview. *Cryopreservation of fish gametes*, 151-175.

Tiersch, T. R., Figiel Jr, C. R., Wayman, W. R., Williamson, J. H., Gorman, O. T., & Carmichael, G. J. (2004). Cryopreservation of sperm from the endangered Colorado pikeminnow. *North American Journal of Aquaculture*, *66*(1), 8-14.

Tiersch, T., R., Huiping Y., Jill A. Jenkins, & Qiaoxiang D. (2007). Sperm cryopreservation in fish and shellfish. *Society of Reproduction and Fertility Supplement, 65*, 493–508.

Tiersch, T. R. and C. C. Green, (Eds.). 2011. *Cryopreservation in Aquatic Species, 2nd Edition*. World Aquaculture Society. Baton Rouge, Louisiana, USA.

Tiersch, T. R., Yang, H., Jenkins, J. A., & Dong, Q. (2007). Sperm cryopreservation in fish and shellfish. *PubMed, 65*, 493–508.

Tiersch, T. R., Goudie, C. A., & Carmichael, G. J. (1994). Cryopreservation of Channel Catfish Sperm: Storage in Cryoprotectants, Fertilization Trials, and Growth of Channel Catfish Produced with Cryopreserved Sperm. *Transactions of the American Fisheries Society*, *123*(4), 580–586.

Tiersch, T. R., & Green, C. C. (2011). Cryopreservation in aquatic species: A Comprehensive Overview of Current Practices, Programmatic Development and Future Directions for Cryopreservation of Gametes Embryos and Larvae of Aquatic Species.

Thilak Pon Jawahar, K., & Betsy, J. (2020). Cryopreservation of fish gametes: An overview. *Cryopreservation of fish gametes*, 151-175.

Trigo, P., Merino, O., Figueroa, E., Valdebenito, I., Sánchez, R., & Risopatrón, J. (2015). Effect of short-term semen storage in salmon (*Oncorhynchus mykiss*)

on sperm functional parameters evaluated by flow cytometry. *Andrologia*, *47*(4), 407-411.

Thirumala, S., Campbell, W. T., Vicknair, M. R., Tiersch, T. R., & Devireddy, R. V. (2006). Freezing response and optimal cooling rates for cryopreserving sperm cells of striped bass, *Morone saxatilis*. *Theriogenology*, *66*(4), 964-973.

Vanderwall, D. K. (2008). How to process a dilute ejaculate of semen for cooled-transported insemination. In *Proceedings of the 54th Annual Convention of the American Association of Equine Practitioners, Dec* (pp. 6-10).

Vishwanath, R., & Shannon, P. (1997). Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. *Reproduction, Fertility and Development*, *9*(3), 321-332.

Viveiros, A. T. M., So, N., & Komen, J. (2000). Sperm cryopreservation of African catfish, *Clarias gariepinus*: cryoprotectants, freezing rates and sperm: egg dilution ratio. *Theriogenology*, *54*(9), 1395-1408.

Viveiros, A. T. M. (2011). Current Status of Sperm Cryopreservation in Siluriform Catfishes. In: Cryopreservation in Aquatic Species, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 387-397.

Viveiros, A., Taffarel, T. R., & Leal, M. C. (2014). Osmolality and composition of the extender during the cold storage of *Prochilodus lineatus* (*Characiformes: Prochilodontidae*) sperm. *Neotropical Ichthyology*, *12*, 643-648.

Vuthiphandchai, V., Chomphuthawach, S., & Nimrat, S. (2009). Cryopreservation of red snapper (*Lutjanus argentimaculatus*) sperm: effect of cryoprotectants and cooling rates on sperm motility, sperm viability, and fertilization capacity. *Theriogenology*, *72*(1), 129-138

Vuthiphandchai, V., Wilairattanadilok, K., Chomphuthawach, S., Sooksawat, T., & Nimrat, S. (2015). Sperm cryopreservation of silver barb (*B arbodes gonionotus*): cryoprotectants, cooling rate and storage time on sperm quality. *Aquaculture Research*, *46*(10), 2443-2451.

Wang, Y., Zhang, L., Sohail, T., Kang, Y., Sun, X., & Li, Y. (2022). Chlorogenic acid improves quality of chilled RAM sperm by mitigating oxidative stress. *Animals*, *12*(2), 163.

Yang, S., Fan, B., Chen, X., Hua, Y., & Meng, Z. (2022). Optimization of a sperm cryopreservation protocol for giant grouper (*Epinephelus lanceolatus*). *Aquaculture*, *555*, 7381-7386.

Yang, S., Fan, B., Chen, X., & Meng, Z. (2022). Evaluation of different sugar extenders and large volume cryotube for sperm cryopreservation of brown-marbled grouper (*Epinephelus fuscoguttatus*). *Aquaculture Research*, *53*(11), 4069-4075.

Yang, S., Han, L., Huang, R., Liufu, Y., Meng, Z., & Lin, H. (2017). Optimization of conditions for the cryopreservation of yellow catfish (*Pelteobagrus fulvidraco*) sperm. *Cryobiology, 76*, 104–110.

Yang, A., Tang, W. S., Si, T., & Tang, J. X. (2017). Influence of physical effects on the swarming motility of Pseudomonas aeruginosa. *Biophysical Journal*, *112*(7), 1462-1471.

Yang, S., Huang, W., Chen, H., Huang, M., Liufu, Y., & Meng, Z. (2020). Effect of chilled storage on sperm quality of basa catfish (*Pangasius bocourti*). *Fish Physiology and Biochemistry*, *46*(6), 2133–2141.

Yang, S., Fan, B., Chen, X., & Meng, Z. (2020). Cryopreservation of sperm in brown-marbled grouper (*Epinephelus fuscoguttatus*). *Aquaculture international*, *28*, 1501-1516

Yang, S., Guan, J., & Hua, Y. (2023). Optimization of sperm cryopreservation protocol for Basa catfish (*Pangasius bocourti*). *Cryobiology, 111*, 89–95.

Young, W. P., Frenyea, K., Wheeler, P. A., & Thorgaard, G. H. (2009). No increase in developmental deformities or fluctuating asymmetry in rainbow trout (*Oncorhynchus mykiss*) produced with cryopreserved sperm. *Aquaculture*, *289*(1-2), 13-18.

Yusoff, M., Hassan, B. N., Ikhwanuddin, M., Sheriff, S. M., Hashim, F., Mustafa, S., & Koh, I. C. C. (2018). Successful sperm cryopreservation of the brown-marbled grouper, *Epinephelus fuscoguttatus* using propylene glycol as cryoprotectant. *Cryobiology*, *81*, 168–173.

Yusoff, M. S., Christianus, A., Ismail, M., Esa, Y., Hassan, M., Hamid, N., Siti Nadia, A., & Zulkifle, M. (2019). Discrimination analysis of hybrid *Pangasianodon hypophthalmus* (Sauvage, 1983) (♀) × *Pangasius nasutus* (♂) (Bleeker, 1976) and its parental species.

Yusoff, S. F. M., Christianus, A., EsaY, I. M., Garba, B., Safiin, N. S. Z., & Hamid, N. H. (2023). Potential enhancement of proximate, fatty acid, and amino acid compositions through the hybridisation of *Pangasianodon hypophthalmus*

(Sauvage, 1983) (♀) and *Pangasius nasutus* (Bleeker, 1976) (♂). *Adv. Anim. Vet. Sci*, *11*(4), 663-671.

Yusof, F., & Nakajima, M. (2019). Morphological Tool to Elucidate Two Closely Related *Pangasius* Catfish. *International Journal of Allied Health Sciences*, *3*(3), 765-765.

Yongsheng, T., Jingjing, Z., Zhentong, L., Ziqi, L., Linna, N., Betsy, J., & Kumar, S. (Editors) (2020). *Cryopreservation of fish gametes*. Springer Nature, 187-210.

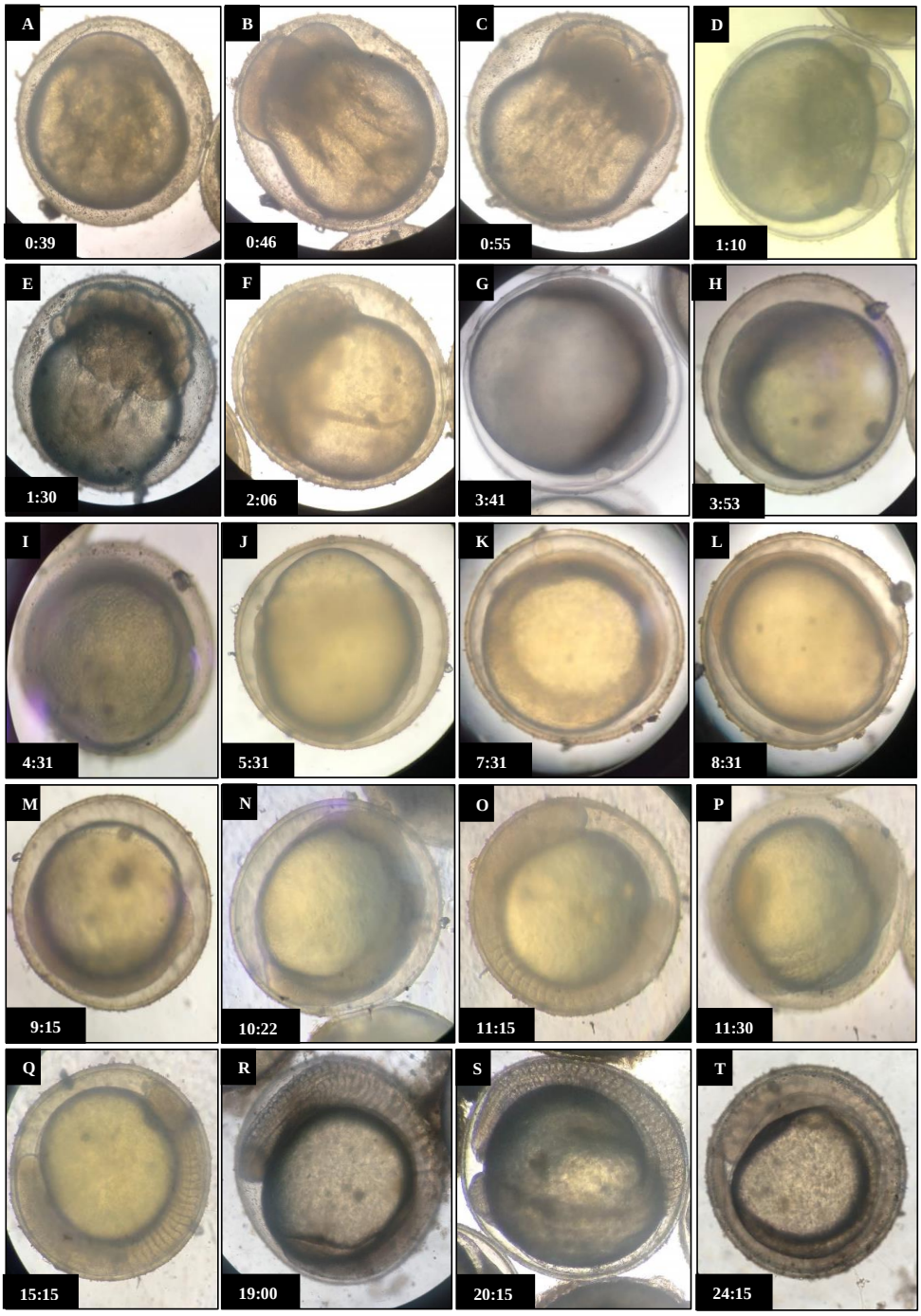
Zhang, T., Isayeva, G., Adams, S. L., & Rawson, D. M. (2005). Studies on membrane permeability of zebrafish (*Danio rerio*) oocytes in the presence of different cryoprotectants. *Cryobiology*, 50(3), 285–293.

Zulkiflee, S. Z., Yusof, M. F., Rahman, N. A. A., & Rostam, M. A. (2020). Moisture, ash and fat composition of *Pangasianodon hypophthalmus* (Sauvage, 1878) and *Pangasius nasutus* (Bleeker, 1863). *International Journal of Allied Health Sciences*, *4*(3), 1486-1495.

APPENDICES

Appendix 1

Egg development of *Pangasius nasutus* using cryopreserved sperm.



A, fertilized egg; **B**, 2-cells stage; **C**, 4-cells stage; **D**, 8-cells stage; **E**, 16-cells stage; **F**, early blastula stage; **G**, mid blastula stage; **H**, more cells divided into smaller cells; **I**, late blastula stage; **J**, early gastrula stage; **K**, yolk covered by blastoderm; **L**, blastopore nearly closed; **M**, begin to form head and tail; **N**, head and tail more visible; **O**, body muscular formed; **P**, vertebrae more formed and visible; **Q**, optical lens formed; **R**, heart beating and tail start to move; **S**, optical lens more visible and tail lifted from egg yolk; **T**, embryo wriggles to break chorion; **U**, embryo hatched from chorion; **V**, *P.nasutus* larvae. Numbers show hours and minutes after insemination.

Appendix 2

Journal publication.

Idris, N., Abduh, M. Y., Noordin, N. M., Abol-Munafi, A. B., & Koh, I. C. C. (2024). Development of sperm cryopreservation protocol for patin buah, *Pangasius nasutus*. *Cryobiology*, 104878.

Cryobiology 115 (2024) 104878

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

Development of sperm cryopreservation protocol for patin buah, *Pangasius nasutus*

Nurizzati Idris [a], Muhammad Yazed Abduh [b], Noordiyana Mat Noordin [a,b], Ambok Bolong Abol-Munafi [a], Ivan Chong Chu Koh [a,b,*]

[a] Institute of Tropical Aquaculture and Fisheries, Universiti Malaysia Terengganu, 21300, Kuala Nerus, Terengganu, Malaysia
[b] Faculty of Fisheries and Food Sciences, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia

ARTICLE INFO

Keywords:
Sperm cryopreservation
Pangasius nasutus
Cryoprotectant
Dilution ratio
Toxicity
Cooling rate

ABSTRACT

The development of sperm cryopreservation for *Pangasius nasutus* is necessary in order to serve the growing demand of this species through artificial fertilization and the preservation of valuable strains of male broodstocks. In the present study, the basic protocol of sperm cryopreservation for *P. nasutus* was established by identifying the optimal conditions for optimum cryoprotectant, toxicity of cryoprotectants, extenders, freezing condition and dilution ratio. Methanol (MeOH) at 10% concentration had the best post-thaw motility (26.3 ± 0.9%) and curvilinear velocity (VCL) compared to dimethyl acetamide and dimethyl sulfoxide. MeOH was the least toxic cryoprotectant; sperm suspended in 5 and 10% MeOH maintained motility up to 50 min. No significant differences were detected between the three types of extenders tested (0.9% sodium chloride, Calcium-free Hanks' Balance salt solution and ringer solution). *P. nasutus* sperm had a narrow range of optimal cooling rate. Significantly higher post-thaw motility was identified when cooling at 9.23 °C min^{-1}, obtained by freezing at height of 14 cm above liquid nitrogen vapor for 7 min, showing lower cooling rate is suitable for this species. However, when cooling below and above the optimal cooling rate, post-thaw motility dropped drastically. There were no significant differences among the dilution ratios investigated, indicating the volume of cryodiluent at all tested ratios (1:9, 1:19 and 1:49) was sufficient for the protection of cells during the cryopreservation process. The development of the protocol for cryopreserved *P. nasutus* sperm will assist artificial seed production and provide an important tool for genetic and breeding research.

1. Introduction

Pangasius nasutus (Bleeker, 1863), locally known in Malaysia as 'Patin buah' is a freshwater fish species grown in Southeast Asian countries that belongs to family Pangasiidae and order Siluriformes. This species is an important aquaculture fish in Malaysia. They are well known for their high quality of white and delicious flesh [1]. The market preferences can be observed not just in Malaysia but also in other possible export markets in Asia, Europe, and North America [2]. However, the availability is still dependent on catches from the wild [3], so it is still difficult to locate a constant supply on the market. Consequently, over harvesting will cause the natural population to decline. Therefore, it is crucial to establish methods for breeding this species in captivity.

Numerous improvements have been achieved for the development of this species in captivity. Enhancements in larval and juvenile nutrition have been developed in several studies [4–6] as well as in larval and fingerlings management [2,7]; and hybridization of this species with others *Pangasius* sp. have been done [8–10]. However, there is still a scarcity of available information in broodstock management, breeding technology and genetics which are necessary to increase the production of this species in commercial aquaculture. Natural spawning for genus *Pangasius* usually occurs once a year [11]. Their eggs and sperm are not available throughout the year because this species is a seasonal spawner. In a captive condition, both females and males gonads are not matured at the same time which means that hatchery production of this species is quite difficult [11,12].

Cryopreservation of sperm would therefore be crucial to enhance the breeding opportunities for *P. nasutus*. Sperm cryopreservation is the process of keeping sperm alive in liquid nitrogen at low temperatures below zero, typically at −196°C [13]. The idea behind this technique is to preserve genetic material and keep cells alive at low temperatures of liquid nitrogen where cells' biological activity is momentarily inhibited

* Corresponding author. Faculty of Fisheries and Food Sciences, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia.
E-mail address: ivankcc@umt.edu.my (I.C.C. Koh).

https://doi.org/10.1016/j.cryobiol.2024.104878
Received 22 October 2023; Received in revised form 14 January 2024; Accepted 29 February 2024
Available online 1 March 2024
0011-2240/© 2024 Society for Cryobiology. Published by Elsevier Inc. All rights reserved.

and can be preserved for a very long time [14]. This technique would help to store male gametes for an extended period of time and the ability to use stored sperm when eggs are available, which helps synchronize gamete availability for both sexes [15–17]. It is also useful for simplified broodstock maintenance [18,19] where only females can be induced during off-season spawning and cryopreserved sperm can be utilized to fertilize the eggs. Desired fish characteristics also can be maintained by storing the genome of valuable strains of male broodstock in order to produce high quality of seed [20].

Cryopreservation of sperm for *Pangasius* species has been investigated since 1995 for *P. hypophthalmus*, *P. larnaudii*, *P. gigas*, *P. bocourti* and Albino pangasius catfish [12,21–26], but sperm cryopreservation for *P. nasutus* has yet to be explored and no studies have been established. In *Pangasius* sp. sperm, investigation on the suitable cryoprotectants and their optimum concentrations were the basis of the development of sperm cryopreservation of *Pangasius* sp. using dimethyl acetamide (DMA), dimethyl sulfoxide (Me_2SO) and methanol (MeOH) at various concentrations. This was followed by identification on the optimum cooling rates, types of extenders and effect of equilibration times. On the other hand, Me_2SO has been successfully used as cryoprotectant for *P. hypophthalmus*, *P. larnaudii*, *P. gigas* and *P. bocourti* except for Albino pangasius catfish where 10% MeOH combined with 10% skim milk shows the best result [21]. However, currently protocols are not applicable to be used as a standard protocol for *P. nasutus* sperm. This is because sperm cryopreservation is species-specific, it is difficult to standardize or optimize this procedure and apply it to all types of fish. This is due to the fact that sperm from various species require various conditions, necessitating the establishment of a specific sperm cryopreservation protocol for each species [27].

For successful sperm cryopreservation, the optimization of several important parameters is necessary. Therefore, the selection of suitable candidates for the cryoprotectant, toxicity of cryoprotectant at different concentration, extender, cooling rate and dilution ratios were investigated in this study in order to develop the protocol for sperm cryopreservation of this species [28,29]. These findings are crucial for future research on the improvement of sperm cryopreservation techniques and the use of artificial fertilization for *P. nasutus* species as well as to increase seed quality and generate aquaculture production.

2. Materials and methods

2.1. Gamete collection

Sperm was collected from male broodstock of *P. nasutus* reared at a private fish farm, Three Oceans Fish Pond located in Rawang, Selangor, Malaysia. Broodstock of *P. nasutus* were cultured outdoors inside the net cages in earthen pond with a freshwater recirculating system. The broodstocks were fed with pellets twice a day, but feeding was discontinued for 12 h before sperm collection.

Three matured males (1.5–2.0 kg) were selected according to sexual maturity criteria where matured males expressed sperm with simple pressure on the abdomen. The broodstocks were anesthetized by immersing them in an anaesthetic bath containing an adequate amount of clove oil for no longer than 10 min. Males were properly dried with a clean towel and placed on a flat surface area with the ventral region facing up. Then, sperm was collected by stripping (gentle abdominal massage), dispensed into separate 5.0 mL tubes for each respective male with care to avoid any contamination of freshwater, blood or urine. Collected sperm was immediately mixed with 0.9% NaCl at ratio 1:1 to avoid drastic decrease in initial motility, then placed on crushed ice in an icebox for further analysis. The sperm was produced naturally without any induction of hormones.

2.2. Sperm quality evaluation

Sperm motility was used as a main parameter in this study. Percentage of sperm motility was recorded by using Dino eye video camera and software (DinoCapture 2.0, version 1.5.45) where a video camera combined with the optical lens of a biological microscope (CX43; Olympus; objective lens, 10 ×) at room temperature. The initial motility of diluted sperm and post-thaw motility of sperm were observed after rapid mixing with freshwater as an activating solution at a sperm to solution ratio of 1:499 and 10:490, respectively in a 1.5 mL microtube. The video timer was recorded starting from sperm sample activation. After mixing, 8 μL of the mixture was taken and immediately put onto 21 well coated glass slide and sperm motility was recorded from 5 s until all sperm stopped moving [30].

Sperm motility (%) was examined by reviewing the video recordings. From the video images, sperm cells were randomly chosen and the motile sperm were counted. Only sperm that swim vigorously in a forward direction were counted as motile [31] and sperm that remained stationary or only vibrated were regarded as non-motile. Sperm motility was presented in percentage (%) which was calculated [motile sperm/total sperm counted] × 100. Measurements were repeated in duplicate for each dilution and the average was used in the data analyses. Duration of sperm motility was assessed by reviewing the video recording with the initial sperm duration considered beginning from the mixing of sperm into freshwater until all the sperm were non motile.

Upon sperm collection, sperm concentration was determined by using a haemocytometer method. Briefly, sperm was diluted at 10,000 times dilution ratio with 0.9% NaCl using 2 step dilution method. After that, 15 μL of the diluted sperm was placed under the cover slip on each side of the haemocytometer. Sperm cell concentration was observed using haemocytometer under biological microscope (CX43; Olympus; objective lens, 40 ×).

Spermatozoa velocity according to the actual path (curvilinear velocity, VCL; $\mu m\ s^{-1}$) was evaluated by using a semi-subjective method. The video recording of sperm motility was reviewed and the movement of sperm was observed until 10 s. The actual length of sperm movement was measured using a stage micrometer. In each condition, at least 50 sperm cells were examined and the average results were used for data analysis. This assessment was limited to the experiment's optimum cryoprotectant and concentration.

2.3. Sperm cryopreservation and thawing

Diluted sperm (fresh sperm to 0.9% NaCl ratio of 1:1) was mixed thoroughly with cryodiluent (cryoprotectant + extender) at dilution ratio of 1:9 (final sperm to cryodiluent ratio 1:19) and equilibrated for 5 min at room temperature. After equilibration, 250 μL of the dilution was loaded into 0.25 mL straw (I.M.V., France) and the open ends of straws were sealed with straw powder. The straws were frozen with liquid nitrogen (LN) vapor in a cylindrical stainless steel jar which was filled with LN to a depth 5–8 cm (Fig. 1) [32]. Straws were placed horizontally on an angular floating frame made of polysterene block at a height of 14 cm from the surface of LN (except for the experiment on optimal freezing rates) to expose the straws to LN vapor for 7 min. After 7 min, the final temperature reached −60 °C (cooling rates of 9.23 °C min^{-1}) and straws were then immediately immersed in the LN. All cryopreserved samples were held in the LN storage tank for at least 24 h before motility assessment.

Thawing was done by removing straws from the LN storage tank and placed right away in a water bath filled with water at room temperature (25 °C) for 15 s. Sperm was expelled into 1.5 mL centrifuge tubes after cutting them using a straw cutter with care to ensure no water was added. Thawed sperm were immediately used for sperm quality observations.

2.4. Determination of optimum cryoprotectant and concentration

Adopting the standard sperm cryopreservation and thawing described in 2.3, the effectiveness of three different cryoprotectants

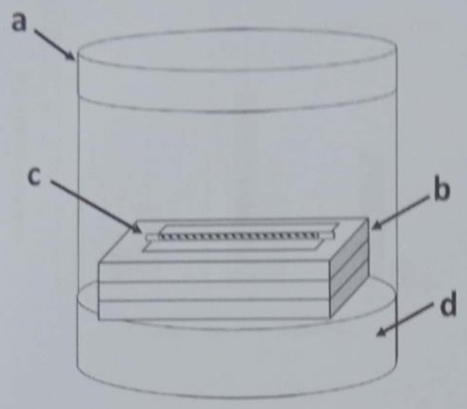


Fig. 1. Freezing setup for cryopreservation process. a) cylindrical stainless steel jar with a lid; b) hollow angular floating frame made of polysterene block in which height can be adjusted; c) 0.25 mL straw (I.M.V., France) was placed horizontally; d) liquid nitrogen was filled to a depth 5–8 cm.

including dimethyl sulphoxide (Me_2SO), dimethyl acetamide (DMA) and methanol (MeOH) at three different concentrations of 5, 10 and 15% with 0.9% NaCl as extender for a final dilution ratio of 1:19 were evaluated.

2.5. Cryoprotectant toxicity

To determine the toxicity of cryoprotectants, diluted sperm was subsequently suspended with the three cryoprotectants, Me_2SO, MeOH and DMA at concentrations of 5, 10, and 15% with the remainder of the solution consisting of 0.9% NaCl extender at final dilution ratio 1:19 and kept on crushed ice at 4 °C. Sperm quality was observed before dilution into cryodiluent and after 10, 25 and 50 min using the method mentioned above (Section 2.2).

2.6. Determination of optimum extender

Three types of extenders, i) 0.9% Sodium Chloride (NaCl), ii) Calcium-free Hanks' Balance salt solution (CF-HBSS: 152 mM NaCl, 6 mM KCl, 0.9 mM MgSO4.7H2O, 0.5 mM Na2HPO4.7H2O, 0.5 mM KH2PO4, 4.6 mM NaHCO3, 6.2 mM Glucose) and iii) ringer solution (128.34 mM NaCl, 2.7 mM KCl, 1.4 mM CaCl, 2.4 mM NaHCO3, 25.3 mM Glucose) were investigated in this study. Upon sperm collection, fresh sperm was diluted with three types of extenders at sperm to diluent ratio of 1:1. Then, diluted sperm was mixed with different cryodiluent (MeOH + respective extender) at a ratio 1:9, to achieve a final dilution ratio of 1:19 and cryoprotectant concentration of 10%. The samples were immersed in LN after being exposed to LN vapor for 7 min at a height of 14 cm. Samples were kept for at least 24 h before sperm quality assessment.

2.7. Determination of optimum cooling rates

Based on results from 2.4 to 2.6, the best cryodiluent was used for this experiment. Diluted sperm was suspended with 10% MeOH + 90% 0.9% NaCl as extender at final dilution ratio of 1:19 and was dispensed into a 250 μL straw. Then, straws were put on the hollow square floating frame and cooled. The cooling rates were controlled by adjusting the height of straws to 10, 12,14 and 16 cm on the floating frame (actual cooling rates of 11.98, 10.57, 9.23 and 6.25 °C min^{-1}, respectively) and cooling for 7 min (corresponding to immersion temperature of −80, −70, −60 and −40 °C, respectively) before immediately immersing the straws in LN. Cooling rates were computed based on the time taken for the temperature to change from 0 °C to final temperature. Samples were kept for at least 24 h before motility assessment.

2.8. Determination of optimum dilution ratios

To determine the optimum ratio, diluted sperm was mixed with 10% MeOH +90% 0.9% NaCl at three dilution ratios (1:4, 1:9 and 1:24), where final solution contained of 1:9, 1:19 and 1:49 dilution ratios of sperm to cryodiluent. The dilutions were exposed to LN vapor for 7 min at 14 cm height before immersed to LN. Samples were kept for at least 24 h before motility assessment. The post-thaw motility of sperm was observed after mixing with freshwater as an activating solution at a sperm to solution ratio of 5:245, 10:490 and 10:390, respectively in a 1.5 mL microtube for final dilution of 1:999.

2.9. Data analysis

All values were expressed as mean ± SE. Prior to analysis, data was normalised using arcsine square root transformation. One-way analysis of variance (ANOVA) was used to determine the best cryoprotectant, toxicity of the cryoprotectants, optimum extender and cooling rate as well as dilution ratios with the interaction on the sperm quality after thawing. Experiments which had significant differences were then evaluated using a post-hoc (Tukey) test to determine significance between treatments. The significance level was set at ($P < 0.05$) for all statistical analysis. All statistical analyses were performed using SPSS 25.0 software (SPSS, Chicago, IL, USA).

3. Results

3.1. Optimal cryoprotectant and concentration

The results on the effect of three types of cryoprotectants (Me_2SO, MeOH and DMA) at various concentrations (5, 10 and 15%) on post-thaw motility are shown in Fig. 2. Initial sperm motility, concentration, duration and velocity were 51.7 ± 2.4%; 2.09 ± 0.67 × 10^{10} cells/mL; 31.3 ± 0.33 s and 58.50 ± 1.97 μm s^{-1}, respectively. The 10% MeOH +90% NaCl treatment resulted in post thaw motility (PTM) of 26.3 ± 0.9% which was higher than all other conditions ($P < 0.05$). The PTM values of 15% MeOH (17.7 ± 3.7%) was the second highest followed by 10% Me_2SO (16.7% ± 2.4%), 5% Me_2SO (15.0 ± 3.4%), 10% DMA (12.0 ± 2.6%), 5% DMA (11.0 ± 2.5%) as well as 5% MeOH (4.7 ± 2.7%). Both 15% Me_2SO and DMA recorded the lowest PTM values at 2.2 ± 0.7% and 2.7 ± 0.3%, which was not significantly different ($P > 0.05$) compared to 5% MeOH.

The concentration of 15% MeOH (38.2 ± 0.4 s) showed the highest motility duration after post-thaw followed by 10% MeOH (35.2 ± 1.0 s) and 10% DMA (31.2 ± 3.2 s). There were no significant differences ($P > 0.05$) between all of these treatments. At 5% concentration, all cryoprotectants had a low motility duration of 25–26 s. The shortest motility duration was detected in 15% Me_2SO (16.2 ± 1.7 s) which was not significantly different with 10% Me_2SO (22.0 ± 0.8 s).

VCL of 10% MeOH (58.04 ± 2.45 μm s^{-1}) was significantly highest compared to all conditions tested here. The second highest VCL was observed in 15% MeOH (46.60 ± 3.10 μm s^{-1}), followed by 5% (39.80 ± 1.8 μm s^{-1}) and 10% DMA (40.30 ± 1.50 μm s^{-1}). The lowest VCL was observed in 15% Me_2SO (18.8 ± 0.8 μm s^{-1}) which was not significantly different to 10% Me_2SO (25.0 ± 1.1 μm s^{-1}).

3.2. Toxicity of cryoprotectants

The initial sperm motility, concentration and duration were 17 ± 1.3%; 2.09 ± 0.67 × 10^{10} cells/mL and 23 ± 1.1 s. 5% MeOH and 10%

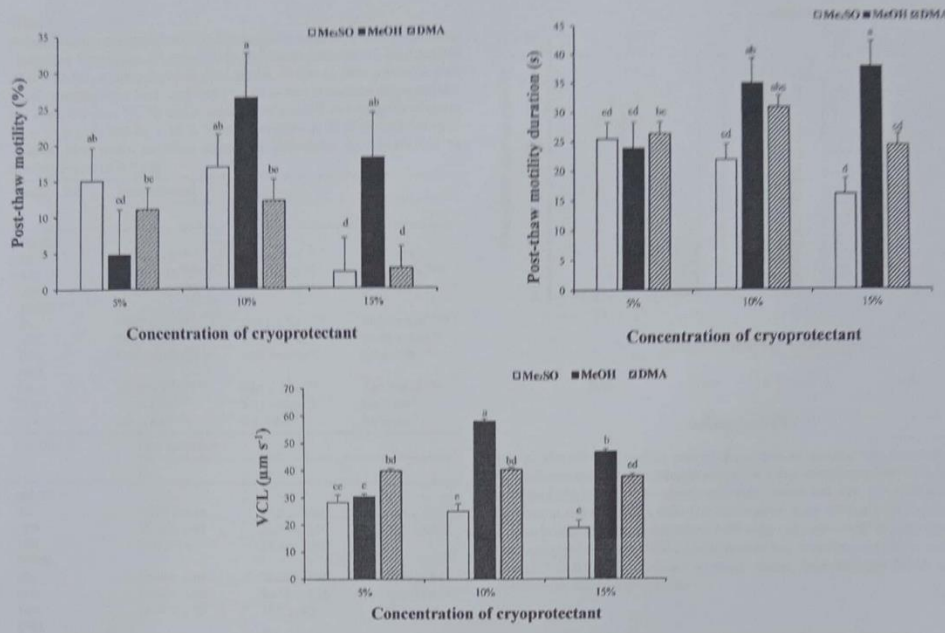


Fig. 2. Post-thaw quality of *Pangasius nasutus* sperm cooled at 14 cm above the surface of liquid nitrogen for 7 min with various cryoprotectant at 3 different concentrations (5, 10 and 15%) and 0.9% NaCl as an extender at a final dilution ratio of 1:19. Diluted sperm was inserted into 0.25 mL straw and equilibrated for 5 min. Me_2SO, dimethyl sulfoxide; MeOH, methanol; DMA, dimethyl acetamide. All values are mean ± SE of sperm from three samples (n = 3). Initial for sperm motility, duration and curvilinear velocity (VCL) were 51.7 ± 2.4%; 31.3 ± 0.33 s and 58.50 ± 1.97 μm s^{-1}, respectively. Values with different letters are significantly different (P < 0.05).

MeOH as well as 5% Me_2SO were least toxic at all intervals compared to other conditions (Table 1). However, there were no significant differences (P > 0.05) detected for 10% Me_2SO up to 5 min and 5% DMA up to 25 min exposure. All cryoprotectants at 15% were considerably more toxic compared to other cryoprotectant concentrations.

3.3. Optimal extender

The initial sperm motility, concentration and duration for this experiment were 35.2 ± 2.7%; 2.09 ± 0.67 × 10^{10} cells/mL and 22.9 ± 0.1 s. The effects of different extenders in the diluents on post-thaw motility are shown in Fig. 3. There were no significant differences (P > 0.05) for post-thaw sperm motility and duration at all types of extenders observed in this study.

3.4. Optimal cooling rate

Initial sperm motility, concentration and duration were 46.7 ± 2.4%; 2.09 ± 0.67 × 10^{10} cells/mL and 22.7 ± 0.9 s. The effects of cooling rates on post-thaw motility were investigated by adjusting the height of straw above LN vapor (10–16 cm), and the time exposed to LN vapor was set to 7 min before plunged into LN. The highest PTM result (17 ± 0.6%) was observed at cooling rate 9.23 °C min^{-1}, at 14 cm height (corresponding to immersion temperature −60 °C) which was significantly higher (P < 0.05) than others (Fig. 4). When cooling below and above the optimal cooling rates, PTM dropped drastically.

3.5. Optimal dilution ratio

Initial sperm motility, concentration and duration were 45.7 ± 1.9%; 2.09 ± 0.67 × 10^{10} cells/mL and 23.7 ± 1.5 s. The results of different dilutions ratios are seen in Fig. 5. There were no significant differences (P > 0.05) for post-thaw motility and duration at all dilution ratios (1:9, 1:19 and 1:49) investigated in this study.

4. Discussion

The most common parameters used to assess the quality of the sperm after cryopreservation is motility, which typically has a positive correlation with fertility [20,33]. However, there is no information about sperm motility and its characteristics for *P. nasutus*. In the present study, the initial motility of fresh sperm for *P. nasutus* was lower than 60% which was 51.67 ± 2.4% and sperm became rapidly immotile within 1 h after stripping. For this study, milt was obtained from male broodstocks belonging to a private fish farm. As milt was obtained during the commercial production phase of the farm, hormonal induction was not considered to increase male *P. nasutus* sperm quality following the protocols of the farm. However, the sperm quality of *P. nasutus* sperm obtained was similar to that our previous trials in which we obtained milt from wild caught fish (unpublished data).

In the present study, we evaluated three different cryoprotectants (Me_2SO, DMA, MeOH) which had previously been used to cryopreserve fish sperm at different concentrations, varying from 5 to 15% [34]. The most successful cryoprotectant with the highest post-thaw motility of 26.3 ± 0.9% was 10% MeOH with 0.9% NaCl when cooled at height of

Table 1
Percent motility and duration of *P. nasutus* sperm suspended with various concentrations of cryoprotectants and 0.9% NaCl as an extender at a final dilution ratio 1:19. Me_2SO, dimethyl sulfoxide; MeOH, methanol; DMA, dimethyl acetamide. Samples were kept on crushed ice in an icebox and the sperm motility was observed after 10, 25 and 50 min. Initial motility and duration of sperm were 17 ± 1.3% and 23 ± 1.1 s. Values are means ± SE of sperm from three samples. (n.d. shows no data). Significant differences are represented by different letters ($P < 0.05$).

Motility (%)	Time (Minutes)		
	10	25	50
ME_2SO			
5%	14.33 ± 1.2 ^{ab}	9.67 ± 0.33 ^{abcd}	12.0 ± 1.15 ^{abcd}
10%	7.33 ± 1.33 ^{abcd}	5.67 ± 0.88 ^{cde}	6.67 ± 1.76 ^{bcde}
15%	5.33 ± 0.88 ^{cde}	0.67 ± 0.67 ^{gh}	0.0 ± 0.0 ^{h}
MEOH			
5%	15.33 ± 1.76^{a}	12.67 ± 0.67 ^{abc}	11.67 ± 1.45 ^{abcd}
10%	15.33 ± 1.2^{a}	14.33 ± 1.45 ^{ab}	14.33 ± 2.19 ^{ab}
15%	8.67 ± 0.67 ^{abcd}	4.67 ± 0.33 ^{def}	2.0 ± 0.58 ^{efg}
DMA			
5%	10.67 ± 1.76 ^{abcd}	9.67 ± 2.0 ^{abcd}	4.67 ± 0.33 ^{def}
10%	5.0 ± 1.73 ^{def}	1.33 ± 0.67 ^{efg}	0.0 ± 0.0 ^{h}
15%	2.0 ± 0.58 ^{efg}	0.0 ± 0.0 ^{h}	0.0 ± 0.0 ^{h}

Duration (S)	Time (minutes)		
	10	25	50
ME_2SO			
5%	18.33 ± 0.89	17.0 ± 0.58	20.0 ± 2.65
10%	17.67 ± 1.85	16.33 ± 2.73	22.0 ± 0.33
15%	11.0 ± 1.0	14.0 ± 1.33	n.d
MEOH			
5%	23.83 ± 1.67	21.00 ± 2.30	21.67 ± 3.84
10%	19.67 ± 2.60	19.50 ± 2.75	21.67 ± 3.17
15%	18.67 ± 3.18	18.0 ± 0.0	17.0 ± 0.0
DMA			
5%	21.00 ± 4.93	21.50 ± 1.80	13.83 ± 7.67
10%	15.67 ± 2.96	15.33 ± 1.45	n.d
15%	11.67 ± 1.67	n.d	n.d

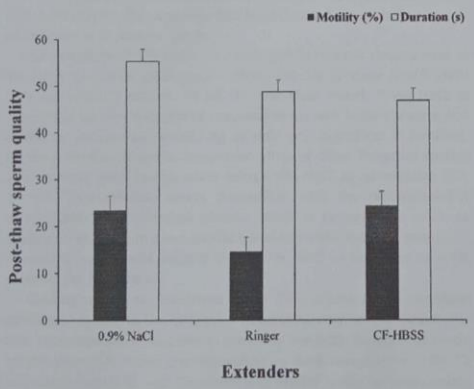


Fig. 3. Post-thaw motility and duration of *Pangasius nasutus* sperm cooled at 14 cm height above the surface of liquid nitrogen for 7 min in 10% MeOH and various extenders. 0.9% NaCl, Ringer and CF-HBSS, Calcium free hanks' balance salt solution. All values are mean ± SE of sperm from three samples (n = 3). Initial for sperm motility and duration were 35.2 ± 2.7% and 22.9 ± 0.1 s. There were no significant differences between values ($P < 0.05$).

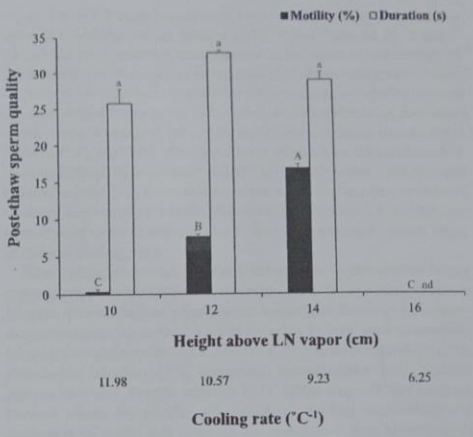


Fig. 4. The effect of cooling rate on the post-thaw motility and duration of *Pangasius nasutus* sperm at different heights of straw above the surface of liquid nitrogen (LN). Sperm were diluted with 10% MeOH and 90% 0.9 NaCl as an extender at a final dilution ratio 1:19 and cooled above LN vapor for 7 min at various heights (10, 12, 14 and 16 cm). All values are mean ± SE of sperm from three samples (n = 3). Initial for sperm motility and duration were 46.7 ± 2.4% and 22.7 ± 0.9 s. (n.d. shows no data). Values with different letters are significantly different ($P < 0.05$).

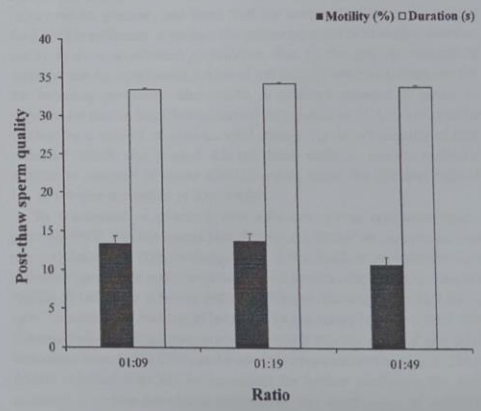


Fig. 5. The effect of different dilution ratios on the post-thaw motility and duration of *Pangasius nasutus* sperm at 14 cm height of straw above the surface of liquid nitrogen for 7 min. Diluted sperm with 0.9% NaCl at ratio 1:1 was suspended with 10% MeOH and 90% 0.9 NaCl as an extender at a final dilution ratio 1:19. Initial for sperm motility and duration were 45.7 ± 1.9% and 23.7 ± 1.5 s. Values are means ± SE of sperm from three samples (n = 3). There were no significant differences between values ($P > 0.05$).

14 cm from LN surface for 7 min. MeOH has been successfully used to cryopreserved sperm for many freshwater fish such as catfish [35], common carp [36], salmonids [37,38] and eels [32,39,40]. This chemical is known as an intracellular cryoprotectant with a low molecular weight that diffuses into the cells more quickly than other cryoprotectants [22]. When applied as a cryoprotectant, MeOH enhances cell survival throughout the cryopreservation process by penetrating quickly into the sperm and enables sperm to achieve osmotic equilibrium readily under freezing [41] without suffering any osmotic shock-related toxicity [40]. Moreover, corresponding to our toxicity results, motility of sperm suspended in 10% MeOH was not affected until 50 min, indicating that

this cryoprotectant has a good permeability and does not cause osmotic stress in *P. nasutus* sperm. This may be explained by the fact that MeOH penetrates into the sperm cells at a rate comparable to the rate of water transport, therefore preventing osmotic stress [42].

Our results also revealed that there were differences with motility duration and VCL when cryopreserved using the different concentration of cryoprotectants. MeOH at 10% concentration resulted in highest motility duration (35.2 ± 1.0 s) and VCL (58.04 ± 2.45 μm s^{-1}) compared to others condition. Motility duration and velocity are closely related to the energy production and propulsion mechanisms within the sperm cell [43]. This is primarily influenced by the presence of ATP (adenosine triphosphate), the role of mitochondria, and the structure and function of the flagella. Mitochondria produce ATP, which serves as the energy source for the flagellum's movement [44]. The efficient production of ATP in the mitochondria is crucial for maintaining the energy levels required for the sperm's rapid and sustained movement [45]. Hence, this indicates that 10% MeOH can avoid depletion of ATP levels or damage to mitochondria in sperm cells during the freezing and thawing process. Consequently, it is important to maintain the motility, duration and velocity of the sperm after the freezing process in order to successfully reach the oocytes and fertilize the eggs. Therefore, utilizing cryopreserved sperm with the highest motility duration and VCL will increase the probability of fertilization success.

Based on previous studies of sperm cryopreservation in *Pangasius* species, Me_2SO is the best cryoprotectant where 10% Me_2SO is the best for *P. bocourti* sperm cells [22] as well as for *P. larnaudii* [23], 9% Me_2SO for *P. gigas* [25] and 12% Me_2SO for *P. hypophthalmus* [12]. Me_2SO is a permeating cryoprotectant [46,47] that is widely used as a cryoprotectant for a variety of aquatic species [48,49]. This substance is distinguished by its tiny molecular size, rapid penetration ability, and interaction with plasma membrane phospholipids [48]. However, Me_2SO has been reported to be toxic and has higher osmolality compared to MeOH [50]. From our toxicity result, a significant reduction in sperm motility was observed in 10% and 15% concentrations of Me_2SO compared to MeOH. Moreover, motility duration and velocity of post-thaw sperm for all concentrations of Me_2SO was significantly lower than 10% MeOH. This suggests that Me_2SO may be unsuitable as cryoprotectant for *P. nasutus* sperm.

All extenders (0.9% NaCl, CF-HBSS and freshwater ringer) used in the study produced good results which did not produce significantly different motility values. Of all the solutions tested, 0.9% NaCl is potentially the best for farm or commercial use as it is the cheapest and easiest to prepare as the mixing of only one ingredient is required. Previous studies in sperm cryopreservation of other *Pangasius* species also reported good results when using 0.9% NaCl as an extender [12, 23–25]. This solution seems compatible with the physiochemical composition of the seminal plasma which is necessary in order to maintain the sperm in a non-motile but viable state until it is used [25]. Therefore, our results suggest that 0.9% NaCl is the most suitable extender for *P. nasutus*.

Cooling rate is an important factor [51] affecting the post-thaw motility of sperm, as the optimal cooling rates vary with different species. The optimum cooling rate in our study was 9.23 °C min^{-1}, at 14 cm height above LN vapor (corresponding to final temperature −60 °C; calculated from 0 to −60 °C) which was similar to the optimal cooling rates in other *Pangasius* sp. where samples were cryopreserved using a computer-controlled freezer at cooling rates of 10 °C min^{-1} [12,22–25]. This indicated that slower cooling rates were more suitable for freezing of *Pangasius* sp. sperm and *P. nasutus* shared the same traits. Additionally, it should be noted that the type of cryoprotectant and effective cooling rates are connected [46]. MeOH is believed to be more effective at slower cooling rates as a cryoprotectant because of the reduced slow cooling damage especially that of intracellular ice [46]. However, dehydration and long-term exposure to high solute concentration might kill cells if the cooling rate is too slow [52]. This was also noted in our study as freezing at a slower cooling rate of 6.25 °C min^{-1} (at height of 16 cm above LN vapor) resulted in total loss of motility in post thaw sperm. Meanwhile, cooling rates of 11.98 °C min^{-1} and 10.57 °C min^{-1} are too fast for *P. nasutus* sperm cells and would result in cell damage by the formation of intracellular ice crystals [53]. Fast cooling rates do not allow sufficient egress of intracellular water to maintain equilibrium and the supercooled water in the cells undergoes intracellular ice formation which causes damage to the cell by disruption of cellular structure and function [54]. Moreover, the formation of intracellular ice is followed by recrystallization during thawing if the thawing rate is slow, which is also harmful to cells [52]. Nevertheless, in our study, fast thawing rates were used and thawing rates probably did not affect the results. Therefore, the present results clearly indicate that *P. nasutus* sperm has a narrow range of optimal cooling rates.

The cooling protocol in our study involved the manipulation height of the straws from the LN surface using the vapor to cryopreserve sperm samples. 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 *P. bocourti* [55], *Clarias gariepinus* [35,56], *Pelteobagrus fulvidraco* [57], *Epinephelus septemfasciatus* [58], *E fuscoguttatus* [30] and *Anguilla japonica* [32]. While usage of the program freezers allows for specific and adjustable cooling rates which the equipment is costly and requires an investment that breeders and farmers are reluctant to commit. The cooling protocol used here is therefore an acceptable alternative to using the program freezer, as it is a convenient and reliable method for field operations or in rural areas where there is no advanced or costly equipment.

In the current study, the application of dilution ratio 1:9 to 1:49 did not show any significant differences among them. Therefore, any ratio between these ranges can be used because the cryodiluent is enough to protect and cover the amount of cells during the cryopreservation process. It has been proven that the concentration of protective chemicals (e.g., protein, glucose, and ions) that are naturally present in seminal fluid [50] is sufficient to protect the sperm membrane at higher dilution ratios. From a application standpoint, due to the greater volume of spermatozoa in cryodiluent, ratios of 1:9 to 1:19 are often adequate for the breeding programs. Meanwhile, a minimal amount of sperm is required for research activities, and dilution ratios of 1:19 to 1:49 can be utilized for a variety of experimental setups. The small amount of milt collected which was around 2.5 mL from male *P. nasutus* makes it difficult to compare at minor dilution ratios, hence the dilution ratio of 1:1 to 1:3 was not tested in this study.

To summarise, a practical and effective sperm cryopreservation protocol for *P. nasutus* sperm was developed. Based on our results, we can conclude that 10% methanol with 0.9% NaCl as an extender is a suitable cryodiluent and cryopreservation process should be conducted within 10 min after dilution with cryodiluent. Cooling rates at 9.23 °C min^{-1} obtained by freezing at height of 14 cm above LN vapor for 7 min (corresponding to final temperature −60 °C) was the optimal and dilution ratios from 1:9 to 1:49 can be used to cryopreserve the sperm. These results obtained here will be important for further studies on the optimization of cryopreservation procedures and application of artificial fertilization for *P. nasutus* species. Additional investigation on varieties of packaging such as different volumes of straws or cryotube and cryovials and low dilution ratios (1:1 to 1:3) are needed to improve the efficiency of sperm cryopreservation protocol for this species. Other evaluation methods such as fertilization efficiency, sperm viability and DNA integrity should also be considered to evaluate sperm quality.

Ethical approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed by the authors.

Declarations of competing interest

None.

BIODATA OF AUTHOR

Name	:	Wan Nurizzati binti W Idris
Hometown	:	Tanah Merah, Kelantan.
Phone number	:	0145053358
Email	:	nurizzatidris@gmail.com
Date of birth	:	21 October 1994

Education background :

Master of Science (Aquaculture) (by research) Research tittle: The development of sperm cryopreservation protocol for *Pangasius nasutus* (Bleeker, 1986) Universiti Malaysia Terengganu	**2020-Present**
Bachelor of Science in Agrotechnology (Aquaculture) (Hons) Universiti Malaysia Terengganu	2015-2018
Diploma of Fisheries Universiti Malaysia Terengganu	2012-2015
Sijil Pelajaran Malaysia (SPM) Sekolah Menengah Kebangsaaan Tanah Merah (1)	2007-2011