Chapter 1

1.0 INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Population of Malaysia is approximately 25 million people and is increasing annually. Consequently, demand for food including from animal proteins such as from fish is also increasing. In 2007, the fisheries sector produced RM 6.47 million, consisting 1,654,217.98 metric tonnes of fish and 558,178,294 of ornamental fish valued at RM 647.05 million. Fisheries activity contributes about 1.2% to our national KDNK in the same year (Department of Fisheries, 2007). However, the supply of fish to meet the country's fish requirement has not been met; and, therefore, there is continuous importation of fish and their products annually. Realising this, the Malaysian Government gives a high priority in fisheries development including aquaculture sector.

Worldwide, development in aquaculture industry over the years has made a continuous and ample impact on fisheries industry and business. Numerous aspects of aquaculture industry including research and development activities have been conducted to improve the technologies related to aquaculture development. In Malaysia, aquaculture sector has involved the culture of freshwater fishes such as tilapia, catfish, carps and mahseer. These fishes are popular for human food consumption in Malaysia due to health awareness among Malaysian whereby many people prefer to eat fish than other animal proteins in their diets.

Mahseer (*Tor tambroides*) fish are a delicacy and valuable fish. Mahseer population in Malaysia is decreasing in spite of its high demand and lucrative business potential. In peninsular Malaysia and Sarawak, *Tor tambroides* is known as *kelah* and *empurau*, respectively, is indigenous, valuable mahseer species, with an aquaculture

potential and of conservational value. Normally, this freshwater fish lives in headwaters of most major river systems and a group of inhabiting fast flowing mountainous streams and rivers in the plains, often preferring clear, swift flowing waters with stony, pebbly or rocky bottoms (Shreshtha, 1997). This species is also found in Peninsular Malaysia and is distributed throughout Southeast Asia from Indonesia to Southern China (Kottelat *et al*., 1993; Zhou and Cui, 1996; Roberts, 1999).

The decline of mahseer natural stock is partly due to anthropogenic activities towards our streams and rivers and over fishing also has dampened its production to a certain extent (Ng, 2004). Juveniles of this species are increasingly sought after by the aquarium industry (Ng, 2004). Now, this species has faced a serious problem of extinction and considered as a threatened species. This species is considered as one of the most expensive freshwater food fish due in part to the decreasing population in the wild. The market value of this fish can exceed US$ 50 per kg in Malaysia.

At same time, most fish breeders feel the ultimate potential to improve the fish production as well the conservation process are still remains within the aquaculture industry. The importance of this species has been recognised and a concerted attempt has been made to evaluate their aquaculture potential, including captive breeding using long-term pond-reared brood stock, commencing in the 1990s (Ingram *et al*., 2005). There has been a strong attempt to artificially propagate several *Tor* species to produce one of the conservation ways to protect this endangered species.

Production of mahseer fingerlings using artificial method is still facing problem (Petr and Swar, 2002; Ingram *et al*., 2005). During the year of 2002-2004, a limited success was obtained through international research collaboration between Australia and

Sarawak whereby they were able to captive breed this species using hormone induction techniques on long-term, pond-reared brood stock (Ingram *et al*., 2005).

Captive breeding of most *Tor* species is based on wild-caught, mature fish (Joshi, 1988; Nandeesha *et al*., 1993; Gurung *et al*., 2002). Some success with pond reared broodstock of a few species has been reported (Ogale, 2002; Ingram *et al*., 2005). Thus, mass production in the hatchery is still unstable since this species is not reliable to spawn naturally in captivity. We need an effort to create suitable techniques to sustain the production of this endangered species. In India, efforts are being made to revive the stocks of *Tor khudree* through *ex situ* conservation measures of which cryopreservation has been the much sought-after technique (Basavaraja and Keshavanath, 2000). However, little information is available on the reproductive biology and no sperm cryopreservation attempt of *Tor tambroides* is apparently insufficient to ensure the suitable and success procedure for this conservation technique.

Sperm cryopreservation can be applied in fish industry to the demand for fish products today. Cryopreservation of sperm has been develop over several decades and it is used widely now in practical programmes. Sperm cryopreservation protocols are now available for over 200 species of finfish and shell fish (Scott and Baynes, 1980; McAndrew *et al*., 1993; Billard *et al*., 1995a). Cryopreservation plays an important role in the production and propagation of new strains/breeds of economically important species and in the conservation of wild stocks of threatened aquatic species. In this context, a few `sperm banks' have been created, notably for grouper, salmonids and Indian cultivated and endangered fish species (Chao *et al*., 1992; Rana, 1995; Ponniah, 1998).

Studies on cryopreservation of invertebrate eggs, embryos and larvae have also been met with encouraging success (McAndrew *et al*., 1993; Subramoniam and Arun,

1999). Cryopreservation of fish sperm is not as complicated when compared to freezing of ova or embryos. Most of research activities in fish sperm cryopreservation have involved the studies on different aspects including different species, different cryopreservation methods, different factors affecting freezeability of sperm, mechanism and physiology of sperm during the freezing process. For example, successful sperm cryopreservation protocol of any fish species may be obtained from procedures with different equilibration durations and cooling rates (Vuthiphandchai *et al*., 2009).

Meanwhile, sperm cryopreservation of mahseer is receiving some attention within the past 20 years ago. In the process of developing sperm cryopreservation procedure, many cryoprotectants have been tested and used on marine and freshwater fish sperm. Basavaraja and Hedge (2004) have successfully cryopreserved the sperm of *Tor khudree* using modified Fish Ringer Solution (mFRS). In mFRS, dimethyl sulfoxide (DMSO) is commonly used in the fish sperm cryopreservation protocol that was found to be effective to prevent the sperm damage of *Tor khudree* (Ponniah *et al*., 1992; Basavaraja and Hedge, 2004) and *Tor putitora* (Ponniah *et al*., 1999a) during the freezing process. Therefore, mFRE extender using DMSO (10%) was the extender of choice for this study.

Ponniah *et al*. (1992), who cryopreserved *Tor khudree* and *Tor* hybrid sperm, obtained 80.00 and 70.00% frozen-thawed motility, respectively, after storage in liquid nitrogen (LN_2). A few years later, Basavaraja and Hedge (1998) and Basavaraja and Hedge (2003) successfully cryopreserved *Tor khudree* sperm and a high motility rate of more than 93.00% was obtained after cryopreservation in LN_2. Both researchers used mFRS extender in their studies. However, Basavaraja and Hedge (2004) have performed the sperm cryopreservation in *Tor khudree* using modified fish Ringer solution and DMSO as cryoprotectant which produced 80.00 to 81.00% and 43.00 to 67.00% of frozen-thawed

cryopreserved sperm after 10 and 70 days of storage, respectively. Basavaraja *et al*. (2002) reported that a good fertility of cryopreserved *Tor khudree* sperm was obtained after fertilising with the eggs.

Ponniah *et al*. (1999a) produced viable hatchlings of *Tor khudree* from one year-old cryopreserved milt. Similarly, Ponniah *et al*. (1999b) obtained viable hatchlings of *Tor putitora* from sperm cryopreserved for one year. Isswara *et al*. (1999) have suggested guidelines for establishing commercial sperm banks in India.

Some of the research regarding this process reported post-thawed motility of sperm depends greatly on the equilibration period before freezing (Basavaraja and Hedge, 1998; Horvath and Urbanyi, 2000; Sansone *et al*., 2002). Thus, in the development of Thai mahseer sperm cryopreservation procedure also required optimal equilibration duration to obtain a successful result in term of viability of the frozen-thawed sperm.

Besides, one of the important phase that involved in the cryopreservation process is vapour exposure phase which the sperm will be expose to liquid nitrogen vapour. How long the duration of the phase and optimal end point temperature should be determined to produce optimal preparation for the sperm before it being plunged into the LN_2. While the mechanism involved in this phase take place, it needs the optimal duration and temperature of the vapour.

With the optimal combinations of factors in this process, we may apply it under real field condition. This technology could be modified for simplification and practicality to be used under field condition. If this is achievable, this technique will be useful to be applied in our effort to prevent the endangered species like Thai mahseer from being extinct.

With this background, this research will be focused on studies involving collection and subsequent freezing of semen of mature male Thai mahseer. This is the fish of choice for consideration and was based on its economic and commercial potential as well as for conservation of this species from extinction.

As conclusion, Thai mahseer stock needs restoration and protection as being a valuable fish of hills both from conservation and commercial food consumption. A meaningful strategy would be the judicious combination of reproduction and conservation. Only then this endangered game fish can be rescued from extinction and the various sanctuaries, rivers, streams, pools and others could be saved from turning into deserts of aquatic. Therefore, in our opinion, it is justifiable that this study on cryopreservation of Thai mahseer's sperm to be embarked in line with the country's efforts in commercialisation as well as conservation programmes of this fish.

1.2 STATEMENT OF PROBLEMS

There are several major questions which could not be answered yet regarding sperm cryopreservation with special reference to *Tor tambroides* which include:

a) What is the standard sperm cryopreservation protocol for *Tor tambroides*?
b) What is the optimal extender for cryopreservation of *Tor tambroides* sperm?
c) What is the optimal equilibration duration and vapour exposure temperature as well as duration during the process of cryopreservation of *Tor tambroides* sperm?
d) What is the optimal combination of factors (equilibration duration, vapour exposure temperature and vapour exposure duration) during cryopreservation process in order to obtain maximal frozen-thawed motility of *Tor tambroides* sperm?

e) Is it any difference on sperm characteristics using different refrigerator/incubator (between normal refrigerator and low temperature incubator) after certain equilibration duration?

f) Is the normal refrigerator is suitable enough to be apply to perform sperm cryopreservation under field condition?

g) Would it possible to perform *Tor tambroides* sperm cryopreservation under field condition?

h) What is the highest frozen-thawed sperm motility for *Tor tambroides* that we could achieve?

i) Is there any significant difference in sperm frozen-thawed characteristics between performing sperm cryopreservation in laboratory and field condition?

j) How is the correlation between body weight and body length on frozen-thawed sperm motility characteristics?

1.3 JUSTIFICATION

To our knowledge, there are no reports in the literature on cryopreservation of *Tor tambroides* sperm. Most of the studies were conducted involving cryopreservation of *Tor khudree* and *Tor putitora* using Fish Ringer Extender. Therefore, development of sperm cryopreservation protocol for *Tor tambroides* is urgently needed due to its low population in the wild as well as this species is at the brink of extinction. In Malaysia, our labarotary at the University of Malaya is undertaking this challenge and has given a high priority in research in an effort to cryopreserve the *Tor tambroides* sperm. Subsequently, this cryopreservation method was designed to test under field condition. If this pioneer study is

successful, it would be very beneficial not only to the wildlife agencies but also possibility of commercialising this species for human food as well as for ornamental purposes.

1.4 APPLICATION

Several ex situ conservation strategies have been suggested for the revival of stocks of *Tor tambroides*, a threatened species. Cryopreservation of sperm is crucial for the conservation of stocks of endangered species so that sustainable production can be ensured (Basavaraja and Hedge, 2004). It is essential that selected broodstocks are able to produce high quality milt to ensure the optimal production of viable sperm. Similarly, development of such a database for fish could benefit commercial aquaculture through increased production capabilities (Christensen and Tiersch, 2005).

Assessment of the sperm quality will give us the bigger picture of the reproductive performance for each fish. This process is good for us to know and differentiate which fish have the higher commercial value which can be used in the future to expand and broaden their population. Successful attempt to perform sperm cryopreservation under field condition will emphasise the possibility of doing this procedure anywhere because of their flexibility.

The great and ample benefits from fish gamete preservation include (Lubzenz *et. al*., 1997):

a) Potentially greater efficiency in selective breeding through storage of gametes from genetically improved fish stocks obtained by classical selective techniques, genetic manipulation (for example, triploids), or the recently obtained transgenic fish, cross-fertilisation in prodantic hermaphrodites (for example, gilthead sea bream, *Sparus aurata*) and extension of the breeding season in fish.

b) Increased protection of stocks from disease by allowing the introduction of new genetic lines with reduced danger of transmitting unknown pathogens to cultured fish.

c) A continuous supply of gametes for optimum utilisation of hatchery facilities or for experimentation.

d) Economy of maintenance of brood stock and providing a safe guard for accidental loss of genetically improved lines.

e) Easy of transport of genetic material between hatcheries within and between countries, thus making genetically protected, cryopreserved gametes and embryos an attractive product *per* se.

1.5 OBJECTIVES

Thus, the objectives of this research were:

a) To determine the effects of equilibration duration, vapour exposure temperature and vapour exposure duration in sperm cryopreservation protocol for Thai mahseer (*Tor tambroides*) fish using the modified Fish Ringer Extender (mFRE).

b) To develop optimal combination of equilibration duration, vapour exposure temperature and vapour exposure duration for sperm cryopreservation in Thai mahseer (*Tor tambroides*) fish.

c) To compare the frozen-thawed sperm characteristics between low temperature incubator and normal refrigerator during cryopreservation process.

d) To attempt the transfer of technology from laboratory to the field condition for the sperm cryopreservation of Thai mahseer (*Tor tambroides*) under field condition using optimal combination of equilibration duration, vapour exposure temperature and vapour exposure duration.

Chapter 2
LITERATURE REVIEW

Chapter 2

2.0 REVIEW OF LITERATURE

2.1 FISH SPERM

2.1.1 General Fish Morphology and Characteristics

Generally in fish, the milt production per year is high (Yaron, 1995). During spermatogenesis of carp fish which is very short, the morphological changes of the spermatids are very limited which consist only of a slight elongation of the head, which becomes elliptical, and a limited degree of histone transformation among the nuclear proteins (Nandi *et al*., 1979). However, in grass carp, there was non-histone proteins observed (Kadura *et al*., 1985). The chromatin is granular and not highly condensed (Stein, 1981; Baccetti *et al*., 1984).

Basically, the spermatogenesis is initiated again when the sperm was released during the spawning season and thus, there were some indications suggested that unreleased sperm from the previous cycle may be found in the old male's testis (Billard and Cosson, 1992). According to Cruea (1969), carp fish was found to have high rate of abnormal sperm that may have explained the ageing of sperm may occur at some degree. This may be due to urine contamination which is frequent after hypophysation carried on outside the reproductive season and induces a hypo-osmotic shock, partial activation and alteration of the sperm structure (Perchec *et al*., 1995).

Most external fertilising teleosts possess immotile sperm on ejaculation and will only become motile and metabolically active after being released into the water (Rurangwa *et al*., 2004). After the activation, generally in most freshwater fish, the sperm usually moves actively for less than 30 seconds and shows gradual lower motility within 2 minutes after activation (Morisawa and Suzuki, 1980; Perchec *et al*., 1993; Billard *et al*., 1995a;

Kime *et al*., 2001). However, there were some fish species that have longer period of motile sperm after activation and could remain in motile condition for 1-2 days, for examples, the spotted wolffish (*Anarhichas minor*) and the 3- as well as 15-spined sticklebacks (*Gasterrosteus aculeatus, Spinachia spinachia* (Kime and Tveiten, 2002; Elofsson *et al*., 2003a,b). Similar characteristics have also been found in some marine sculpins (Koya *et al*., 1993), and the ocean pout (*Macrozoarces americanus*) (Yao and Crim, 1995). In ocean pout, within 5 days after activation, the sperm could remain motile in seminal fluid or in well-designed milt diluent. (Yao *et al*., 1999a). These sperm characteristics were suggested to be considered as important criteria when developing a protocol for fish sperm cryopreservation (Cosson *et al*., 2000).

2.1.2 Fish Spermatogenesis

A process of forming the mature sperm is related to spermatogenesis which is a well-organised and coordinated mechanism that involves the proliferation and differentiation of diploid spermatogonia (Schulz *et al*., 2009). Longer duration was observed in spermatogenesis of mammalian sperm compared to duration of fish sperm production which is also influenced by the water temperature (Nobrega *et al*., 2009). In principle, Schulz *et al*. (2009) and Nobrega *et al*. (2009) suggested that the process of spermatogenesis in fish germ cell is similar to that of the mammalian species. It can be divided into three different phases: the mitotic or spermatogonial phase with the different generations of spermatogonia (i.e., undifferentiated spermatogonia including the stem cells, and differentiated or differentiating spermatogonia); the meiotic phase with the primary and secondary spermatocytes; and the spermiogenic phase with the haploid

spermatids emerging from meiosis and differentiating without further proliferation into motile, flagellated genome vectors, the sperm (Figure 2.1).

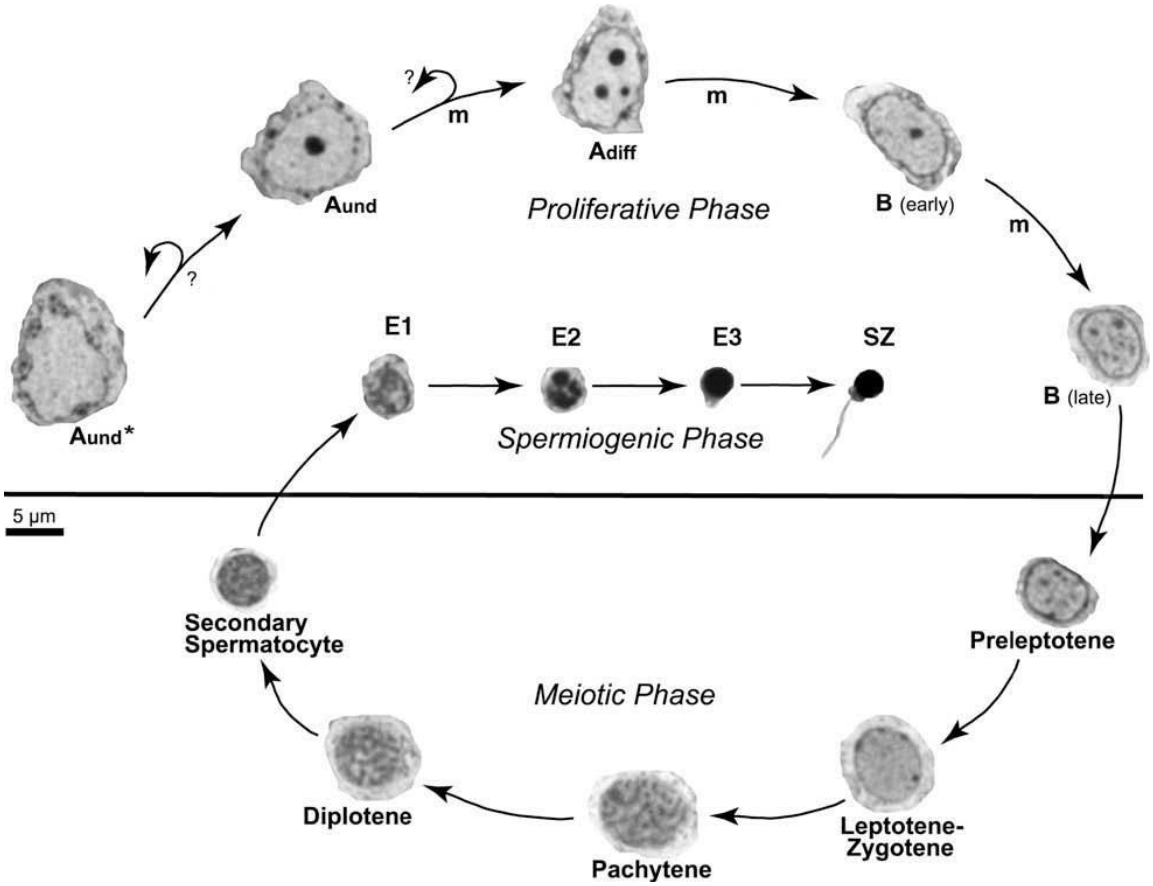

Figure 2.1: Step-by-step representation of zebrafish spermatogenesis from undifferentiated spermatogonia to sperm, throughout the three phases of the spermatogenic process: proliferative or spermatogonial, meiotic or spermatocytary and spermiogenic. The spermatogonial micrographs show the most characteristic features of each cell type, although there is a range of their morphology and size. Type A undifferentiated* spermatogonia (Aund*); type A undifferentiated spermatogonia (Aund); type A differentiated spermatogonia (Adiff); type B spermatogonia (B); self-renewal (curve arrows); mitosis (m); early spermatids (E1); intermediate spermatids (E2); final spermatids (E3); and sperm (SZ) Aund* and Aund are single cells, whereas Adiff, B (early) and B (late) are grouped. (Adapted from Schulz *et al*., 2005; Leal *et al*., 2009).

2.2 FACTORS AFFECTING THE QUALITY OF SPERM

It is important to have quality fresh semen before proceed to sperm cryopreservation process and various factors may affect it. The quality of sperm is depending on the biotic and abiotic factors that may influence the successful of the sperm cryopreservation results.

2.2.1 Biotic Factor

2.2.1.1 Individual genetic inheritance and species-dependent fish

Generally, individual fish could provide variations on their quality of sperm as it depends on the genetic inheritance of each fish. Rana (1995) reported a high individual variation of milt quality that was caused by individual fish. Level of lipid composition of the sperm plasma membrane has caused significant variation (Parks and Graham, 1992). Similar reports were made by Parks and Lynch (1992) and Drokin (1993) who suggested that specific composition of membranes such as the cholesterol: phospholipid (Cho/PL) ratio could affect the quality of milt. According to Chowdhury and Joy (2007), there are clear relationships between seminal vesicle plasma consituents, osmolarity and the duration of fish sperm motility, however, no relationship was shown between the individual components of seminal vesicle plasma and sperm motility.

Some researchers reported that the success of sperm cryopreservation such as sperm survivability is dependent on fish species and type of fish (marine or freshwater) involved. Billard *et al*. (1995a) stated that sperm cryopreservation of marine fish sperm is easier to perform as compared to freshwater species. This statement may be related to level of freezing resistance which freshwater fish sperm from marine fish species showed higher resistance to cryopreservation process (Scott and Baynes, 1980). Even though it is yet to be experimentally tested, Drokin (1993) stated the ratio of Cho/PL in fish sperm could be

affected by the habitat salinity. According to Medeiros *et al*. (2002), different procedures of sperm cryopreservation are required for different species of fish with special considerations to variation in their cell shapes, cell organelles size and composition.

2.2.1.2 Intra-testicular aging of sperm

Intra-testicular aging of sperm has been reported for many fish species and it has affected sperm quality at the end of the milting period (Rana, 1995). In the 3-spined stickleback *Gasterrosteus aculeatus*, the amount of sperm in the testes and the size of the ejaculate were reduced in males that had mated several times (Zbinden *et al*., 2001).This statement is supported by Hafez and Hafez (2000) that semen quality are influenced by size of testicular and other factors such as the degree of sexual stimulation, frequency of ejaculation, age and methods of semen collection.

2.2.1.3 Spawning season

As the spawning season progressed, the concentration of marine fish sperm such as sea bass decreased as well (Fauvel *et al*., 1998) which the sperm showed longer duration of swimming at the beginning of the milting season compared with at the end of the season (Sorbera *et al*., 1996). Similar finding obtained by Shangguan and Crim (1999) that reported the highest motility rates of winter flounder (*Pleuronectes americanus*) sperm were found at the beginning of the milting period.

Futhermore, this statement may be related to the lower motility and fertilisation at the end of the reproduction period showed lower motility and fertilisation rates as well as reduced the short-term storage capacity such as in Atlantic halibut (*Hippoglossus hippoglossus*) (Methven and Crim, 1991; Shangguan, 1998) and turbot (Suquet *et al*.,

1998). Senescence of sperm may cause a decreasing of sperm freezability during the freezing process.

2.2.1.4 Gradient of ionic and sperm motility induction

Generally, most of the fish sperm are in immotile form during the spermatogenesis occurs in seminal vesicle and storage period in the testis (Chowdhury and Joy, 2007). The maintenance of this situation is influenced by the unique microenviroment and biochemical composition in the seminal vesicle plasma such as the ionic composition, osmotic pressure (osmolality) and pH which as well related to the sperm motility (Chowdhury and Joy, 2001; Mansour *et al*., 2002; Mazzoldi *et al*., 2005). During the spawning season or artificial reproduction, the induction of sperm motility is induced after it has been released to the aqueous environment (Alavi and Cosson, 2005). The fresh sperm motility may be affected by various factors such as osmotic pressure, temperature, pH, ionic composition (Na^+, K^+, Ca^{2+} and Mg^{2+}) and dilution rate (Chowdhury and Joy, 2007).

Typically, fish sperm are quiescent in the testis or in isotonic solution to seminal plasma, and unless affected by the concentration of specific ions or other factors, the motility of sperm of freshwater fishes is initiated by exposure to hypotonic solutions (Morisawa and Suzuki, 1980; Morisawa *et al*., 1983). The seminal fluid is rich with many nutrients and ions and one important function of their present is maintaining the sperm quality when stored in an immotile state in the genital tract (Rurangwa *et al*., 2004). This statement is supported by Morisawa *et al*. (1999) that pH or ions present may cause the polarisation of cell membrane and the stimulation of fish sperm motility.

Some researchers demonstrated the sperm activation using two-steps procedure due to simultaneously movement of the sperm after been activated which first dilution is for

mixing between the viscous milt and activation medium but still remain the sperm in immotile form while second dilution is for initiating the sperm motility for accurate quality assessment (Billard and Cosson, 1992).

In carp fish, osmolality-dependent permeabilisation and structural changes are induced in the sperm membrane by hyposmolality and reorganisation of lipid structure has been proposed as a possible mechanism (Figure 2.2) (Marian *et al*., 1993). According to Takai and Morisawa (1995), different osmolality needed to induce the initiation of sperm motility during spawning; seawater fish sperm exposes to the hypertonicity and hypotonicity exposure for the freshwater teleosts, respectively. As for trout fish, the inhibition of sperm motility is mainly due to K^+ ion concentration (Gatti *et al*., 1990; Billard and Cosson, 1992) and anaerobiosis process that produced high CO_2 content within the genital tract (Dreanno *et al*., 1995).

Billard and Cosson (1992) and Cosson *et al*. (2000) reported and suggested the usage of buffered media instead of water as activation medium although nature of fish spawning in either freshwater or seawater. This statement may explain the sperm activation using buffered media instead of water showed significant higher sperm motility of paddlefish (*Polyodon spathula*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) (Cosson *et al*., 2000) which prolonged the time of motility and less damage to sperm cell occur.

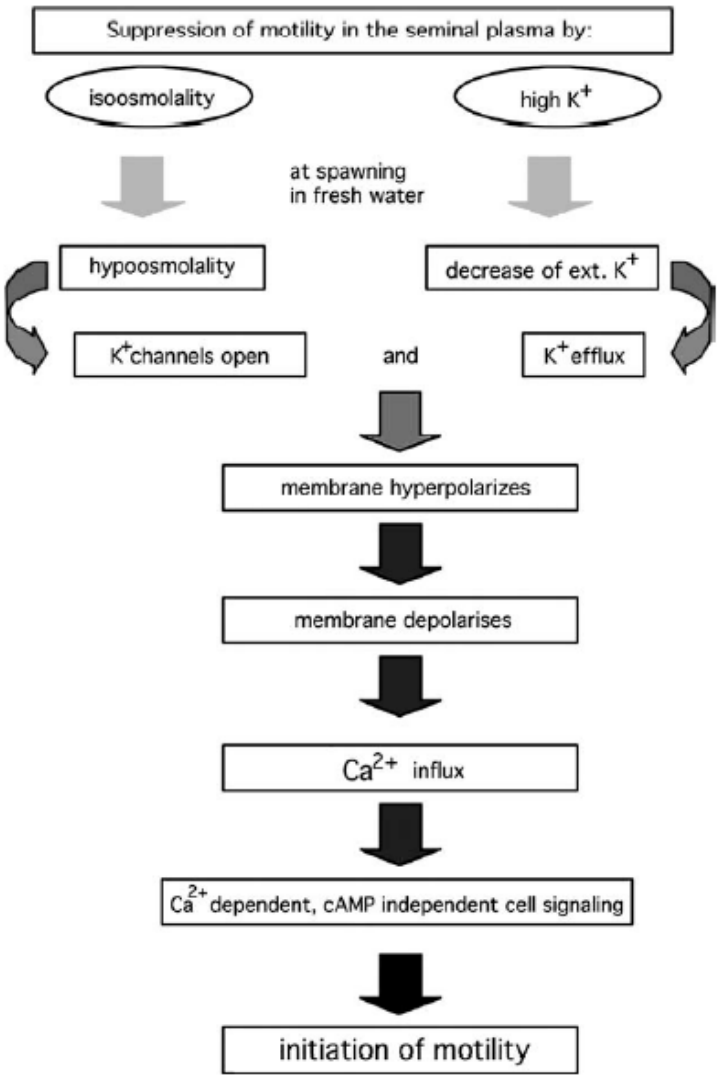

Figure 2.2: Suggested cell-signaling pathways for the mechanism of initiation of carp sperm motility (Adapted from Krasznai *et al*., 2000).

2.2.2 Abiotic Factor

2.2.2.1 Nutrition

According to Ciereszko *et al*. (1996), they stated the dietary ascorbic acid plays important role in fish male fertility in rainbow trout which has been supported from the their previous findings (Ciereszko and Dabrowski, 1995) that the antioxidant function of vitamin C provides a protection for the sperm cells by reducing the risk of lipid peroxidation and

ascorbic acid deficiency reduces both sperm concentration and motility and consequently the fertility.

According to Mansour *et al*. (2002), the endogenous respiration of glycolysis, tricarboxylic acid cycle, intra-cellular lipids, oxidative phosphorylation and β-oxidation during the immotile stage of the fish sperm provides the energy which is similar mechanism happens in mammalian sperm. In general, the internally fertilisation fish use energy obtain from the glycolysis process while fish that fertilise their eggs via externally use energy obtain from all possible mechanisms such as glycolysis, tricarboxylic acid cycle, intra-cellular lipids, oxidative phosphorylation and β-oxidation (Mounib, 1967; Hamlett *et al*., 2002).

However, Rinchard *et al*. (2000) found nutrition could cause some negative effect in decreasing the sperm motility such as in male lamprey (*Petromyzon marinus*) after been injected with gossypol acetic acid.

2.2.2.2 Temperature and photoperiod

Unfortunately, there are little reports on the effect of temperature on sperm quality (Bobe and Labbe, 2009) although some recent researches reported the temperature-dependent sex determination when using different temperature of broodstock rearing medium (Rurangwa *et al*., 2004). However, sperm of rainbow trout (*Oncorhynchus mykiss*) showed better result in ability to withstand the cryopreservation by rearing at high temperature during spermatogenesis followed by transfer to colder water (Labbe and Maisse, 1996). Moreover, as been reviewed by Hazel and Williams (1990), the temperature changes in lipid composition could cause some reaction on erythrocytes, brain, liver or muscle membranes. For example, some slightly different on lipid composition from that of warm-acclimated

fish was found in rainbow trout sperm after the fish been transferred to a lower temperature than their rearing temperature (cold acclimation) (Labbe and Maisse, 1996). Although the positive temperature effect was revealed by Muller *et al*. (2008), the effect was still unknown on fresh sperm and its cholesterol role (Bobe and Labbe, 2009).

Another important rearing factor is photoperiod which could have effect on the gonadal recrudescence and important in aquaculture manipulation to determine the suitable time for spawning season (Nash, 1999). After shifting the photothermal cycles and been exposed, there were some different in production of sperm concentration, duration of motility and seminal fluid pH differed among males on the different cycles in sunshine bass (Tate and Helfrich, 1998). However, there were no effects of photoperiod on sperm production in goldfish (Iigo and Aida, 1995) and wolffish (Pavlov *et al*., 1997).

2.2.2.3 Stress

Gamete quality is affected by the broodstock stress which is totally different levels of intensity which depend on the type of stressor, the species and the duration of the stress (Bobe and Labbe, 2009). Repeated acute stress induced by exposure to emersion during the 9-month period prior to spawning in rainbow trout resulted in lower egg volume and sperm density in milt as well as lower survival at the eyed stage, hatching and swim-up (Campbell *et al*., 1992). In contrast, sperm counts did not significantly vary between stressed and control groups (Bobe and Labbe, 2009).

During the breeding season, male sockeye salmon (*Oncorhynchus nerka*) respond to confinement stress with elevated levels of cortisol and glucose and decreased levels of reproductive steroids (T and 11-KT) (Kubokawa *et al*., 1999). Stress may also act by inducing changes in plasma osmolarity which in turn can affect sperm quality in fish

(Rurangwa *et al*., 2004). As an example, white bass (*Morone chrysops*) transported for 5 hours in freshwater had reduced seminal fluid osmolalities and motility at activation (10–25% motile cells in 38% of sperm samples) (Allyn *et al*., 2001). In striped and white bass, male broodstock captured from the wild during the spawning season and moved to captivity produce milt with non-motile sperm (Berlinsky *et al*., 1997).

In aquaculture, the process of capture and transportation could affect the sperm motility of fish cause via stress occur during the process which observed in white bass (*Morone chrysops*) (Allyn *et al*., 2001). In rainbow trout, we also observed that males confined alone in small tanks had cortisol levels 4 times higher than the controls, and sperm motility decreased to less than 10% of the control values (Bobe and Labbe, 2009).

2.2.2.4 Urine contamination

Usually, sperm contamination with urine is related to the close position between the sperm ducts and urinary ducts which well found in freshwater fish species (Rana, 1995). The negative effect of this phenomenon is sperm initiation spontaneously which could be found in some farmed fish species such as in tench (*Tinca tinca*) (Linhart and Kvasnicka, 1992), European catfish (*S. glanis*) (Linhart and Billard, 1994), paddlefish (*P. spathula*) (Linhart *et al*., 1995), common carp (*Carpius carpio*) (Billard *et al*., 1995b; Perchec *et al*., 1995; Billard, 1998), turbot (*P. maxima*) (Dreanno *et al*., 1998), tilapia (*Oreochromis mossambicus*) (Linhart *et al*., 1999). Labbe and Maisse (2001) suggested the bladder of fish can be emptied by gentle pressure in order to prevent any urine contamination.

In species with high sperm densities such as sea bass (*Dicentrarchus labrax*), urine contamination is more easily detected because of the lower viscosity and colour changes of the portions of sperm samples containing urine (Fauvel *et al*., 1999). Based on urea

concentration test, a mean contamination rate of 15.3% (urine volume: sperm volume) was reported in turbot by Dreanno *et al*. (1998). Therefore, this kind of contamination should be prevented due to harmful effects on the sperm quality.

2.3 FISH SPERM CRYOPRESERVATION

2.3.1 Sperm Cryopreservation

Generally, cryopreservation means preservation of any live cell under super low temperature condition and at the same time maintains the viability of the cell. Thus, sperm cryopreservation means preservation of live sperm for a certain period which the process involved several phase such as temperature reduction, cellular dehydration, freezing and thawing and it is catogerised as one of cryobiology branch (Medeiros *et al*., 2002). During the freezing and thawing process, Mazur (1977) reported that the cells suspended in a medium can be super-cooled to temperatures below 0^{o}C and when heat is removed from an aqueous solution, its temperature decrease past its freezing point and under-cools before ice formation or freezing is initiated.

Prevention or minimising the formation of intra-cellular and extra-cellular ice crystals are the crucial target when carried out the freezing process. Therefore, a researcher has to think and choose the best combination of variables such as sufficient cell dehydration involving in the freezing process to ensure the optimal freezeability and survivability of the frozen-thawed cells.

One of the major factors influencing the dehydration of the cell during freezing process is cooling rates. For example, the molecular motion is reduced due to the reducing of the temperature. It is this cessation of cellular activity that forms the basis for indefinite storage of cryopreserved material (Ashwood-Smith, 1980). Storage duration is only limited

by external influences such as background irradiation which theoretically limits the period of storage to somewhere between 200-32,000 years (Ashwood-Smith, 1980).

Each of the cryopreservation phases produces various degrees of severity on the cellular damage stimulated by different distinct mechanisms and the functional state of the frozen-thawed cells is the result of the injuries accumulated throughout the freezing process (Medeiros *et al*., 2002). Nevertheless, a phase of cooling and freezing in cryopreservation could be categorised as traumatic venue for sperm which the extent of effects varies among the different species (Maldjian *et al*., 2004). Sperm will face through dramatic changes in their intra-cellular and extra-cellular environments owning to exposure to cryoprotectants, cooling, freezing and thawing during cryopreservation (Guruprasad *et al*., 2007). The most predominant effects of cryopreservation on sperm are lost of viability, reduction in motility, morphologic changes and alteration in chromatin structure (Critser *et al*., 1987; Cheek *et al*., 1991). Damage to plasma membranes has been observed when sperm were exposed to cryoprotectants before freezing (Tadei *et al*., 2001) with a greater percentage of sperm losing their integrity and normal function of their plasma membranes during the freezing and thawing processes. Generally, a substantial number (approximately 50%) are damaged during cryopreservation of sperm process occur (Watson, 2000).

In the past few years, cryopreservation procedure has been improved as cryobiological studies focusing on the adaptation of cooling rates to biophysical properties of sperm, changes of sperm packaging systems as well as the accurate and consistent freezing of large numbers of samples (Roca *et al*., 2006). Therefore, improvement of semen cryopreservation technologies requires in depth knowledge of the gamete physiology and the biochemical processes occurring during semen collection, processing and freezing-thawing (Yoshida, 2000).

2.3.2 History of Fish Sperm Cryopreservation

In 1930s, the initial investigations involved sperm and ova as experimental materials have been started. A group of scientists has pioneered the cryopreservation in sperm led by Polge *et al*. (1949). This early research discovered some vital and valuable clues of freezeability potential of sperm at low temperature with other important factors affecting during the process. As consequent of this finding, progresses in sperm cryopreservation process of various species has been developed. Different type of cryopreservation has been design and developed depending on suitability and optimal condition for each procedure.

In Cyprinidae, semen cryopreservation has been investigated in some South and Southeast Asian cyprinids such as in Indian carp (*Labeo rohita*) (Routray *et al*., (2006), tawes carp (*Puntius gonionotus*), grass carp (*Ctenopharyngodon idella*), zebra fish (*Brachidanio rerio*) (Harvey *et al*., 1982), common carp (*Cyprinus carpio*) (Kurokura *et al*., 1984; Koldras and Bieniarz, 1987; Lubzens *et al*., 1993; Magrary *et al*., 1996), asp (*Aspius aspius*) (Babiak and Glogowski, 1998), deccan mahseer (*Tor khudree*) (Ponniah *et al*., 1992, 1999a; Basavaraja *et al*., 2002; Basavaraja and Hedge, 2004), golden mahseer (*Tor putitora*) (Ponniah *et al*., 1999b) and bleak (*Chalcalburnus chalcoides*) (Lahnsteiner *et al*., 2000). Although several findings have been reported on the sperm cryopreservation, the knowledge is still too little especially in *Tor* species. Moreover, some aspects of the process such as interactions of several variables have yet to be revealed as it showed ample impact on the survivability of the sperm. These variables which include type and concentration of cryoprotectant agents, dilution ratio between extender and semen, equilibration duration and optimal temperature of exposure, cooling and warming rates required to be considered. Several cryoinjury for frozen-thawed sperm of many freshwater

fish due to cryopreservation has been reported (Rana, 1995). Thus, the early findings in this area of research are important for future references of similar related studies.

2.3.3 Advantage of Sperm Cryopreservation

Stimulation of semen cryopreservation in fish has been induced by several objectives and goals in various diverse areas (Alvarez *et al*., 2003; Viveiros *et al*., 2009). Many practical methodologies have been developed as it required fulfilling the utilisation of fish semen instead of wastage situation.

Sperm cryopreservation of fish plays a huge and important role along with other biotechniques method in conservation of endangered and wildlife species. It is a valuable procedure that could be applied and emphasised in order to save and to prevent threatened species as well as to sustain production of this species and as an alternative breeding strategy such as *in vitro* fertilisation beside natural breeding in normal habitat and reproductive manipulation in genetic improvements in fish. Moreover, the gene bank could be developed using this reproductive technique. Through long term storage, availability of sperm for fertilisation may be extended. Also, continuous supplies of gametes ensure optimum utilisation of hatchery facilities or for experimentation (Lubzens *et al*., 1997).

Gamete cryopreservation and artificial fertilisation offer benefits for genetic improvement of commercially produced fish (Christensen and Tiersch, 2005). A greater potential of efficiency in selective breeding through storage of gametes from genetically improved fish stocks obtained by classical techniques, genetic manipulation (for example, triploids), or the recently obtained transgenic fish, cross-fertilisation in protandric hermaphrodites (for example, gilthead sea bream, *Sparus aurata*) and extension of the breeding season in fish (Lubzens *et al*., 1997).

A lot of potential sperm from great sources such as superior animals or valuable strains of fish species could be saved against possible loss through the spread of diseases or natural disaster. It also may increase the protection of stocks from disease by allowing the introduction of the new genetic lines with reduced danger of transmitting unknown pathogens to cultured fish (Lubzens *et al*., 1997). Expensive animal facilities that were partially devoted to the management of animals could be eliminated if the cryopreservation is put into practice and providing a safeguard for accidental loss of genetically improved lines (Lubzens *et al*., 1997). In worldwide transportation, it make easier, cheaper and safer in transporting the genetic material in liquid nitrogen tank rather than bring the normal broodstock (Wilmut and Rowson, 1973).

2.4 SPERM CRYOPRESERVATION PROCEDURE DEVELOPMENT

The development of sperm cryopreservation protocol has a tremendous improvement over the years and this is considered to be one of the important steps for animal breeding, conservation of endangered species and to overcoming aspects of male infertility in humans (Watson, 2000). Tiersch (2000) stated that basically, the cryopreservation process involved a series of steps including sample collection, sperm extension, cryoprotectant selection, cooling, storage, thawing, and viability detection. Various variables interaction included in each phase of freezing process that requires suitable factors to obtain an excellent results and care should be taken to reduce or eliminate sources of uncontrolled variation because errors at each step can accumulate and lead to considerable losses of viable cells (Leibo, 2000). Futhermore, development of each procedure in sperm cryopreservation can vary depending on species-specific differences as considering it has

various sperm sizes, sperm shapes, and biochemical characteristics (Yang and Tiersch, 2008).

2.4.1 Sperm Collection

Usually, fish sperm collection was performed by gentle stripping at abdomen muscle or by humanised killed and crushing of dissected testis. Collection by stripping of sperm samples avoids the killing of valuable fish, and individual males can be sampled repeatedly. However, to maximise the volume of sperm available, especially to allow experimental replication, crushing of dissected testis has been used for sperm collection. Practically, it is depends on the morphology and anatomy of abdomen muscle which nearer to the genital papilla of the fish. Some of the fish such as African catfish (*Clarias gariepinus*) might have lipid structure in that area, thus it hard to collect the semen by stripping. Therefore, the fish needed to be killed before the testis could be dissected out.

2.4.2 Extender Selection

Basic ingredients in making suitable extender are important for cryopreservation process. Normally, the elements involved in extender are cryoprotectant agent (CPA), sugar, salt, buffer, and antibiotics. In general, sperm cryopreservation medium includes a penetrating cryoprotectant (for example; glycerol, ethylene glycol or dimethyl sulfoxide), a non-penetrating cryoprotectant (for example; milk or egg yolk), one or more sugars (for example; glucose, lactose or sucrose), salts (for example; sodium citrate or citric acid), a buffer (for example; Tris or Test) and antibiotics (for examples; penicillin or streptomycin) (Evans and Maxwell, 1987). The role of the extender is to retain the functional capability and fertilising ability of sperm by controlling the pH, osmolality, ion concentration, and in

sources of energy during cooling, freezing and thawing (Vishwanath and Shannon, 2000; Holt, 2000). As fish semen are highly viscous, producing a suitable extender require serious consideration about sperm activation and motility which normally an extender require salt-balanced buffers with certain osmolality and pH are used to inhibit the activation of the sperm motility (Yang and Tiersch, 2008).

Varieties in chemical composition of extender were greatly designed elsewhere and simpler extender containing only two or three substances, with NaCl, KCl and $CaCl_2$ as the commonly salts in the fish sperm cryopreservation (McAndrew *et al*., 1993). An extender containing NaCl (0.4%), sodium citrate (0.8%), and egg yolk (10%) was used for cryopreservation of mahseer sperm which produced up to 80% post-thawed sperm motility (Ponnian *et al*., 1992). For sperm cryopreservation of *Tor putitora*, there were significant different between six different extenders used in term of percentage of motile sperm and hatchlings produced (Ponniah *et al*., 1999a,b). In a subsequent study, the same extender, but with the addition of antibiotics, Penicillin (500 IU/ml) and Streptomycin (6000 IU/ml) produced viable hatchlings of *Tor khudree* from one-year-old cryopreserved milt; however, the frozen-thawed sperm motility was only 50% (Ponniah *et al*., 1999a).

Fish Ringer Solution also produced the good results of fertility using *Tor khudree* cryopreserved sperm (Basavaraja *et al*., 2002). In another study, six different extenders were screened for cryopreservation of the golden mahseer (*Tor putitora*) sperm and the extender composition was found to have a significant effect on frozen-thawed sperm motility and hatchability (Ponniah *et al*., 1999b). The extender adopted by them (Ponniah *et al*., 1999a) also gave the best frozen-thawed motility (up to 90%) and a hatching rate of 12.1% of control in *Tor putitora*, after 366 days of cryostorage of sperms (Ponniah *et al*.,

1999b). Among the seven extenders, egg yolk citrate produced the highest frozen-thawed sperm motility and fertilising ability of cryopreserved Indian major carp (Kumar, 1988).

In zebrafish, extender used for sperm cryopreservation need to be held in extender that is isotonic to the plasma osmolality (~300 mOsmol/kg) to prevent activation of sperm cause once sperm was activated by hypotonic osmolality, sperm have a short burst of motility (30 seconds to 5 minutes) (Yang *et al*., 2007). Three different extenders (Ginsburg extender, buffered sperm motility-inhibiting solution (BSMIS), Hank's balanced salt solution (HBSS) have been found suitable extenders for freezing process and maintain the fertility ability of the sperm (Harvey *et al*., 1982; Morris *et al*., 2003; Draper *et al*., 2004; Yang *et al*., 2007). However, basically the Ginsburg extender contains a specific brand of powdered skimmed milk (Harvey *et al*., 1982; Draper *et al*., 2004) which is not readily available worldwide and could impedes the observation of sperm motility and morphology, especially after thawing. Therefore, HBSS and BSMIS seem to be chosen as suitable extender for cyropreservation of zebrafish sperm due to their composition which does not contain powdered skimmed milk (Yang and Tiersch, 2008).

Different situation for *Xiphophorus* fishes which the sperm activation can be induces by isotonic solutions (~310 mOsmol/kg), rather than hypotonic or hypertonic, and upon activation sperm can remain continuously motile in longer period (approximately around one week) (Huang *et al*., 2004b; Yang *et al*., 2006). Thus, in sperm cryopreservation of *Xiphophorus couchianus* (Huang *et al*., 2004a) and *Xiphophorus helleri* (Huang *et al*., 2004c), HBSS at 310 mOsmol/kg was selected as suitable extender and yielded frozen-thawed sperm motility at 78±3% and 58±7%, respectively. Alternatively, HBSS at an osmolality of 500 mOsmol/kg was also used as extender for *Xiphophorus helleri* because sperm immobilised at this osmolality could be re-activated by

changing into isotonic osmolality which produced around 55% of frozen-thawed sperm motility (Yang *et al*., 2006).

Usually, the basic study on milt: extender ratio on aquatic organism was determined using several dilution ratios as preliminary test before proceed to further step (McAndrew, 1993). Several researchers found that extreme dilution of animal samples may cause the reduction of sperm motility such as mammals (Harrison *et al*., 1978) and rainbow trout (Scott and Baynes, 1980). To get the best results, milt is normally diluted 1:3-9 (milt:diluent) prior to cryopreservation such as in salmonid fish (Scott and Baynes, 1980; Lahnsteiner *et al*., 1995). According to Basavaraja and Hedge (2004), milt dilution ratio of 1:10-20 were found the suitable ratio for the cryopreservation of *Tor khudree* sperm using modified Fish Ringer Solution (mFRS). Higher percentages of motile sperm and motility duration were obtained when *Tor khudree* sperm were diluted at 1:2 or 1:3, in comparison to 1:1 or dilutions higher than 1:3 (Ponniah *et al*., 1999b). For tilapia, ratios exceeding 1:5 showed no improvement in post-thawed sperm motility of *Oreochromis mossambicus* (Harvey, 1983). However, according to Asmad *et al.* (2008), ratio of 1:9 was the best ratio for cryopreservation of *Oreochromis niloticus*. Therefore, the optimal condition is still unclear because of various factors involved depending on species of the fishes.

2.4.3 Cryoprotectant Agent

The main purpose of including cryoprotectant agent in extender composition for sperm cryopreservation is to protect the sperm from suffering the cryoinjury due to intracellular ice crystal formation and excessive dehydration (Yang and Tiersch, 2008). Therefore, the composition of the extender, suitable cryoprotectants and the optimum freezing and

thawing rates are important factors for successful semen cryopreservation (Hammerstedt *et al*., 1990; Curry *et al*., 1994). In general, cryoprotectants agent are divided into two categories: permeating cryoprotectants (for example, propylene glycol, dimethyl sulfoxide (DMSO), methanol, and glycerol) and non-permeating cryoprotectants (for example, sucrose, egg yolk, milk, and proteins) (Yang and Tiersch, 2008).

Cell death occurs in rapid freezing due to the formation of intracellular ice; in contrast, slow freezing causes cell death due to 'solution effects', a combined effect of the concentration of solutes in the extracellular environment and their osmotic and chemical consequences on the cells, so called 'the factor theory of cryoinjury' (Mazur *et al*., 1972). According to Gao *et al*. (1997) and Karow (1997), the solution effect can be restricted by the presence of the permeating cryoprotectant. According to www.wikipedia.com, (30.6.2009), mechanism of several cryoprotectants involved the decreasing of solution's or material's glass transition temperature which the biological material structure and function would remain its native due to hydrogen bonds form and bind with biological molecules as it is important for protection of protein and DNA function.

Different species usually require different suitable cryoprotectant as variuos cryoprotectants have been tested and it will be determined experimentally (Full *et al*., 2004). According to Lovelock and Bishop (1959), DMSO has faster penetration into most cells when compare with glycerol. This statement was supported by Merryman (2007) that stated the advantage of large molar volume presence in these two cryoprotectants.

Common cryoprotectants that have been used for sperm cryopreservation of mahseer sperm are DMSO, glycerol, and methanol which gave moderately good motility and fertility rates (Ponniah *et al*., 1992; Ponniah *et al*., 1999a,b). However, in sperm cryopreservation of *Tor khudree* using glycerol failed to protect the sperm from the

cryoinjury (Basavaraja and Hedge, 2003). Comparison between DMSO, methanol and propylene glycol, DMSO proved to be best suitable cryoprotectant in order to protect the sperm during the freezing process using modified Fish Ringer Solution (Basavaraja and Hedge, 2004). However, the usage of DMSO and glycerol as cryoprotectant produced no significant difference in motility and hatching rates (Ponniah *et al*., 1999a,b). According to Basavaraja and Hedge (2003), various cryoprotectants were tested and better frozen-thawed sperm motility (up to 96%) was found in usage of DMSO.

DMSO was selected as suitable cryoprotectant for sperm cryopreservation of *Chalcalburnus calcoides* and found N-N dimethyl acetamide, 1, 2-propanediol and glycerol were unsuitable to be used due to their toxic and damage the sperm before freezing (Lahnsteiner *et al*., 2000). Moreover, Lahnsteiner *et al*. (2000) also stated that unsuitable usage of methanol and ethylene glycol as cryoprotectant because of the failure to protect sperm during freezing and thawing although it was not toxic. Furthermore, DMSO was found to be suitable internal cryoprotectant in carp fish (Cognie *et al*., 1989) and it was supported by some other researchers (Withler, 1981; Kurokura *et al*., 1984; Lubzens *et al*., 1993; Magrary *et al*., 1996; Babiak and Glogowski, 1998).

In barramundi fish, DMSO and glycerol were more suitable as cryoprotectant than methanol (Leung, 1987). In common snook fish, glycerol was found to be unsuitable cryoprotectant (Tiersch *et al*., 2004). DMSO and methanol provided sufficient cryoprotection in sterlet sperm, whereas other cryoprotectants (glycerol, propylene glycol, sucrose, lactose) were either toxic to sperm before freezing or decreased the frozen-thawed sperm viability (Lahnsteiner *et al*., 2004). However, usage of propylene glycol as cryoprotectant produced better results in frozen-thawed sperm motility although there were

no different in term of fertilisation rates as compared to usage of glycerol and DMSO (Rideout *et al*., 2003).

Generally, most cryoprotectants are toxic to sperm and the necessary concentration of cryoprotectant is important to know as high concentration of cryoprotectants can be toxic or lethal to sperm cells (Jamieson, 1991). In sperm cryopreservation of zebrafish, the toxicity of DMSO, N,N-dimethyl acetamide (DMA), methanol, and glycerol at concentrations of 5, 10 and 15% were evaluated with sperm cells which analysis of frozen-thawed motility showed that methanol at a concentration of 8% was the best choice (Yang *et al*., 2007). This statement was supported by earlier researchers that found methanol was the suitable cryoprotectants (Harvey *et al*., 1982; Draper *et al*., 2004).

High post-thawed motility of Pacific bluefin tuna sperm was observed from three cryoprotectants (DMSO, glycerol and methanol) when 1% NaCl was used as an extender; 10% methanol and glycerol were suitable at two equilibration durations (5 and 10 minutes), whereas 10% and 20% DMSO were effective for cryopreservation at equilibration times between 10 to 20 and 5 to 10 minutes, respectively (Gwo *et al*., 2005). The cryopreservation of medaka sperm using 10% DMSO and 10% dimethylformamide (DMF) as cryoprotectants produced frozen-thawed sperm motility at 78–100% and hatching rate at 82–100% (Aoki *et al*., 1997; Krone and Wittbrodt, 1997). Huang *et al*. (2004a) and Huang *et al*. (2004c) reported that 14% glycerol was found suitable in cryopreservation *Xiphophorus helleri* and *Xiphophorus couchianus* sperm, respectively.

2.4.4 Samples Packaging for Freezing

In order to optimise the sperm cryopreservation protocol, samples packaging is crucial for cooling rate standardisation and sample identification. Various kinds of containers which

form in different materials, sizes and shapes can be used as storage medium. Each type of container shows different heat transfer properties that plays ample influence in cooling and thawing rates and different results can be obtained in using the same style of containers but comes from different manufactures (Yang and Tiersch, 2008). Standard packaging protocol needs to be established and then becomes a reference for other studies. According to Yang and Tiersch (2008), French straw is favourable to be used instead of capillary tubes or cryovials since it has some advantages such as potential for use with automated straw filling and sealing equipment, sample identification by permanent printing of alphanumeric labels or barcodes on straws, sample biosecurity by complete sealing of the straw and standardisation of the cooling process (Yang and Tiersch, 2008).

2.4.5 Equilibration Duration

Generally, equilibration period allows the cryoprotectants to penetrate through sperm cell which may be influenced by the type and concentration of cryoprotectant and the permeability of sperm cells. For example, Basavaraja *et al*. (2002) developed a practical protocol for the cryopreservation of *Tor khudree* sperm using modified FRS which showed high frozen-thawed motility rates (92 to 98%) were obtained with 5 and 10% DMSO, equilibrated for 10, 20 and 30 minutes, while 15% DMSO at three equilibration periods significantly reduced ($P<0.05$) frozen-thawed motility of sperm stored up to 70 days. Based on toxicity analysis, 10 minutes equilibration duration was selected as the best duration in zebrafish (Yang *et al*., 2007). Best frozen-thawed sperm motility of *Xiphophoprus helleri* and *Xiphophoprus couchianus* was obtained from sperm equilibrated less than 30 minutes but within 10 to 120 minutes of equilibration duration, no consistent difference was observed (Huang *et al*., 2004a,b). However, Aoki *et al*. (1997) and Krone

and Wittbrodt (1997) reported that some freezing process of fish sperm which does not have specific equilibration duration as the sperm going directly to the freezing process after mixing with the cryoprotectants.

2.4.6 Freezing phase

Basically, the freezing process involved the cooling rate that may cause some changes on the osmotic and pH balance of intracellular and extracellular solutions. Yang and Tiersch (2008) explained that during freezing process with an excessively slow cooling rate, the osmotic equilibrium is maintained and much of the freezable water leaves the cell resulting in excessive dehydration. With an excessively fast cooling rate, little or no freezable water leaves the cell, and thus large intracellular crystals can form, causing damage to the cell. They highlighted the need of balance situation between the duration of exposure and minimising the formation of intracellular ice crystal for sperm survival. Thus, they suggested many tests with various cryoprotectants have to be done to determine the optimal cooling rates since different species have specific sperm physiology.

Due to cost factor, dry ice and liquid nitrogen were usually used since it is cheap and easy to use, but the cooling rates are difficult to quantify and control compared to programmable freezers, which offer high levels of control and reproducibility, but are expensive and difficult to use in the field (Yang and Tiersch, 2008). Usage of dry ice (Harvey *et al*., 1982; Morris *et al*., 2003; Draper *et al*., 2004) and a programmable freezer (Yang *et al*., 2007) in sperm freezing has been used widely in zebrafish. Other studies on zebrafish reported that the suitable cooling rate at 10°C/minute (Yang *et al*., 2007) using HBSS extender plus 8% methanol and at 16°C/minute using Ginsburg buffer plus powdered milk (Harvey *et al*., 1982).

An optimal cooling rate of striped bass sperm with DMSO ranged from 14 to 20°C/minute (Thirumala *et al*., 2006). Optimal cooling rates for green swordtail sperm varied from 5 to 45°C/minute for cooling from 5 to -80°C (Huang *et al*., 2004a). Optimal cooling rate for sperm of black grouper was reported to be 20 to 154°C/minute (Gwo, 1993). Huang *et al*. (2004a,c) performed the sperm cryopreservation of *Xiphophorus* fish using programmable freezer to determine the optimal cooling rate which resulted that 20 to 30°C/minute was optimum when sperm were cryopreserved with 14% glycerol. However, the highest frozen-thawed motility in some species may be obtained from using a combination of two-steps cooling rates (Linhart *et al*., 2000).

Study on freezing end points before plunging into liquid nitrogen in red snapper fish showed that -80°C gave better results in frozen-thawed sperm motility compared to -40°C due to less cryoinjury. He explained that the sperm samples at -40°C are plunged into liquid nitrogen before reaching optimal end point dehydration of the sperm may not be completed, and more intracellular ice may be formed (Vuthiphandchai *et al*., 2009). This finding supports the theoretical concept of ice crystal nucleation occurrence within cells at -40°C (Friedler *et al*., 1988).

2.4.7 Storage of Cryopreserved Sperm

Suitable and proper storage is required to remain the immotile form of the frozen sperm and it fertility potential for further application such as source of fertilisation process. Usually, cryopreserved sperm was hold and stored in liquid nitrogen (-196°C) in a storage tank which is a standard method for cryogenic storage of samples from fishes since it helps to prevent from any potential and easier for inventory and future identification (Yang and Tiersch, 2008). The use of plastic or French straws for packaging, especially the newer

forms with high safety and durability, offers the advantages of permanent labeling by printer, and complete sealing of the straws which minimises or prevents transfer of materials (for example, sperm cells or bacteria) among samples stored in the same dewar (Morris, 2005). In *Tor khudree*, it was found that Fish Ringer Solution could be an ideal extender for this species as has yielded up to 93.75% of post-thawed cryopreserved sperm after 10 weeks of storage (Basavaraja and Hedge, 1998).

2.4.8 Thawing Phase

The survivability of frozen-thawed sperm may be influenced by thawing factors such as its temperature and duration of the process (Morris, 1981). Leung (1991) explained the thawing mechanism theoretically that it is vise versa to cooling mechanism and same negative effect through formation of ice crystallisation between −40°C and 0°C also may happen in thawing process. In order to prevent and minimise the formation of the ice crystal, it required a rapid and appropriate thawing process.

In sperm cryopreservation of *Tor khudree* using modified Fish Ringer Solution plus DMSO and sperm was thawed for 10 seconds at 37°C and retained 55% motility for about 77 seconds (Basavaraja and Hedge, 2004). The highest results may be obtained using three different thawing parameters; 50°C for 5 or 10 seconds and within 10 seconds at 40°C in channel catfish (Christensen and Tiersch, 2005). Generally, for frozen samples packaged in 0.25 ml French straws, a 5 seconds exposure within a 40°C water bath is practical and yields suitable motility and fertility after thawing in zebrafish and *Xiphophorus* fishes (Huang *et al*., 2004a,c; Yang *et al*., 2006; Yang *et al*., 2007).

2.4.9 Semen Analysis

Viable frozen-thawed sperm is the main target to achieve after performing the sperm cryopreservation. Quality of the sperm including their fertility potential can be analysed using various methods and one of them is quantitative analysis using sperm analyser machine such as Integrated Visual Optical System (IVOS) sperm analyser, Computer-Assisted Semen Analysis (CASA) and Automated Sperm Morphology Analysis (ASMA). Sperm morphology evaluation is influenced by numerous factors, such as the fixation and staining technique (Gravance and Davis, 1995; Pena *et al*, 1999), sperm handling procedures and the most probably important, the evaluator's skills (Yeung *et al*., 1997).

Generally, parameters in evaluation of the viability of cryopreserved sperm include total and progressive motility, velocity distribution, morphology evaluation, membrane integrity, ability to bind to oocytes, and fertilisation ability (Yang and Tiersch, 2008). However, the most parameter used in evaluating the sperm quality is sperm motility but fertilisation performance is considered to be the most informative (Yang and Tiersch, 2008).

Basically, sperm move spontaneously after activated in a straight or slightly curved trajectory and the various velocity patterns are schematically represented in Figure 2.3 (Rurangwa *et al*., 2004). Several findings using computer-assisted sperm analyser in various fish such as on African catfish, carp, goldfish, roach, Eurasian perch, trout and lake sturgeon stated that, the most useful parameters of velocity are the curvilinear velocity (VCL, the actual velocity along the trajectory) and the straight line velocity (VSL, the straight line distance between the start and end points of the track divided by the time of the track) (Ciereszko *et al*., 1996; Kime *et al*., 2001; Rurangwa *et al*., 2001, 2002; Jobling *et al*., 2002).

Rurangwa *et al*. (2004) suggested if the trajectory is a straight line, then VCL and VSL are identical and the angular path velocity (VAP, the velocity along a derived smoothed path) is generally of little use in most fish. However, a gelatinous egg mass was a medium of fertilisation that made the sperm swam in more erratic path and VCL and VAP were both useful measurements in both stickleback (*G. aculeatus*) and wolffish (*A. minor*) (Kime and Tveiten, 2002; Elofsson *et al*., 2003a). They concluded that the VCL/VAP ratio gives a good estimate of the ‘‘wiggliness’’ of the trajectory. In several situations, the trajectory can become increasingly curved and eventually become tight concentric circles and the best parameters to be used as indicator for sperm quality are the linearity (LIN, the ratio of net distance moved to total path distance (VSL/VCL)) or straightness (STR, the ratio of net distance moved to smoothed path distance (VSL/VAP)) (Rurangwa *et al*., 2004).

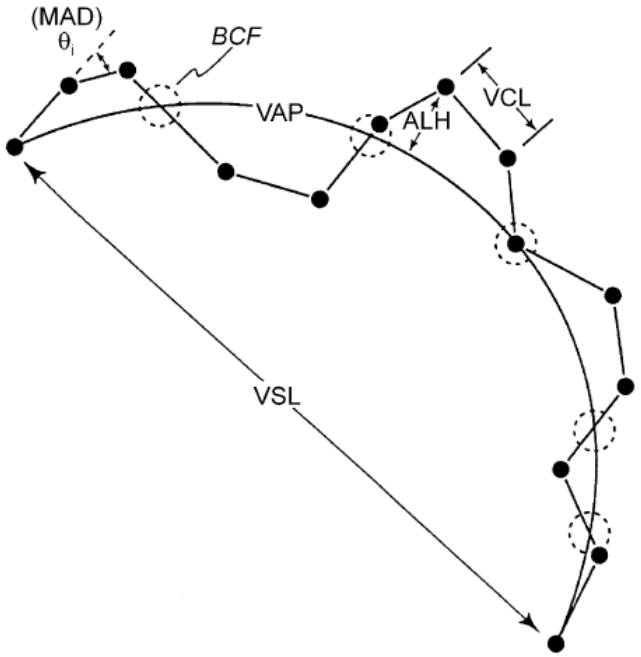

Figure 2.3: Schematic representation of some of the motility patterns measured by the CASA system. Black circles represent successive images of the head of a motile sperm and are joined by straight path lines. Curved line indicates a smooth path fitted through sperm track. MAD: mean angular displacement, BCF: beating cross frequency, VAP: average path velocity, ALH: amplitude of lateral head displacement, VCL: curvilinear velocity, VSL: straight line velocity (Adapted from Boyer *et al*., 1989).

2.5 SIGNIFICANT MILESTONES IN SPERM CRYOPRESERVATION OF FISH

Development of protocols for sperm cryopreservation has been studied over the past 20 years for fishes. Table 2.1 shows the significant milestones of fish sperm cryopreservation. Nowadays, more than 200 species of fishes have been studied about their sperm (Suquet *et al*., 2000; Zhang *et al*., 2003).

Table 2.1: Significant milestones of fish sperm cryopreservation in various types of fishes

Year	Author	Significant findings
1992	Ponniah *et al.*	Reported the sperm cryopreservation of *Tor khudree*

		with 80% motility post-thawed cryopreserved sperm when protected with glycerol up to 11 months as compared to DMSO, which resulted in 50% motility.
1994	Tiersch *et al.*	Studied in channel cat fish on the storage in cryoprotectants, fertilisation trial and growth of the fry produced using cryopreserved sperm.
1995	Lahnsteiner *et al.*	Performed the study on uniform method for cryopreservation of semen of the salmonoid fish
1996	Conget *et al*.	Successfully cryopreserved the rainbow trout which yielded approximately 63% of live sperm and the average fertilisation potential of frozen-thawed sperm was 58%.
1997	Dreanno *et al.*	Demonstrated the sperm cryopreservation of turbot (*Scopthalamus maximus*) that showed the best results obtained when sperm was diluted at 1:2 ratio with a modified Mounid extender, supplemented with 10% BSA and 10% DMSO.
1998	Cabrita *et al*.	Stated that samples frozen with DMSO in the extender no. 6 from Erdhal and Graham displayed the best results in cryopreservation of rainbow trout semen.
1999b	Ponniah *et al.*	Stated that no significant difference between different cryoprotectants or equilibration durations (45 to 175 min) in golden mahseer (*Tor putitora*).

1999	Richardson *et al*.	Performed the semen cryopreservation of yellowtail flounder (*Pleuronectes ferrugineus*) using plaice Ringer with 10% propylene glycol and found there were no significant different in fertilisation and hatching rates between semen frozen in 0.25 ml and 1.7 ml straws.
1999b	Yao *et al.*	Evaluated the motility, fertility and ultra structural changes of ocean pout (*Macrozoarces Americanus*) on post-thawed cryopreserved sperm which yielded 20-25% of post-thawed cryopreserved sperm using 20% of DMSO and produced higher post-thawed cryopreserved sperm motility at 9°C/minute of freezing rate as compared to 6°C/minute and 18°C/minute.
2000	Babiak *et al.*	Reported the sperm cryopreservation of rainbow trout (*Oncorhynchus mykiss*) yielded the highest fertilisation rates of post-thawed cryopreseved sperm using two extender; Erdahl and Graham's with 10% egg yolk, 0.3M glucose and 10% DMA.
2000	Lahnsteiner *et al.*	Performed the sperm cryopreservation in the bleak, *Chalcalburnus chalcoides* which stated the optimal extender composition was a buffered physiological sperm motility-inhibiting saline solution containing 10% DMSO and 0.5% glycine and the optimal sperm equilibration duration in extender was ≤ 5 minutes.

2000	Linhart *et al.*	Stated that there were significant differences between fresh and frozen-thawed sperm regarding fertilisation rate (68±11% and 56±10%, respectively) and insignificant differences between fresh and frozen/thawed sperm on the hatching rate (50±18 and 52±9%, respectively) in common carp (*Cyprinus carpio*) using Kurokura medium and DMSO as their extender and cryoprotectant agent, respectively.
2000	Yao *et al*.	Reported that 20% of DMSO yielded the highest frozen-thawed sperm motility (20–25% of the total cells) in sperm cryopreservation of ocean pout (*Macrozoarces americanus*) using ocean pout semen diluent (Yao *et al.*, 1995).
2001	Cabrita *et al*.	Stated that cryopreservation of rainbow trout (*Oncorhynchus mykiss*) sperm using large straw volumes (1.8 ml) could be useful for hatchery purposes which sperm was diluted at 1:3 ratio in no. 6 Erdahl and Graham extender (Erdahl and Graham, 1980) with 7% DMSO and 10% egg yolk).
2001	Labbe and Maisse	Studied the characteristics and freezing tolerance of the sperm of brown trout (*Salmon trutta*) according to rearing water salinity.
2001	Rurangwa *et al*.	Stated that Mounib's extender with DMSO or glycerol provided the best cryoprotection to the sperm of

		African catfish (*Clarius gariepinus*) and concluded the sperm motility was positively related to fertility.
2001	Viveiros *et al*.	Concluded that with slow cooling rates of 22 to 25°C/minute, hatching rates in sperm cryopreservation of African catfish can be maximised by plunging as soon as temperature of semen reaches -38°C using Ginzburg Fish Ringer and methanol as extender and cryoprotectant agent, respectively.
2002	Basavaraja *et al.*	Developed a simple practical protocol for the cryopreservation of *Tor khudree* sperm which showed high post-thawed motility rates (92 to 98%) were obtained with 5 and 10% DMSO, equilibrated for 10, 20 and 30 minute, while 15% DMSO at three equilibration periods significantly reduced ($P < 0.05$) post-thaw motility of sperm stored up to 70 days.
2002	Lahnsteiner *et al.*	Managed to cryopreserve the sperm of turbot, *Lota-lota* which showed the highest motility rate of the post-thawed cryopreserved sperm (46.6±8.0%) using 10% methanol, 1.5% glucose and 7% hen egg yolk were used as cryoprotectant agents.
2003	Alvarez *et al*.	Reported the sperm cryopreservation of silver carp (*Hypophthalmichthys molitrix*) using the solution of NaCl 68.38 mmol/litre, sodium citrate 27.20 mmol/litre, and dextrose 11.01 mmol/litre which

		showed DMSO was more suitable cryoprotectant than methanol and glycerol.
2004	Basavaraja and Hedge	Performed the sperm cryopreservation in *Tor khudree* using modified fish Ringer solution and DMSO as cryoprotectant which produced 80-81% and 43-67% of post-thawed cryopreserved sperm after 10 and 70 days of storage, respectively.
2004	Chen *et al*.	Stated that cryopreservation technique using extender TS-2 (Mounib, 1978) with 10% DMSO for turbot (*Scophthalmus maximus*) sperm in 1.8 ml cryovial could be used for hatchery purposes that resulted average fertilisation rate (70.1±8.9%) and hatching rates (46.8±5.2%).
2004	He and Woods III	Demonstrated the sperm cryopreservation of striped bass (*Morone saxatilis*) which found no effect of different concentration of DMSO (2.5, 5 or 10%) on plasma membranes after sperm were exposed for 10 min. However, mitochondria function decreased ($P<0.01$) with increased of DMSO concentration.
2004b	Huang *et al.*	Stated the sperm cryopreservation in a live bearing fish, the platyfish (*Xiphophorus couchianus*) which produced that highest average sperm motility (78±3%) at 10 minutes after thawing was obtained when sperm were suspended in HBSS with 14% glycerol as

		cryoprotectant, diluted at a ratio of sperm to HBSS: glycerol of 1:20, equilibrated for 10 minutes, cooled at 25°C/minute from 5 to -80°C before plunging into liquid nitrogen, and thawed at 40°C in a water bath for 7 seconds.
2004c	Huang *et al.*	Performed the cryopreservation of green swordtail (*Xiphophorus helleri*) using HBBS as extenders with 10% DMSO and suggested that the optimal rate with the presence of CPA ranged from 20 to 35°C/minute.
2004	Muchlisin *et al*.	Performed sperm cryopreservation of tropical bagrid catfish (*Mystus nemurus*) using Fish Ringer Solution and DMSO as extender which produced 58% of frozen-thawed sperm.
2004	Rideout *et al.*	Reported the development of sperm cryopreservation techniques in Atlantic cod and haddock with the effect of sperm age on cryopreservation success.
2004	Riley *et al*.	Demonstrated the sperm cryopreservation of red snapper (*Lutjanus campechanus*) using HBBS with 10% DMSO that produced the highest motilities after thawing (71±16%).
2005	Christensen and Tiersch	Performed the cryopreservation of channel catfish (*Ictalurus punctatus*) sperm with 5% methanol (43±17%) which resulted in a lower frozen-thawed cryopreserved sperm motility reduction than 10%

		methanol (67±14%) and using HBBS as extender.
2005	Horvath *et al*.	Stated that the highest frozen-thawed sperm motility (18±10%), fertilisation (18±11%) and hatching rates (17±12%) were observed motility in North-American sturgeon with Modified Tsvetkova's extender (MT) used in combination with 5% methanol.
2005	Linhart *et al*.	Managed to cryopreserve the European catfish (*Silurus glanis*) sperm which produced the best hatching rate of 82-86% using sperm stored for 5 hours before freezing and frozen with DMSO in concentration of 8, 10 and 12% or with a mixture of 5% DMSO and 5% propandiole.
2006	Liu *et al.*	Evaluated the quality of cryopreserved sperm in red seabream (*Pagrus major*) using computer-assisted sperm analysis (CASA) that reported the total motility of frozen-thaw sperm (72.3±6.3%) 30 seconds after activation.
2006	Maria *et al*.	Reported that frozen-thawed sperm motility (66%) of piracanjuba, *Brycon orbignyanus* was observed in semen cryopreserved in 154 mM NaCl with egg yolk and methylglycol.
2006	Routray *et al*.	Suggested that germ cells of Indian major carp, *Labeo rohita* such as sperm of dead fishes can be cryopreserved and utilised for restoration of a species.

2006	Sarvi *et al*.	Performed the sperm cryopreservation of Caspian brown trout (*Salmo trutta caspius*) using the extender of 0.3 M glucose with 10% methanol and 10% egg yolk and 0.6 M sucrose with 10% DMSO and 10% egg yolk, yielded 66.6%±2.2 and 59.8%±5.1 eyeing rates, respectively.
2006	Thirumala *et al.*	Suggested that the optimal rate of cooling for striped bass (*Morone saxatilis*) sperm cells in the presence and absence of DMSO range from 14 to 20°C/minute.
2006	Yang *et al.*	Stated the high frozen-thawed cryopreserved sperm (~55%) of *Xiphophorus helleri* was obtained in sperm after thawing after cryopreserved with 10-15% glycerol and dilution of thawed sperm in fresh HBSS at 1:4 ratio has decreased the post-thawed cryopreserved sperm motility significantly.
2007	Horvath *et al.*	Reported the sperm cryopreservation of common carp (*Cyprinus carpio*) which yielded the highest hatching rate (69±16%) for 1.2 ml straws at freezing duration of 4 minutes and 39±27% for 5 ml straws at 5 minute.
2008	Pan *et al*.	Demonstrated the first report on successful cryopreservation of yellow catfish sperm using Fish Ringer Extender and 10% methanol as cryoprotectant which yielded 65±5% of frozen-thawed sperm motility.
2008	Taitson *et al*.	Performed the sperm cryopreservation in neotropical

		fish (*Leporinus obtusidens*) under field condition using dry-shipper and a simple solution (composed of 10% DMSO) was used as sperm diluent which and activated by 119 mM $NaHCO_3$ gave the highest motility rate (62±14%).
2009	Ding *et al*.	Stated that after thawed at 37°C for 60 seconds, the sperm had the highest frozen-thawed motility (96.00±1.73%). At the sperm/egg ratio of 100,000:1, the fertilisation rate and the hatching rate of the frozen sperm cryopreserved for 1 week or 1 year in liquid nitrogen (66.01±5.14% and 54.76±4.40% & 62.97±14.28% and 52.58±11.17%) were similar to that of fresh sperm (69.42±8.11% and 59.82±5.27%).
2009	Penaranda *et al*.	Suggested that sperm activation caused by addition of DMSO can be prevented using high $NaHCO_3$ concentrations, improving the cryopreservation process. This effect seems be based on some of the products dissociated from $NaHCO_3$ in aqueous solution, affecting the intracellular pH, essential in the sperm motility.
2009	Vivieros *et al.*	Showed the sperm cryopreservation of curimba semen can be successfully done using a simple glucose solution combined with methylglycol as cryoprotectant, in 0.5 ml straws, yielding motility rates between 86%

		and 95% and fertilisation rates between 47% and 83%.
2009	Vuthiphandchai *et al.*	Performed the cryopreservation of *Lutjanus argentimaculatus* sperm using Fish Ringer Solution which equilibrated in 10% DMSO and cooled at a rate of 10°C/minute to a final temperature of -80°C had the highest motility (91.1±2.2%) and viability (92.7±2.3%) after thawing. The fertilisation rate of frozen-thawed sperm (72.4±2.4%) was not different (P > 0.05) from that of fresh sperm (75.5±2.4%).

Chapter 3
MATERIALS AND METHODS

Chapter 3

3.0 MATERIALS AND METHODS

3.1 LOCATION OF STUDY

The present study was performed at the Institute of Biological Sciences (ISB) Mini Farm (Livestock), the University of Malaya for experiments that were carried out under laboratory ambient (Figure 3.1). As for research activities that were designed under field condition, the experiments were performed at the Taman Negara Sg. Relau, Merapoh, Pahang Darul Makmur (Figure 3.2). This study was carried out from July 2007 until October 2009 for duration of approximately 26 months.

3.2 MATERIALS

3.2.1 Animal Experiment and Maintenance

A total of 20 Thai mahseer fishes (400-1000 g body weight) procured from the local fish farm at Hulu Langat, Selangor Darul Ehsan were used as experimental animals and were reared at the fish house of the ISB Mini Farm (Livestock). The broodstocks were maintained in clean dechlorinised water, oxygenated and flow-through tanks. The fish was fed 5% of body weight once per day with diet pellet (ingredients: crude protein (34%), crude fat (6%), crude fibre (5%) and moisture (11%). While at the Taman Negara Sg. Relau, Merapoh, Pahang Darul Makmur, a total of 17 Thai mahseer fishes (435-1125 g body weight) were used. Hormone induction using Ovaprim (0.5 ml per kg body weight) was carried out on all experimental fishes within 24 hours before the sperm collection.

3.2.2 Equipment, Instruments and Consumables

Various equipment, instruments and consumables involved in this study were beaker, stirrer, syringe, needle, normal thermos, scissor, glove, tissue paper, centrifuge tube, dark clothes, micropipette, micropipette tip, microscope slide, French straw, scaple, goblet, digital weighing balance, weighing machine, Integrated Visual Optical System sperm analyser (IVOS), semen collection styrofoam box, freezing styrofoam box for ambient and field condition, plastic film sealer, special rack, laminar flow work station, light compound microscope, low temperature incubator, normal refrigerator, liquid nitrogen storage tank, liquid nitrogen carrier tank, ultra-pure water system (Milli-Q UF Plus), vortex mixer, pH meter and water bath. The detailed list of equipment, instruments and consumables used in this study is given in Appendix 1.

3.2.3 Chemicals, Solutions and Reagents

Meanwhile, the chemicals, solutions and reagents included in this research were Ovaprim, modified Fish Ringer Solution (mFRS), dimethyl sulfoxide (DMSO), tank water and liquid nitrogen. The detailed list of chemicals, solutions and reagents is given in Appendix 2.

Figure 3.1: Fish house at the ISB Mini Farm (Livestock), the University of Malaya.

Figure 3.2: Taman Negara Sg. Relau, Merapoh, Pahang Darul Makmur.

3.3 METHODS

3.3.1 Modified Fish Ringer Solution (mFRS) Preparation

Table 3.1 shows the chemicals and solution used in modified Fish Ringer Solution (mFRS) that was used as an extender for the cryopreservation of mahseer sperm in this study.

Table 3.1: Chemicals and solution used in modified Fish Ringer Solution (mFRS)

Chemical/Solution	Weight (g)
NaCl	0.750
KCl	0.100
$CaCl_2$	0.016
$MgSO_4$	0.023
NaH_2PO_4	0.041
Glucose	0.100
Milli-Q water (ml)	100.000

The preparation of the mFRS was based on Basavaraja and Hedge (2004). Briefly, all the above chemicals were purchased from Sigma Chemical Company, USA and were weighed accurately using digital weighing balance (Mettler Toledo, USA). Milli-Q water (50 ml) was poured into a beaker (250 ml) after which the chemicals were mixed with the water in the beaker using a stirrer. The milli-Q water was then added until it reached a volume of 100 ml and mixed thoroughly using a stirrer. The pH of the mixture was adjusted using NaOH (100%) to a value of 7.48-7.50 and measured using pH meter (Eutech Instruments Pte. Ltd., Singapore).

3.3.2 Ovaprim Injection

Before the injection of reproductive hormone, individual male body weight was recorded. Ovaprim (Syndel Laboratories Ltd, Canada) was used to stimulate the male reproductive system to enhance the sperm production in the male fish. The matured male fish which have started producing semen were evaluated using IVOS and only fish with good sperm motility characteristics of fresh semen were selected and used. The fish were injected once with Ovaprim (0.5 ml/kg) intramuscularly at the back of the dorsal fin 24 hours before the collection of the semen. As a precaution, it is important to inject the needle with the hormone into the body part that gives minimal swelling effect resulted from the injection as well as to facilitate the ease of injection with less stress to the fish. After injection, the fish were kept in the storage tank before the collection of semen to be carried out 24 hours later.

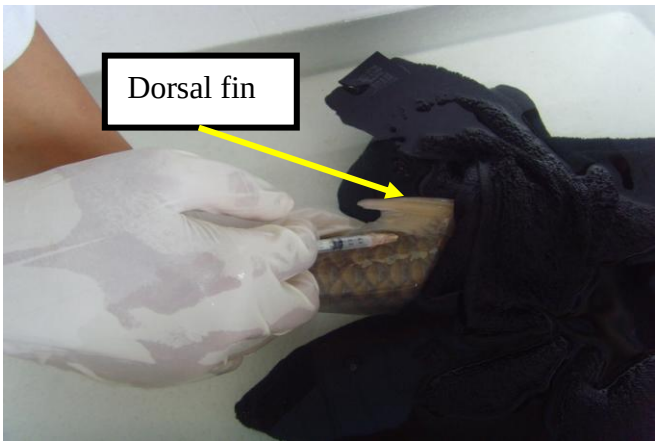

Figure 3.3: Hormonal injection at the back of the dorsal fin.

3.3.3 Collection of Milt

After 24 hours of hormonal injection, the collection of milt was carried out. Two persons were needed to perform this collection whereby one person with gloves would hold the fish using both hands while the other to collect the semen. Briefly, the semen was collected by gently squeezing the fish abdomen whereby the ejaculated semen was collected using a centrifuge tube (15 ml). The collection of semen was carefully carried out and proper care was taken in order to avoid contamination of milt by urine and/or waste material which may kill the sperm or water that may cause sperm activation. In this study, any samples of semen showing auto-activation (signs of sperm motility) were discarded because the sperm will only survive for several hours. Thus, it will affect the viability of frozen-thawed sperm later. Therefore, sufficient training in handling and familiarisation of semen collection technique was needed (learning curve) before the actual experimentation was carried out. For example, it was quite common for the beginners to contaminate the semen with the urine and other waste materials that consequently resulted in poor sperm motility. After the collection, the semen was immediately brought back to the semen processing laboratory at the ISB mini farm (Livestock) for further processing, evaluation and freezing procedures.

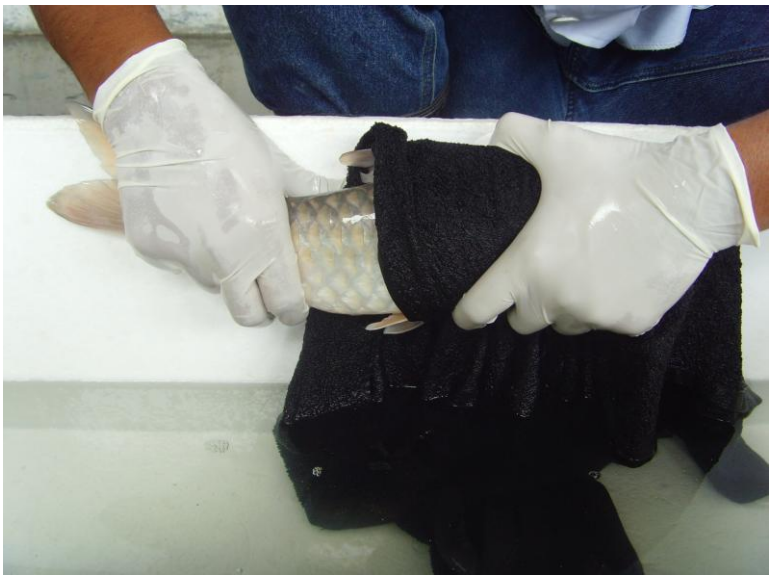

Figure 3.4: The correct technique of holding the fish before semen collection.

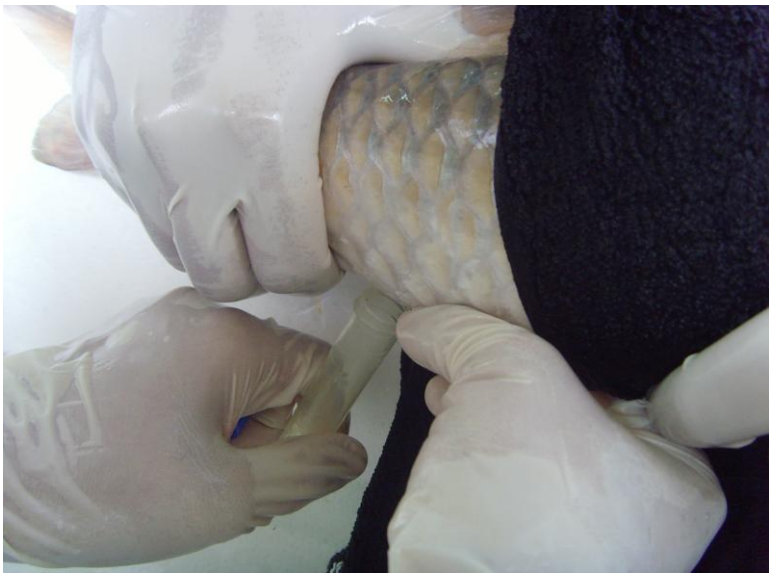

Figure 3.5: The squeezing of fish abdomen to facilitate semen ejaculation into the centrifuge tube.

3.3.4 Pre-sperm Viability Checking

Semen was processed immediately upon arrival to the laboratory to avoid its degradation in quality. Immediately after the collection, semen sample (5 µl) was placed on the slide (Pearl, China), activated using tank water (10 µl) and observed under the light compound

microscope (10x, Nikon, Japan) to make sure the sperm remain alive before processing into further steps.

3.3.5 Diluted Sperm

The semen was diluted with mFRS (1:10, semen:mFRS) in a centrifuge tube (15 ml, Becton, Dickinson of Company, USA). The tube containing the semen mixture was shaken slowly to mix the extender and sperm thoroughly. Then, the mixture in the tube was added with dimethyl sulfoxide (DMSO) as cryoprotectant (10%) (i.e. 90% semen-mFRS mixture: 10% DMSO) and was shaken slowly. In this research, the addition of DMSO (10%) to the final mixture (semen-mFRS without the semen) is referred to modified Fish Ringer Extender (mFRE).

3.3.6 Sperm Enveloping

The diluted sperm was aspirated into a French straw (0.25 ml or 0.50 ml, Kruuse, Denmark), leaving an air space at the tip (without the cotton wool) of the straw (Figure 3.6). The end of straw without cotton wool was electrically heat-sealed. Then these straws were arranged on a special rack (12 cm x 29.5 cm) for further processing (Figure 3.7a). As for Experiments 3 and 4, smaller special rack (10.5 cm x 17 cm) was used to accommodate experimental facilities using the smaller refrigerator under field condition (Figure 3.7b).

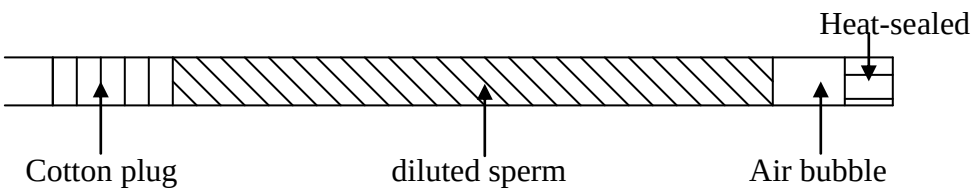

Figure 3.6: Diagrammatic presentation of straw containing the diluted sperm.

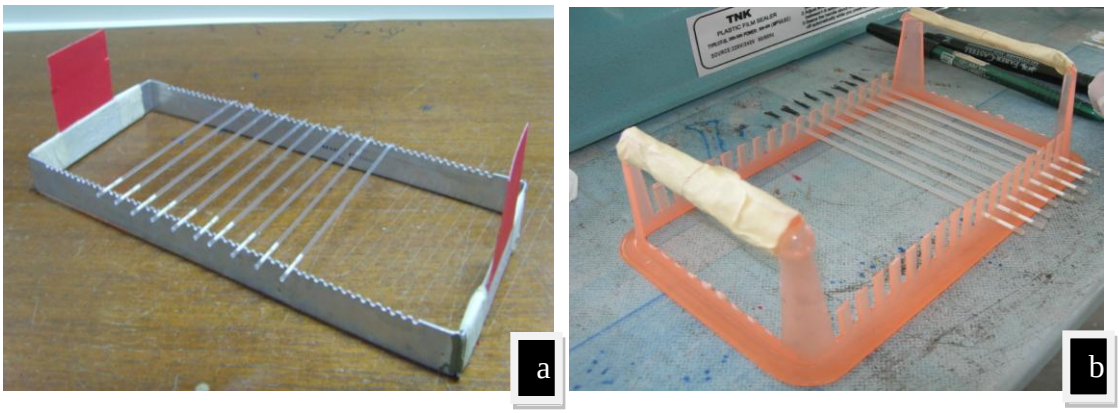

Figure 3.7: Special rack. (a) For freezing under normal laboratory ambient (12.0 cm x 29.5 cm); (b) For freezing under field condition using smaller refrigerator (10.5 cm x 17.0 cm).

3.3.7 Equilibration

The diluted sperm in the straws were equilibrated in a refrigerator (Figure 3.8) in order to reduce the sperm temperature to enable cryoprotectant to diffuse extracellularly from the sperm cell. In other words, the rack of straw filled with sperm was placed into the low temperature incubator (Sheldon Manufacturing Inc., USA) at 4°C (Figure 3.8a) for different equilibration durations under laboratory condition (30, 45 or 60 minutes). This range of duration was choosen due to standardisation of freezing procedure in our laboratory (Asmad, 2008). While for field experiments, a normal refrigerator, which was smaller than low temperature incubator, was used (Figure 3.8b) to facilitate convenient working condition. A thermo-couple was used to make sure the normal refrigerator temperature maintains within a range of 3.5-4.5ºC.

Figure 3.8: Equilibration phase. (a) Equilibrated using low temperature incubator under laboratory ambient; (b) Equilibrated using normal refrigerator under field condition.

3.3.8 Freezing

Freezing process involved two stages whereby the straws in rack containing the sperm were placed in styrofoam box containing liquid nitrogen (Figure 3.9). The first stage was carried out using different liquid nitrogen vapour temperatures (-100, -110 or -120°C) with different vapour exposure durations (5, 10 or 15 minutes) by placing the rack in the

stryofoam box (36 cm x 25.5 cm x 22 cm) with the liquid nitrogen (Figure 3.9a). However, in Experiment 4, smaller polystyrene box (25.5 cm x 19 cm x 23 cm) was used for vapour exposure phase (Figure 3.9b). The second stage was when the straw was completely submerge into the liquid nitrogen (-196°C) for 5 minutes whereby the straws were kept in the liquid nitrogen storage tank for 1 week (Chart Industries Inc., USA) (Figure 3.10a). Under field condition, the straws were kept in temporary liquid nitrogen carrier tank (Chart Industries Inc., USA) (Figure 3.10b) before being transferred into the liquid nitrogen storage tank (Figure 3.10a).

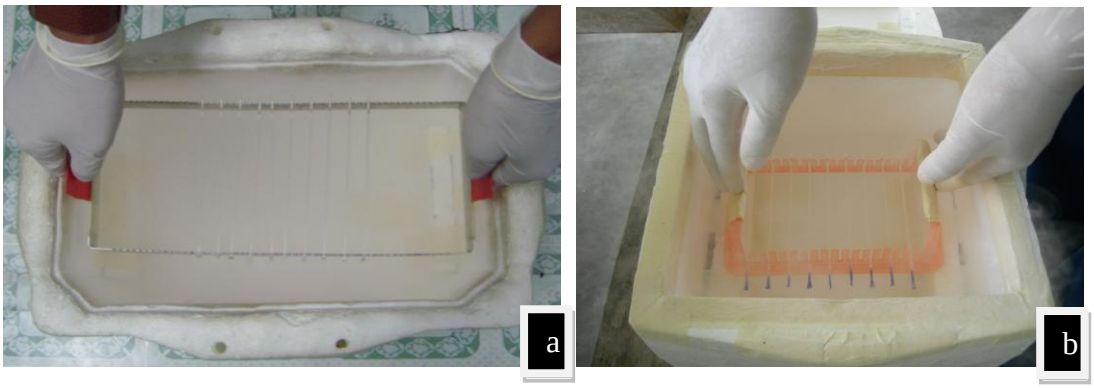

Figure 3.9: Freezing phase under vapour temperature using styrofoam box containing liquid nitrogen. (a) Performed under laboratory ambient; (b) Carried out under field condition.

Figure 3.10: Storage of frozen sperm in LN_2 tank. (a) Storage in LN_2 storage tank under laboratory ambient; (b) Temporary storage in LN_2 carrier tank of frozen sperm after undergo freezing process under field condition.

3.3.9 Thawing

This process was done by transferring the straws from liquid nitrogen tank into water bath (Memmeth, Germany) at 30-32°C for 30 seconds (Figure 3.11). This is a routine procedure under laboratory condition. The straws were wiped dry with tissue paper and both sealed ends were cut to dispense the sperm out for evaluation using the IVOS. As for Experiment 4 under field condition, the frozen sperm were transported in liquid nitrogen carrier tank before being transferred to the permanent liquid nitrogen storage tank at the University of Malaya for further sperm analysis using the IVOS.

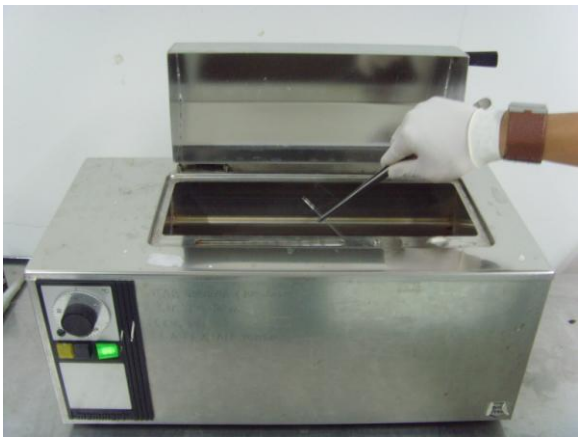

Figure 3.11: Thawing the frozen sperm using water bath.

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3.3.10 Analysis of Frozen-Thawed Sperm

Frozen-thawed sperm were analyzed using 'Integrated Visual Optical System' sperm analyser (IVOS, Hamilton-Thorn, USA). IVOS evaluated the survivability and quality of the sperm were analysed for sperm motility characteristics.

3.4 STATISTICAL ANALYSIS

All the analysis was done by using the software package SPSS (Statistical Package for Social Science) for window, version 16.0. Mean and Standard Error of the Means for each traits were estimated using one-way ANOVA. Means comparison of different traits was done by using Duncan's multiple range tests. Correlations between two dependent variables were analysed using Bivariate Pearson correlation. Correlations were significant at ($P<0.05$) and ($P<0.01$) (2-tailed). There were several factors (independent variables) and parameters (independent variables) involved in this study.

The factors involved were:

a) Equilibration duration - Duration for mixture sperm and extender to equilibrate in the incubator/refrigerator at 4°C.

b) Vapour exposure temperature - Temparature for semen exposure to vapour of LN_2 during freezing process before submerged into the LN_2.

c) Vapour exposure duration - Duration of sperm exposure to LN_2 vapour as preparation before submerging step.

While, the parameters measured were:

a) Total motility - total motility of sperm cells.

b) Progressive motility - total progressive motility of sperm cells.

c) Sperm velocity distributions - rapid, medium, slow and static of sperm movement.

d) Sperm motion characteristics - VAP, VSL, VCL, ALH, BCF, STR and LIN.

3.5 EXPERIMENTAL DESIGN

Thai mahseer, *Tor tambroides* males were selected from the local fish farm at Hulu Langat, Selangor and reared at the fish house of the ISB Mini Farm (Livestock). Mature males (n=20, 400-1000 g) were pre-determined for semen ejaculation and were being selected to be used in this study. Briefly, a single intramuscular injection of gonadotrophin release hormone (GnRH) (Ovaprim; 0.5 ml/kg body weight) was employed 24 hours before the semen collection. Semen was hand-stripped directly into a centrifuge tube. Immediately after the collection, 5 µl of sample was placed on the slide, activated using 10 µl of tank water and observed under the light microscope (10x). Any samples of semen showing

auto-activation (signs of sperm motility) was discarded. Samples were diluted in a ratio of 10% semen and 90% mFRS and the mixture was immediately added with 10% DMSO as suggested by Basavaraja and Hedge (2004). The mixture of milt-extender was loaded into 0.25 or 0.50 ml French straws, sealed and placed on the special rack. The straws were equilibrated at 4°C. The interval between collection of milt and addition of milt to extender ranged between 1 and 2 hour (Basavaraja and Hedge (2004). Subsequently, the straws were frozen in polystyrene box for vapour exposure phase and then stored in a liquid nitrogen storage tank -196°C. Within 1-2 weeks, the straws were thawed in water bath at 30-32°C for 30 seconds and frozen-thawed sperm characteristics were analysed.

3.5.1 Determination of the Effects of Equilibration Duration, Vapour Exposure Temperature and Vapour Exposure Duration in Sperm Cryopreservation Protocol for Thai mahseer (*Tor tambroides*) Fish using the Modified Fish Ringer Extender (mFRE) (Experiment 1)

A total of 20 mature males were involved in this experiment. The method of hormonal stimulation, semen collection, semen dilution and semen processing prior to freezing was previously described in this chapter. The straws were equilibrated in low temperature incubator at 4°C for 30, 45 or 60 minutes. The interval between collection of milt and addition of milt to extender ranged between 1 and 2 hour (Basavaraja and Hedge (2004)). Subsequently, the straws were frozen in polystyrene box at -100, -110 or -120°C of vapour exposure temperature for 5, 10 or 15 minutes of vapour exposure durations. Subsequently, the straws were submerged into the LN_2 and stored in a liquid nitrogen storage tank at -196°C. Factors included in this experiment were equilibration duration, vapour exposure temperature and vapour exposure duration. Within 1-2 weeks, the straws were thawed in

water bath at 30-32°C for 30 seconds and frozen-thawed sperm characteristics were analysed. Three replicates and 15 observations (straws) were performed per each treatment. Paramaters such as total motility, progressive motility, sperm velocity distributions and sperm motion characteristics of frozen-thawed sperm obtained from IVOS analysis were printed and recorded. The best three combinations of equilibration duration, vapour exposure temperature and vapour exposure duration were used for subsequent experiment (Experiment 2). The effects of equilibration durations, vapour exposure temperatures and vapour exposure durations on the sperm motility characteristics were determined.

3.5.2 Optimisation of Combination of Equilibration Duration, Vapour Exposure Temperature and Vapour Exposure Duration for Sperm Cryopreservation in Thai mahseer (*Tor tambroides*) Fish (Experiment 2)

A total of 20 mature males were involved in this experiment. The fish were reared at the fish house of the ISB Mini Farm (Livestock). Basically, the same procedure as in Experiment 1 was performed in this experiment. However, this experiment was an attempt to utilise the three best combinations of factors of equilibration duration, vapour exposure temperature and vapour exposure duration on sperm motility characteristics and sperm motion characteristics. These combinations were 30 minutes, -110°C and 10 minutes; 30 minutes, -110°C and 15 minutes and; 30 minutes, -120°C and 15 minutes; respectively. This experiment involved 3 replicates with 15 straws per combination treatment. All the straws containing the sperm for each treatment underwent freezing process until storage in the liquid nitrogen tank and were subsequently thawed 1-2 weeks after storage for sperm motility characteristics using the IVOS. Frozen-thawed sperm characteristics were

analysed using IVOS. Factors included in this experiment were equilibration duration, vapour exposure temperature and vapour exposure duration. The best combination of equilibration duration, vapour exposure temperature and vapour exposure duration for frozen-thawed sperm characteristics was used for subsequent experiments (Experiment 4).

3.5.3 Comparison between the Low Temperature Incubator and the Normal Refrigerator on the Frozen-thawed Sperm Characteristics with reference to Equilibration Duration (Experiment 3)

Performance of low temperature incubator and normal refrigerator for freezing the sperm was compared based on frozen-thawed sperm characteristics. This experiment was carried out under laboratory condition at the ISB Mini Farm (Livestock) involving 20 mature males and three equilibration durations i.e. 30, 45 or 60 minutes. After the equilibration process, the quality of the sperm was analysed using IVOS. Three replicates and 15 observations (straws) were performed per treatment. The viability of the sperm were compared among the equilibration duration for both incubator/refrigerator. The findings obtained from this experiment will be used as a basis for subsequent experiment (Experiment 4).

3.8.4 An Attempt to Transfer of Technology from Laboratory to the Field Condition for the Sperm Cryopreservation of Thai mahseer (*Tor tambroides*) under Field Condition (Experiment 4)

In this attempt, a total of 17 mature males were involved and reared at the fish tank in Taman Negara Sg. Relau, Merapoh, Pahang Darul Makmur. Basically, the same procedure as in Experiment 2 was used in this experiment. However, under field condition, a normal

refrigerator with smaller special rack design was used for equilibration whereby 30 minutes equilibration duration was applied as suggested from the results of Experiment 3. A smaller styrofoam box was used to facilitate the vapour exposure phase. Other factors such as incubator/refrigerator temperature, equilibration duration, vapour exposure temperature and vapour exposure duration were followed as recommended from Experiment 2. These were 4°C, 30 minutes, -110°C and 15 minutes, respectively. Similar hormonal stimulation, semen collection, semen dilution and semen processing prior to freezing were used as previously described in this chapter. A total of 17 replicates and 15 straws sample were evaluated for frozen-thawed sperm characteristics after cryopreservation attempt under field condition. At the end of the field work, the frozen sperm from the field were transported in liquid nitrogen carrier tank before being transferred to the permanent liquid nitrogen storage tank at the University of Malaya for further sperm analysis using the IVOS. The sperm parameters measured in this experiment were sperm motility characteristics.

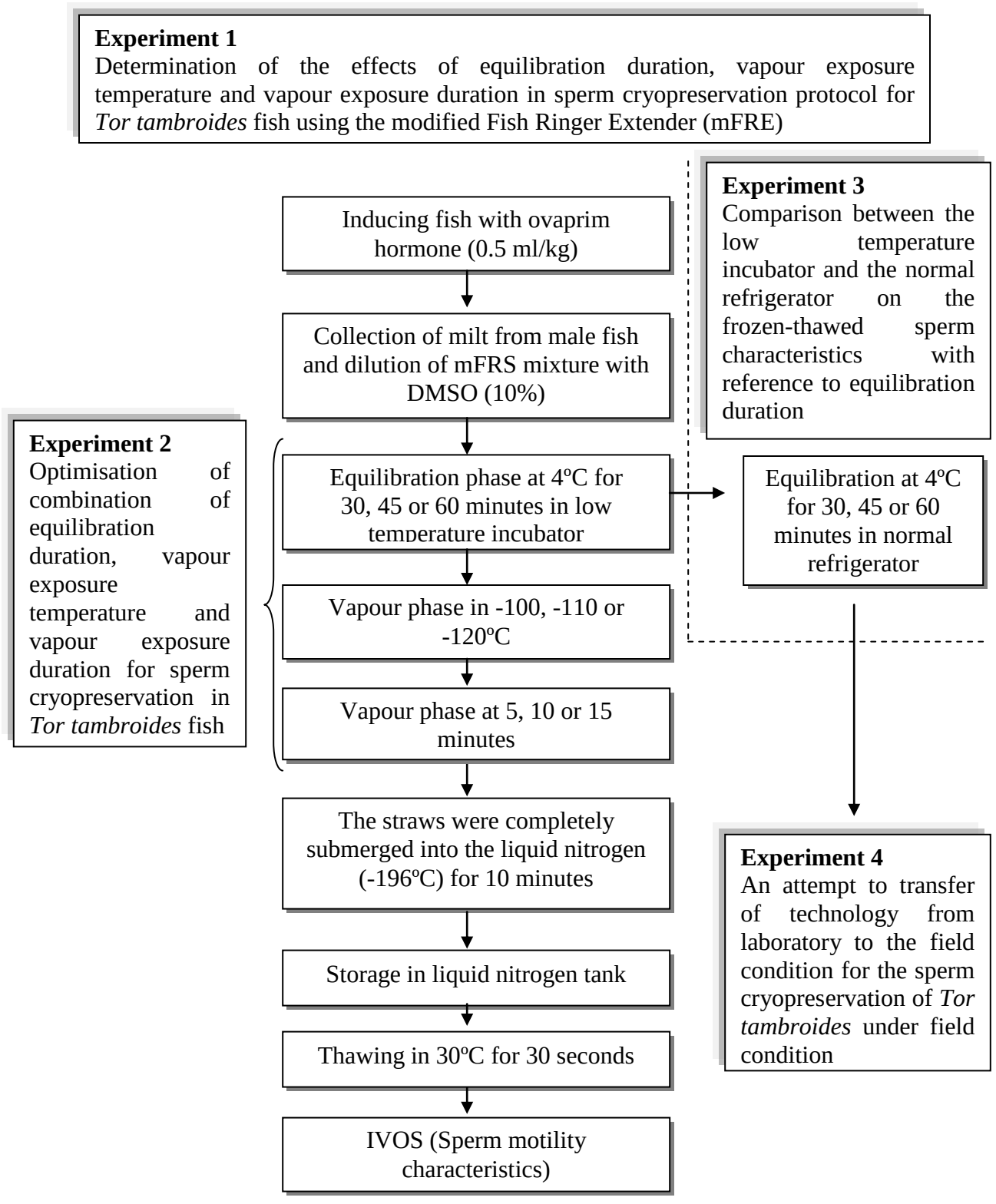

Figure 3.12: Flow chart of experimental design.

Chapter 4
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4.0 RESULTS

4.1 EFFECTS OF EQUILIBRATION DURATION ON FROZEN-THAWED SPERM MOTILITY CHARACTERISTICS IN THAI MAHSEER (*Tor tambroides*) (EXPERIMENT 1)

Frozen-thawed sperm motility characteristics were the main parameters analysed using the IVOS in this experiment. Table 4.1 shows that equilibration duration of 30 minutes was significantly higher than that of 45 minutes in total motility (67.31±1.27% and 61.93±1.31%, respectively) and progressive motility (13.94±0.65% and 11.76±0.58%, respectively). However, there were no significant differences in total motility and progressive motility (ranging from 65.09±1.02% to 67.31±1.27% and from 13.94±0.65% to 14.27±0.68%, respectively) between 30 minutes of equilibration duration and 60 minutes of equilibration duration. Similar situations were observed in rapid and slow velocities which are shown in Table 4.2. There were no significant differences between all equilibration durations in medium velocity, ranging from 9.39±0.34% to 9.96±0.35%. The lowest velocity of sperm movement was the static sperm which was found to be significantly higher in 45 minutes of equilibration duration (38.60±1.32%) as compared to 30 and 60 minutes of equilibration duration (32.73±1.27% and 31.16±1.17%, respectively).

In Table 4.3, 30 minutes of equilibration durations were significantly different with 45 minutes of equilibration duration in sperm motion characteristics of VAP, VSL VCL, STR and LIN (36.78±0.66µm/s and 33.63±0.66µm/s, 32.40±0.59µm/s and 29.51±0.56 µm/s, 53.15±0.85µm/s and 49.35±1.05µm/s, 82.89±1.29% and 77.93±1.99%, and 57.16±0.84% and 54.05±1.12%, respectively). However, there were no significant differences between 30 minutes of equilibration duration and 60 minutes of equilibration

duration in VAP, VSL VCL, STR and LIN values, ranging from 36.78±0.66µm/s to 37.62±0.63µm/s, from 32.40±0.59µm/s to 32.97±0.58µm/s, from 53.15±0.85µm/s to 55.07±0.66µm/s, from 82.89±1.29% to 86.29±0.27% and from 57.16±0.84% to 59.02±0.56%, respectively. In ALH, there was no significant difference between 30 minutes of equilibration duration and 45 minutes of equilibration duration, ranging from 3.06±0.07µm to 3.16±0.09µm. However, 45 minutes of equilibration duration (2.89±0.09µm) was significantly different with 60 minutes of equilibration duration (3.16±0.09µm). Meanwhile, there were no significant differences in BCF between all equilibration durations (30, 45 and 60 minutes), ranging from 28.24±0.42Hz to 28.34±0.31Hz.

Table 4.1: Total motility and progressive motility (mean±SEM) of frozen-thawed sperm for different equilibration durations

Equilibration duration (min)	N	Total motility (%)	Progressive motility (%)
30	135	67.31±1.27[b]	13.94±0.65[b]
45	135	61.93±1.31[a]	11.76±0.58[a]
60	135	65.09±1.02[ab]	14.27±0.68[b]

N = no. of observation.
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.2: Velocity distribution (mean±SEM) of frozen-thawed sperm for different equilibration durations

Equilibration duration (min)	N	Rapid (%)	Medium (%)	Slow (%)	Static (%)
30	135	18.23±0.83[b]	9.96±0.35[a]	39.11±0.70[ab]	32.73±1.27[a]
45	135	14.56±0.67[a]	9.39±0.34[a]	37.45±0.77[a]	38.60±1.32[b]
60	135	18.07±0.82[b]	9.67±0.37[a]	41.06±0.77[b]	31.16±1.17[a]

N = no. of observation.
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.3: Sperm motion characteristics (mean ± SEM) of frozen-thawed sperm for different equilibration durations

Equilibration duration (min)	N	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
30	135	36.78 ±0.66^{b}	32.40 ±0.59^{b}	53.15 ±0.85^{b}	3.06 ±0.07^{ab}	28.24 ±0.42^{b}	82.89 ±1.29^{b}	57.16 ±0.84^{b}
45	135	33.63 ±0.66^{a}	29.51 ±0.56^{a}	49.35 ±1.05^{a}	2.89 ±0.09^{a}	26.86 ±0.45^{ab}	77.93 ±1.99^{a}	54.05 ±1.12^{a}
60	135	37.62 ±0.63^{b}	32.97 ±0.58^{b}	55.07 ±0.66^{b}	3.16 ±0.09^{b}	28.34 ±0.31^{b}	86.29 ±0.27^{b}	59.02 ±0.56^{b}

N = no. of observation.
^{ab}Means with different superscripts within a column were significantly different ($P<0.05$).

Table 4.4 shows the correlation of frozen-thawed sperm motility characteristics using 30 minutes of equilibration. There were positive correlations ($P<0.05$) between total motility and progressive motility, rapid and total motility, rapid and progressive motility, medium and total motility, medium and progressive motility, medium and rapid, slow and total motility, slow and medium, VAP and progressive motility, VAP and rapid, VSL and progressive motility, VSL and rapid, VSL and VAP, VCL and progressive motility, VCL and rapid, VCL and rapid, VCL and VAP, VCL and VAP, VCL and VSL, ALH and total motility, ALH and slow, ALH and VAP, ALH and VSL, ALH and VCL, BCF and medium, BCF and slow, STR and static, STR and VAP, STR and VSL, STR and VCL, STR and ALH, STR and BCF, LIN and VAP, LIN and VSL, LIN and VCL, LIN and BCF, and LIN and STR. Meanwhile, there were some negative correlations among the characteristics such as between static and total motility, static and progressive motility, static and rapid, static and medium, static and slow, VAP and medium, VSL and medium, VCL and medium, ALH and static, BCF and progressive motility, BCF and progressive motility, BCF and rapid, BCF and VSL, STR and total motility, STR and progressive motility, STR and rapid, and LIN and medium.

Positive correlations among frozen-thawed sperm motility characteristics were obtained in Thai mahseer sperm cryopreservation using 45 minutes (Table 4.5) such as between total motility and progressive motility, rapid and total motility, rapid and progressive motility, medium and total motility, medium and progressive motility, medium and rapid, slow and total motility, slow and progressive motility, slow and rapid, slow and medium, VAP and progressive motility, VAP and rapid, VSL and progressive motility, VSL and rapid, VSL and VAP, VCL and VAP, VCL and VSL, ALH and VAP, ALH and VSL, ALH and VCL, BCF and VAP, BCF and VSL, BCF and VCL, STR and static, STR and VAP, STR and VSL, STR and VCL, STR and ALH, STR and BCF, LIN and VAP, LIN and VSL, LIN and VCL, LIN and BCF, and LIN and STR. However, there were also negative correlations in this table such as between static and total motility, static and progressive motility, static and rapid, static and medium, static and slow, VAP and medium, VSL and medium, VCL and medium, ALH and progressive motility, ALH and rapid, ALH and medium, STR and total motility, STR and progressive motility, STR and rapid, and LIN and medium.

Table 4.6 shows the correlations of frozen-thawed sperm motility characteristics of cryopreservation of *Tor tambroides* using 60 minutes of equilibration duration whereby there were some positive correlations between total motility and progressive motility, total motility and rapid, total motility and medium, total motility and slow, total motility and VAP, total motility and VSL, total motility and LIN, progressive motility and rapid, progressive motility and medium, progressive motility and VAP, progressive motility and VSL, progressive motility and VCL, progressive motility and STR, progressive and LIN. Rapid and medium, rapid and VAP, rapid and VSL, rapid and VCL, rapid and LIN,

medium and BCF, slow and ALH, VAP and VSL, VAP and VCL, VAP and LIN, VSL and VCL, VSL and LIN, and STR and LIN.

Besides, there were also negative correlations among parameters such as total motility and static, progressive motility and slow, progressive motility and static, progressive motility and ALH, rapid and slow, rapid and static, rapid and ALH, medium and static, medium and VAP, medium and VSL, medium and VCL, medium and LIN, slow and static, static and VAP, static and VSL, static and LIN, VAP and BCF, VSL and BCF, ALH and BCF, ALH and STR, and ALH and LIN.

For overall correlations in frozen-thawed sperm characteristics between all equilibration durations used for this experiment, some positive correlations were found between total motility and progressive motility, total motility and rapid, total motility and medium, total motility and slow, total motility VAP, total motility and VSL, progressive motility and rapid, progressive motility and medium, progressive motility and VAP, progressive motility and VSL, progressive motility and VCL, progressive motility and LIN, rapid and medium, rapid and VAP, rapid and VSL, rapid and VCL, rapid and STR, medium and slow, medium and BCF, slow and ALH, slow and BCF, static and STR, VAP and VSL, VAP and VCL, VAP and ALH, VAP and STR, VAP and LIN, VSL and VCL, VSL and ALH, VSL and STR, VSL and LIN, VCL and ALH, VCL and BCF, VCL and STR, VCL and LIN, ALH and STR, BCF and STR, BCF and LIN, and STR and LIN.

Meanwhile, negative correlations were found between total motility and static, total motility and STR, progressive motility and static, progressive motility and ALH, rapid and static, rapid and ALH, rapid and STR, medium and static, medium and VAP, medium and VSL, medium and VSL, medium and VCL, medium and ALH, medium and STR, medium and LIN, slow and static, static and VAP, and static and VSL.

Table 4.4: Correlations between frozen-thawed sperm motility characteristics using 30 minutes of equilibration duration in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.695**	.758**	.625**	.603**	-.999**	.161	.151	.117	.174*	.054	-.237**	-.114
Progressive Motility	1.000	.972**	.312**	-.052	-.695**	.422**	.472**	.231**	-.117	-.227**	-.194*	.065
Rapid		1.000	.353**	.003	-.757**	.407**	.433**	.243**	-.055	-.227**	-.257**	-.017
Medium			1.000	.221*	-.626**	-.353**	-.350**	-.264**	.056	.206*	-.150	-.246**
Slow				1.000	-.605**	-.017	-.065	.056	.366**	.273**	-.046	-.057
Static					1.000	-.161	-.152	-.118	-.176*	-.054	.238**	.114
VAP						1.000	.962**	.895**	.262**	-.168	.352**	.505**
VSL							1.000	.816**	.182*	-.178*	.301**	.491**
VCL								1.000	.462**	.069	.562**	.541**
ALH									1.000	-.036	.176*	.004
BCF										1.000	.516**	.271**
STR											1.000	.845**
LIN												1.000

No. of observation = 135
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.5: Correlations between frozen-thawed sperm motility characteristics using 45 minutes of equilibration duration in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	.727**	.729**	.606**	.777**	-.983**	-.001	.075	-.154	-.100	-.063	-.235**	-.151
Progressive Motility	1.000	.957**	.316**	.266**	-.726**	.244**	.321**	-.016	-.291**	-.005	-.178*	.016
Rapid		1.000	.332**	.252**	-.743**	.253**	.304**	.012	-.241**	-.027	-.230**	-.059
Medium			1.000	.312**	-.605**	-.315**	-.299**	-.276**	-.212*	.068	-.121	-.180*
Slow				1.000	-.795**	-.091	-.019	-.158	.145	-.038	-.169	-.155
Static					1.000	.003	-.071	.156	.089	.022	.247**	.166
VAP						1.000	.965**	.884**	.358**	.308**	.550**	.643**
VSL							1.000	.755**	.281**	.232**	.472**	.625**
VCL								1.000	.524**	.438**	.708**	.649**
ALH									1.000	.066	.276**	.144
BCF										1.000	.706**	.640**
STR											1.000	.936**
LIN												1.000

No. of observation = 135

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.6: Correlations between frozen-thawed sperm motility characteristics using 60 minutes of equilibration duration in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.603**	.633**	.517**	.514**	-.940**	.182*	.181*	.100	-.096	.149	.049	.184*
ProgressiveMotility	1.000	.982**	.345**	-.206*	-.658**	.521**	.543**	.236**	-.360**	.033	.240**	.608**
Rapid		1.000	.380**	-.195*	-.690**	.539**	.536**	.310**	-.294**	.017	.100	.508**
Medium			1.000	.003	-.584**	-.311**	-.322**	-.295**	-.159	.321**	-.086	-.208*
Slow				1.000	-.513**	-.095	-.091	-.005	.270**	.043	.003	-.126
Static					1.000	-.207*	-.203*	-.115	.083	-.136	-.040	-.199*
VAP						1.000	.982**	.857**	.169	-.380**	-.007	.540**
VSL							1.000	.768**	.079	-.368**	.167	.659**
VCL								1.000	.485**	-.429**	-.418**	.061
ALH									1.000	-.339**	-.490**	-.431**
BCF										1.000	.036	-.126
STR											1.000	.751**
LIN												1.000

No. of observation = 135

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.7: Overall correlations between frozen-thawed sperm motility characteristics using 30, 45 and 60 minutes of equilibration duration in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.671**	.708**	.580**	.638**	-.971**	.134**	.159**	.025	-.003	.048	-.165**	-.048
Progressive Motility	1.000	.972**	.328**	.009	-.694**	.421**	.471**	.167**	-.242**	-.051	-.085	.192**
Rapid		1.000	.359**	.032	-.733**	.428**	.454**	.204**	-.177**	-.060	-.133**	.116*
Medium			1.000	.173**	-.597**	-.308**	-.306**	-.254**	-.112*	.187**	-.098*	-.187**
Slow				1.000	-.653**	-.031	-.022	-.010	.262**	.105*	-.059	-.077
Static					1.000	-.159**	-.181**	-.050	-.014	-.072	.145**	.028
VAP						1.000	.970**	.875**	.280**	-.002	.408**	.576**
VSL							1.000	.775**	.197**	-.038	.357**	.581**
VCL								1.000	.495**	.168**	.584**	.539**
ALH									1.000	-.059	.173**	-.007
BCF										1.000	.569**	.400**
STR											1.000	.872**
LIN												1.000

No. of observation = 405.

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4.2 EFFECTS OF VAPOUR EXPOSURE TEMPERATURE ON FROZEN-THAWED SPERM MOTILITY CHARACTERISTICS IN THAI MAHSEER (*Tor tambroides*) (EXPERIMENT 1)

The second factor studied in this experiment was the vapour exposure temperature which consists of three different temperatures i.e. -100, -110 and -120°C. Table 4.8 shows there were significant differences between three temperatures used whereby -110°C showed the highest significant value in total motility (68.53±1.13%) and progressive motility (15.75±0.61%) compared to -100 (60.74±1.41% and 11.65±0.68%, respectively) and -120°C (65.07±0.99% and 12.56±0.56%, respectively). Meanwhile, -100°C (60.74±1.41%) was significantly different from that of -120°C (65.07±0.99%) in total motility but both temperatures were not significantly different in progressive motility, ranging from 11.65±0.68% to 12.56±0.56%.

As for velocity distribution analysis (Table 4.9), -110°C of vapour exposure temperature (19.73±0.71) was significantly different compared to -100 and -120°C of vapour exposure temperatures (15.27±0.84% and 15.86±0.75%, respectively) in rapid sperm velocity. In medium and slow velocities, similar situations were obtained that showed -110°C (10.30±0.31% and 40.67±0.68%, respectively) was significantly different to -100°C (7.99±0.31% and 37.38±0.92%, respectively) but -110°C was not significantly different to -120°C, ranging from 10.30±0.31% to 10.71±0.39%. All vapour exposure temperatures (-100, -110 and -120°C) were found to be significantly different in static sperm velocity values (39.30±1.41%, 29.40±1.18% and 39.57±1.10%, respectively).

Meanwhile, in sperm motion characteristics (Table 4.10), similar conditions were obtained in VAP, VCL, ALH, STR and LIN values which showed -110°C of vapour exposure temperature (35.93±0.68%, 51.20±1.01%, 2.89±0.08µm, 78.84±1.93% and

55.56±1.15%, respectively) was significantly different to that of -100°C of vapour exposure temperature (.97±0.64%, 55.31±0.74%, 3.24±0.10µm, 85.76±0.29% and 59.07±0.55%, respectively). Conversely, -110°C was not significantly different to that of -120°C (ranging from 34.13±0.63% to 35.93±0.68%, from 51.07±0.86% to 51.20±1.01%, from 2.89±0.08µm to 2.97±0.09µm, from 78.84±1.93% to 82.50±1.40% and from 55.56±1.15% to 55.61±0.83%, respectively). However, there was no significant difference between -100°C and -110°C (ranging from 33.16±0.60% to 29.88±0.55%) in VSL but -100°C (33.16±0.60%) was found significantly different to -120°C (31.84±0.60%). In addition, there was no significant difference between all vapour exposure temperatures (-100, -110 and -120°C) in BCF values ranging from 27.35±0.39Hz to 27.94±0.37Hz.

Table 4.8: Total motility and progressive motility (mean±SEM) of frozen-thawed sperm for different vapour exposure temperatures

Vapour exposure temperature (°C)	N	Total motility (%)	Progressive motility (%)
-100	135	60.74±1.41[a]	11.65±0.68[a]
-110	135	68.53±1.13[c]	15.75±0.61[b]
-120	135	65.07±0.99[b]	12.56±0.56[a]

N = no. of observation.
[abc]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.9: Velocity distribution (mean±SEM) of frozen-thawed sperm for different vapour exposure temperatures

Vapour exposure temperature (°C)	N	Rapid (%)	Medium (%)	Slow (%)	Static (%)
-100	135	15.27±0.84[a]	7.99±0.31[a]	37.38±0.92[a]	39.30±1.41[c]
-110	135	19.73±0.71[b]	10.30±0.31[b]	40.67±0.68[b]	29.40±1.18[a]
-120	135	15.86±0.75[a]	10.71±0.39[b]	39.57±0.61[b]	39.57±1.10[b]

N = no. of observation.
[abc]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.10: Sperm motion characteristics (mean±SEM) of frozen-thawed sperm for different vapour exposure temperatures

Vapour exposure temperature (°C)	N	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
-100	135	37.97 ±0.64[b]	33.16 ±0.60[b]	55.31 ±0.74[b]	3.24 ±0.10[b]	27.94 ±0.37[a]	85.76 ±0.29[b]	59.07 ±0.55[b]
-110	135	35.93 ±0.68[a]	31.84 ±0.60[b]	51.20 ±1.01[a]	2.89 ±0.08[a]	28.15 ±0.44[a]	78.84 ±1.93[a]	55.56 ±1.15[a]
-120	135	34.13 ±0.63[a]	29.88 ±0.55[a]	51.07 ±0.86[a]	2.97 ±0.09[a]	27.35 ±0.39[a]	82.50 ±1.40[ab]	55.61 ±0.83[a]

N = no. of observation.
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Correlation analyses for frozen-thawed sperm motility characteristics of Thai mahseer cryopreservation using -100°C were evaluated (Table 4.11). Positive correlations were observed such as between total motility and progressive motility, total motility and rapid, total motility and medium, total motility and slow, total motility and VAP, total motility and VSL, total motility and VCL, progressive motility and rapid, progressive motility and medium, progressive motility and VAP, progressive motility and VSL, progressive motility and VCL, progressive motility and STR, progressive motility and LIN, rapid and medium, rapid and VAP, rapid and VSL, rapid and VCL, rapid and LIN,

medium and slow, medium and BCF, slow and ALH, VAP and VSL, VAP and VSL, VAP and VCL, VAP and LIN, VSL and VCL, VCL and ALH, and STR and LIN.

However, there were negative correlations were obtained such as between total motility and static, progressive motility and static, progressive motility and ALH, rapid and static, medium and static, slow and static, static and VAP, static and VSL, static and VCL, VAP and BCF, VSL and LIN, VCL and BCF, VCL and STR, ALH and BCF, ALH and STR, and ALH and LIN.

For sperm cryopreservation of Thai mahseer using -110°C of vapour exposure temperature (Table 4.12), some positive correlations were obtained such as between total motility and progressive motility, total motility and rapid, total motility and medium, total motility and slow, progressive motility and rapid, progressive motility and medium, progressive motility and VAP, progressive motility and VSL, progressive motility and LIN, rapid and medium, rapid and VAP, rapid and VSL, rapid and VCL, medium and slow, VAP and VSL, VAP and VCL, VAP and ALH, VAP and BCF, VAP and STR, VAP and LIN, VSL and VCL, VSL and ALH, VSL and STR, VSL and LIN, VCL and ALH, VCL and BCF, VCL and STR, VCL and LIN, ALH and STR, BCF and STR, BCF and LIN, and STR and LIN. However, there were also some negative correlations were found such as between total motility and static, total motility and STR, progressive motility and static, progressive motility and ALH, rapid and static, medium and static, medium and VAP, medium and VSL, medium and VCL, and slow and static.

Table 4.13 shows the correlation between frozen-thawed sperm motility characteristics in sperm cryopreservation of Thai mahseer using -120°C of vapour exposure temperature in which some positive correlations were attained such as between total motility and progressive motility, total motility and rapid, total motility and rapid,

total motility and medium, total motility and slow, total motility and VAP, progressive motility and rapid, progressive motility and medium, progressive motility and VAP, progressive motility and VSL, medium and BCF, rapid and medium, rapid and VAP, rapid and VSL, slow and ALH, slow and BCF, static and STR, VAP and VSL, VAP and VCL, VAP and ALH, VAP and STR, VAP and LIN, VSL and VCL, VSL and ALH, VSL and STR, VSL and LIN, VCL and ALH, VCL and BCF, VCL and STR, VCL and LIN, ALH and STR, BCF and STR, BCF and LIN, and STR and LIN.

There were some negative correlations obtained such as between total motility and static, total motility and STR, progressive motility and static, progressive motility and ALH, progressive motility and STR, rapid and static, rapid and STR, medium and static, medium and VAP, medium and VSL, medium and VCL, medium and LIN, slow and static, static and VAP, and static and BCF.

Overall correlations of frozen-thawed sperm motility characteristics for all vapour exposure used in sperm cryopreservation of Thai mahseer are shown in Table 4.14. There were some positive correlations such as between total motility and progressive motility, total motility and rapid, total motility and medium, total motility and slow, total motility and VAP, total motility VSL, progressive motility and rapid, progressive motility and medium, progressive motility and VAP, progressive motility and VSL, progressive motility and VCL, progressive motility and LIN, rapid and medium, rapid and VAP, rapid and VSL, rapid and VCL, rapid and LIN, medium and slow, medium and BCF, slow and ALH, slow and BCF, static and STR, VAP and VSL, VAP and VCL, VAP and ALH, VAP and STR, VAP and LIN, VSL and VCL, VSL and ALH, VSL and STR, VSL and LIN, VCL and ALH, VCL and BCF, VCL and STR, VCL and LIN, ALH and STR, BCF and STR, BCF and LIN, and STR and LIN.

There were also some negative correlations obtained such as between total motility and static, total motility and STR, progressive motility and static, progressive motility and ALH, rapid and static, rapid and ALH, rapid and STR, medium and static, medium and VAP, medium and VSL, medium and VCL, medium and ALH, medium and ALH, medium and STR, medium and LIN, slow and static, static and VAP, and static and VSL.

Table 4.11: Correlations between frozen-thawed sperm motility characteristics using -100°C of vapour exposure temperature in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.646**	.695**	.612**	.688**	-.999**	.314**	.337**	.245**	.115	.082	.038	.156
Progressive Motility	1.000	.979**	.413**	-.052	-.645**	.598**	.637**	.359**	-.210*	-.062	.217*	.550**
Rapid		1.000	.435**	-.004	-.694**	.622**	.635**	.417**	-.161	-.068	.104	.479**
Medium			1.000	.201*	-.613**	-.161	-.103	-.151	-.037	.253**	.053	-.107
Slow				1.000	-.690**	-.036	-.033	.042	.342**	.109	-.057	-.168
Static					1.000	-.315**	-.337**	-.246**	-.117	-.082	-.034	-.154
VAP						1.000	.960**	.848**	.077	-.289**	-.075	.489**
VSL							1.000	.735**	.011	-.226**	.134	.612**
VCL								1.000	.385**	-.280**	-.441**	-.004
ALH									1.000	-.211*	-.362**	-.444**
BCF										1.000	.083	-.122
STR											1.000	.688**
LIN												1.000

No. of observation = 135

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.12: Correlations between frozen-thawed sperm motility characteristics using -110°C of vapour exposure temperature in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.684**	.723**	.531**	.645**	-.955**	.017	.046	-.083	-.137	-.056	-.173*	-.031
Progressive Motility	1.000	.977**	.181*	.130	-.719**	.349**	.411**	.134	-.243**	-.121	.010	.226**
Rapid		1.000	.229**	.143	-.753**	.347**	.383**	.171*	-.154	-.139	-.030	.156
Medium			1.000	.173*	-.507**	-.383**	-.423**	-.249**	-.108	.142	-.062	-.140
Slow				1.000	-.705**	-.004	.023	-.082	.108	.006	-.128	-.023
Static					1.000	-.103	-.129	.012	.061	.045	.111	-.040
VAP						1.000	.972**	.893**	.452**	.174*	.568**	.620**
VSL							1.000	.798**	.349**	.084	.485**	.590**
VCL								1.000	.610**	.426**	.744**	.664**
ALH									1.000	.076	.292**	.133
BCF										1.000	.780**	.639**
STR											1.000	.913**
LIN												1.000

No. of observation = 135

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.13: Correlations between frozen-thawed sperm motility characteristics using -120°C of vapour exposure temperature in

cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.659**	.700**	.543**	.453**	-.920**	.175*	.165	.068	.074	.107	-.222**	-.156
Progressive Motility	1.000	.958**	.337**	-.164	-.682**	.407**	.427**	.149	-.223**	.008	-.229**	.008
Rapid		1.000	.378**	-.133	-.743**	.380**	.379**	.156	-.161	-.004	-.294**	-.098
Medium			1.000	.051	-.631**	-.264**	-.297**	-.228**	-.094	.232**	-.106	-.203*
Slow				1.000	-.484**	.024	.007	.124	.392**	.239**	.096	-.008
Static					1.000	-.177*	-.158	-.091	-.069	-.203*	.190*	.148
VAP						1.000	.977**	.890**	.318**	.027	.415**	.600**
VSL							1.000	.798**	.246**	-.037	.363**	.600**
VCL								1.000	.487**	.188*	.617**	.623**
ALH									1.000	-.037	.198*	.082
BCF										1.000	.548**	.395**
STR											1.000	.908**
LIN												1.000

No. of observation = 135
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.14: Overall correlations between frozen-thawed sperm motility characteristics using -100, -110 and -120°C of vapour exposure

temperature in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.674**	.710**	.567**	.634**	-.966**	.140**	.165**	.028	.002	.041	-.169**	-.047
Progressive Motility	1.000	.972**	.328**	.009	-.694**	.421**	.471**	.167**	-.242**	-.051	-.085	.192**
Rapid		1.000	.359**	.032	-.733**	.428**	.454**	.204**	-.177**	-.060	-.133**	.116*
Medium			1.000	.173**	-.597**	-.308**	-.306**	-.254**	-.112*	.187**	-.098*	-.187**
Slow				1.000	-.653**	-.031	-.022	-.010	.262**	.105*	-.059	-.077
Static					1.000	-.159**	-.181**	-.050	-.014	-.072	.145**	.028
VAP						1.000	.970**	.875**	.280**	-.002	.408**	.576**
VSL							1.000	.775**	.197**	-.038	.357**	.581**
VCL								1.000	.495**	.168**	.584**	.539**
ALH									1.000	-.059	.173**	-.007
BCF										1.000	.569**	.400**
STR											1.000	.872**
LIN												1.000

No. of observation = 405

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4.3 EFFECTS OF VAPOUR EXPOSURE DURATION ON FROZEN-THAWED SPERM MOTILITY CHARACTERISTICS IN THAI MAHSEER (*Tor tambroides*) (EXPERIMENT 1)

As shown in Table 4.15, sperm cryopreservation of Thai mahseer using three different vapour exposure durations, even though insignificant, 15 minutes of vapour exposure duration produced the highest value of total motility (65.25±1.25%) compared to 5 (63.87±1.31%) and 10 minutes (65.21±1.09%) of vapour exposure durations. In progressive motility, 5 minutes of vapour exposure duration (12.31±0.58%) was significantly different compared to 15 minutes of vapour exposure duration (14.83±0.71%), however, there was no significant difference between 5 and 10 minutes of vapour exposure duration with a range from 12.31±0.58% to 12.82±0.58%.

Velocity distribution of frozen-thawed sperm characteristics were demonstrated in Table 4.16. In rapid velocity, there was significant difference between 5 and 15 minutes of vapour exposure duration (15.67±0.72% and 18.44±0.88%, respectively). However, 5 minutes of vapour exposure duration was not significant different to 10 minutes of vapour exposure duration with a range from 15.67±0.72% to 16.75±0.74%. Similar situations were observed in medium, slow and static in which there were no significant differences in 5, 10 and 15 minutes of vapour exposure duration (9.85±0.38%, 9.47±0.31% and 9.67±0.37%, 39.31±0.78%, 39.54±0.70% and 38.77±0.79%, 35.09±1.36%, 34.25±1.10% and 33.14±1.36%, respectively).

Table 4.17 shows similar situations obtained in VAP and VSL in which longer vapour exposure duration resulted higher values within 5 to 15 minutes (34.07±0.62µm, 36.10±0.66µm and 37.86±0.68µm, and 29.84±0.53µm, 31.78±0.55µm and 33.26±0.64µm, respectively). Meanwhile, VCL values showed similar trends in which 5 minutes of vapour

exposure duration (50.55±0.88µm) was significantly different to 15 minutes of vapour exposure duration (54.73±0.89µm). However, 10 minutes of vapour exposure duration was not significantly different to 5 and 15 minutes of vapout exposure duration with a range from 50.55±0.88µm/s to 52.29±0.87µm/s and from 52.29±0.87µm/s to 54.73±0.89µm/s, respectively.

Other sperm motion characteristics such as ALH, STR and LIN values showed similar patterns in which no significant differences were found in all three different vapour exposure durations (5, 10 and 15 minutes). The values are as follows: 2.96±0.09µm, 3.02±0.09µm and 3.11±0.08µm, 82.54±1.40%, 81.87±1.51% and 82.70±1.32% and 55.84±0.89%, 56.91±0.90% and 57.47±0.86%, respectively. However, 5 minutes of vapour exposure duration (28.59±0.36Hz) yielded significantly higher BCF values compared to 10 and 15 minutes of vapour duration (27.70±0.43Hz and 27.14±0.40Hz, respectively).

Table 4.15: Total motility and progressive motility (mean±SEM) of frozen-thawed sperm for different vapour exposure durations

Vapour exposure duration (min)	N	Total motility (%)	Progressive motility (%)
5	135	63.87±1.31[a]	12.31±0.58[a]
10	135	65.21±1.09[a]	12.82±0.58[a]
15	135	65.25±1.25[a]	14.83±0.71[b]

N = no. of observation.
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.16: Velocity distribution (mean±SEM) of frozen-thawed sperm for different vapour exposure durations

Vapour exposure duration (min)	N	Rapid (%)	Medium (%)	Slow (%)	Static (%)
5	135	15.67±0.72[a]	9.85±0.38[a]	39.31±0.78[a]	35.09±1.36[a]
10	135	16.75±0.74[ab]	9.47±0.31[a]	39.54±0.70[a]	34.25±1.10[a]
15	135	18.44±0.88[b]	9.67±0.37[a]	38.77±0.79[a]	33.14±1.36[a]

N = no. of observation.
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.17: Sperm motion characteristics (mean±SEM) of frozen-thawed sperm for different vapour exposure durations

Vapour exposure duration (min)	N	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
5	135	34.07 ±0.62[a]	29.84 ±0.53[a]	50.55 ±0.88[a]	2.96 ±0.09[a]	28.59 ±0.36[b]	82.54 ±1.40[a]	55.84 ±0.89[a]
10	135	36.10 ±0.66[b]	31.78 ±0.55[b]	52.29 ±0.87[ab]	3.02 ±0.09[a]	27.70 ±0.43[ab]	81.87 ±1.51[a]	56.91 ±0.90[a]
15	135	37.86 ±0.68[b]	33.26 ±0.64[b]	54.73 ±0.89[b]	3.11 ±0.08[a]	27.14 ±0.40[a]	82.70 ±1.32[a]	57.47 ±0.86[a]

N = no. of observation.
[ab]Means with different superscripts within a column were significantly different (P<0.05).

For sperm cryopreservation with 5 minutes of vapour exposure duration, some positive correlations were obtained as shown in Table 4.18, namely between total motility and progressive motility, total motility and rapid, total motility and slow, total motility and ALH, progressive motility and rapid, progressive motility and medium, progressive motility and VAP, progressive motility and VSL, progressive motility and VCL, rapid and medium, rapid and VAP, rapid and VSL, rapid and VCL, medium and slow, medium and BCF, slow and ALH, slow and BCF, static and STR, VAP and VSL, VAP and VCL, VAP and ALH, VAP and STR, VAP and LIN, VSL and VCL, VSL and ALH, VSL and STR, VSL and LIN, VCL and ALH, VCL and STR, VCL and LIN, ALH and STR, BCF and

STR, BCF and LIN, and STR and LIN. However, there were also some negative correlations obtained, namely between total motility and static, total motility and STR, progressive motility and static, progressive motility and BCF, progressive motility and STR, rapid and static, rapid and BCF, rapid and STR, medium and static, medium and VAP, medium and VSL, medium and VCL, medium and LIN, slow and static, and static and ALH.

Meanwhile, in Table 4.19, there were some positive correlations in frozen-thawed sperm motility characteristics for 10 minutes of vapour exposure duration, namely between total motility and progressive motility, total motility and rapid, total motility and medium, total motility and slow, progressive motility and rapid, progressive motility and medium, progressive motility and slow, progressive motility and VAP, progressive motility and VSL, rapid and medium, rapid and slow, rapid and VAP, rapid and VSL, medium and slow, static and STR, VAP and VSL, VAP and VCL, VAP and ALH, VAP and BCF, VAP and STR, VAP and LIN, VSL and VCL, VSL and ALH, VSL and BCF, VSL and STR, VSL and LIN, VCL and ALH, VCL and BCF, BCF and STR, BCF and LIN, ALH and STR, BCF and STR, BCF and LIN, and STR and LIN. Conversely, the negative correlations were also obtained, namely between total motility and static, total motility and STR, progressive motility and static, progressive motility and ALH, progressive motility and STR, rapid and static, rapid and ALH, rapid and STR, medium and static, medium and VAP, medium and VSL, medium and VCL, medium and ALH, medium and LIN, and slow and static.

Correlation between sperm motility characteristics in sperm cryopreservation of Thai mahseer with 15 minutes of vapour exposure duration were tabulated in Table 4.20. Positive correlations were obtained between total motility and progressive motility, total

motility and rapid, total motility and medium, total motility and slow, total motility and VAP, total motility and VSL, total motility and LIN, progressive motility and rapid, progressive motility and medium, progressive motility and VAP, progressive motility and VSL, progressive motility and VCL, progressive motility and STR, progressive motility and LIN, rapid and medium, rapid and VAP, rapid and VSL, rapid and VCL, rapid and LIN, medium and BCF, slow and ALH, VAP and VSL, VAP and VCL, VAP and VCL, VAP and LIN, VSL and VCL, VSL and LIN, VCL and ALH, and STR and LIN. However, there were also negative correlations, namely between total motility and static, progressive motility and slow, progressive motility and static, progressive motility and ALH, rapid and slow, rapid and static, rapid and ALH, medium and static, medium and VAP, medium and VSL, medium and VCL, medium and LIN, slow and static, static and VAP, static and VSL, static and LIN, VAP and BCF, VSL and BCF, VCL and BCF, VCL and STR, ALH and BCF, ALH and STR, and ALH and LIN.

Overall correlation of frozen-thawed sperm motility characteristics in cryopreservation of Thai mahseer using three different vapour exposure durations are shown in Table 4.21. Positive correlations are as follows: between total motility and progressive motility, total motility and rapid, total motility and medium, total motility and slow, total motility and VAP, total motility and VSL, progressive motility and rapid, progressive motility and medium, progressive motility and VAP, progressive motility and VSL, progressive motility and VSL, progressive motility and LIN, rapid and VAP, rapid and VSL, rapid and VCL, rapid and LIN, medium and slow, medium and BCF, slow and ALH, slow and BCF, static and STR, VAP and VSL, VAP and VCL, VAP and ALH, VAp and STR, VAP and LIN, VSL and VCL, VSL and ALH, VSL and STR, VSL and LIN, VCL and ALH, VCL and BCF, VCL and STR, VCL and LIN, ALH and STR, BCF and

STR, BCF and LIN, and STR and LIN. Meanwhile, there were also negative correlations obtained, namely between total motility and static, total motility and STR, progressive motility and static, progressive motility and ALH, rapid and medium, rapid and static, rapid and ALH, rapid and STR, medium and static, medium and VAP, medium and VSL, medium and VCL, medium and ALH, medium and STR, medium and LIN, slow and static, static and VAP, and static and VSL.

Table 4.18: Correlation between frozen-thawed sperm motility characteristics using 5 minutes of vapour exposure duration in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.695**	.758**	.625**	.603**	-.999**	.161	.151	.117	.174*	.054	-.237**	-.114
Progressive Motility	1.000	.972**	.312**	-.052	-.695**	.422**	.472**	.231**	-.117	-.227**	-.194*	.065
Rapid		1.000	.353**	.003	-.757**	.407**	.433**	.243**	-.055	-.227**	-.257**	-.017
Medium			1.000	.221*	-.626**	-.353**	-.350**	-.264**	.056	.206*	-.150	-.246**
Slow				1.000	-.605**	-.017	-.065	.056	.366**	.273**	-.046	-.057
Static					1.000	-.161	-.152	-.118	-.176*	-.054	.238**	.114
VAP						1.000	.962**	.895**	.262**	-.168	.352**	.505**
VSL							1.000	.816**	.182*	-.178*	.301**	.491**
VCL								1.000	.462**	.069	.562**	.541**
ALH									1.000	-.036	.176*	.004
BCF										1.000	.516**	.271**
STR											1.000	.845**
LIN												1.000

No. of observation = 135

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.19: Correlation between frozen-thawed sperm motility characteristics using 10 minutes of vapour exposure duration in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.727**	.729**	.606**	.777**	-.983**	-.001	.075	-.154	-.100	-.063	-.235**	-.151
Progressive Motility	1.000	.957**	.316**	.266**	-.726**	.244**	.321**	-.016	-.291**	-.005	-.178*	.016
Rapid		1.000	.332**	.252**	-.743**	.253**	.304**	.012	-.241**	-.027	-.230**	-.059
Medium			1.000	.312**	-.605**	-.315**	-.299**	-.276**	-.212*	.068	-.121	-.180*
Slow				1.000	-.795**	-.091	-.019	-.158	.145	-.038	-.169	-.155
Static					1.000	.003	-.071	.156	.089	.022	.247**	.166
VAP						1.000	.965**	.884**	.358**	.308**	.550**	.643**
VSL							1.000	.755**	.281**	.232**	.472**	.625**
VCL								1.000	.524**	.438**	.708**	.649**
ALH									1.000	.066	.276**	.144
BCF										1.000	.706**	.640**
STR											1.000	.936**
LIN												1.000

No. of observation = 135

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.20: Correlation between frozen-thawed sperm motility characteristics using 15 minutes of vapour exposure duration in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.619**	.646**	.471**	.510**	-.931**	.217*	.215*	.135	-.078	.125	.051	.209*
Progressive Motility	1.000	.982**	.345**	-.206*	-.658**	.521**	.543**	.236**	-.360**	.033	.240**	.608**
Rapid		1.000	.380**	-.195*	-.690**	.539**	.536**	.310**	-.294**	.017	.100	.508**
Medium			1.000	.003	-.584**	-.311**	-.322**	-.295**	-.159	.321**	-.086	-.208*
Slow				1.000	-.513**	-.095	-.091	-.005	.270**	.043	.003	-.126
Static					1.000	-.207*	-.203*	-.115	.083	-.136	-.040	-.199*
VAP						1.000	.982**	.857**	.169	-.380**	-.007	.540**
VSL							1.000	.768**	.079	-.368**	.167	.659**
VCL								1.000	.485**	-.429**	-.418**	.061
ALH									1.000	-.339**	-.490**	-.431**
BCF										1.000	.036	-.126
STR											1.000	.751**
LIN												1.000

No. of observation = 135.
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.21: Overall correlation between frozen-thawed sperm motility characteristics using 5, 10 and 15 minutes of vapour exposure duration in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.674**	.710**	.567**	.634**	-.966**	.140**	.165**	.028	.002	.041	-.169**	-.047
Progressive Motility	1.000	.972**	.328**	.009	-.694**	.421**	.471**	.167**	-.242**	-.051	-.085	.192**
Rapid		1.000	.359**	.032	-.733**	.428**	.454**	.204**	-.177**	-.060	-.133**	.116*
Medium			1.000	.173**	-.597**	-.308**	-.306**	-.254**	-.112*	.187**	-.098*	-.187**
Slow				1.000	-.653**	-.031	-.022	-.010	.262**	.105*	-.059	-.077
Static					1.000	-.159**	-.181**	-.050	-.014	-.072	.145**	.028
VAP						1.000	.970**	.875**	.280**	-.002	.408**	.576**
VSL							1.000	.775**	.197**	-.038	.357**	.581**
VCL								1.000	.495**	.168**	.584**	.539**
ALH									1.000	-.059	.173**	-.007
BCF										1.000	.569**	.400**
STR											1.000	.872**
LIN												1.000

No. of observation = 405.

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4.4 EFFECTS OF COMBINATIONS OF EQUILIBRATION DURATION, VAPOUR EXPOSURE TEMPERATURE AND VAPOUR EXPOSURE DURATION ON FROZEN-THAWED SPERM MOTILITY CHARACTERISTICS IN THAI MAHSEER (*Tor tambroides*) (EXPERIMENT 1)

The results shown in Table 4.22, 4.23 and 4.24 demonstrate 27 combinations of equilibration duration, vapour exposure temperature and vapour exposure duration. Combinations of 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration and combination of 60 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration produced the significantly highest values in total motility and progressive motility (77.27±2.49% and 20.60±2.19%, respectively) compared to other combinations. The lowest values were observed in combination of 45 minutes of equilibration duration, -100°C of vapour exposure temperature and 15 minutes of vapour exposure duration in total motility and progressive motility (44.00±4.16% and 7.40±1.22%, respectively).

In velocity distribution, the highest values were obtained in rapid, medium and slow velocities from different combinations of equilibration durations, vapour exposure temperatures and vapour exposure temperatures, that is 60 minutes, -110°C and 15 minutes (25.47±2.81%), 60 minutes, -120°C and 15 minutes (15.00±0.85%) and 60 minutes, -110°C, and 5 minutes (45.13±1.97%), respectively. The combination of 45 minutes of equilibration duration, -100°C of vapour exposure temperature and 15 minutes of vapour exposure duration yielded the highest value in static velocity (56.00±4.16%) and the lowest values in rapid, medium and slow velocities (9.40±1.60%, 6.20±1.04% and 28.47±2.33%, respectively). In static velocity, the lowest value was produced in combination of 60

minutes of equilibration duration, -110°C of vapour exposure temperature and 5 minutes of vapour exposure duration (22.00±1.17%).

Table 4.24 shows the frozen-thawed sperm motion characteristics using different combination of equilibration duration, vapour exposure temperature and vapour exposure temperature. Combination of 30 minutes, -100°C and 10 minutes, respectively gave the highest VAP and VSL values compared to other combinations (42.93±1.31μm/s and 37.41±1.17μm/s, respectively). The highest values of VSL, LIN and STR were obtained from the similar combination, that is 60 minutes, -110°C and 15 minutes (60.85±1.12μm/s, 87.67±0.83% and 64.13±1.34%, respectively). The combinations of 60 minutes, -100°C and 15 minutes, respectively and 30 minutes, -110°C and 5 minutes, respectively produced the highest values of ALH (3.79±0.40μm) and BCF (30.42±0.94Hz). Similar combinations yielded the lowest values in VAP, VSL, VCL, ALH, BCF, STR and LIN (26.43±1.61μm/s, 24.09±1.12μm/s, 36.37±3.51μm/s, 2.25±0.22μm, 23.63±1.94Hz, 52.07±9.69% and 40.13±4.72%, respectively) as shown in combination of 45 minutes, -110°C and 10 minutes, respectively.

Table 4.22: Total motility and progressive motility (mean±SEM) of frozen-thawed sperm for different combinations of equilibration duration, vapour exposure temperature and vapour exposure duration

Equilibration duration (min)	Vapour temperature (˚C)	Vapour duration (min)	N	Total motility (%)	Progressive motility (%)
30	-100	5	135	65.60±2.55^{bc}	12.27±1.15^{abcdefg}
		10	135	61.87±4.06^{bc}	11.47±1.11^{abcde}
		15	135	69.47±4.74^{bcd}	17.20±3.20^{efgh}
	-110	5	135	62.80±4.96^{bc}	11.73±1.55^{abcdef}
		10	135	73.53±1.99^{cd}	16.33±1.40^{cdefgh}
		15	135	77.27±2.49^{d}	17.73±1.96^{gh}
	-120	5	135	60.93±4.34^{b}	11.33±2.39^{abcde}
		10	135	64.13±4.36^{bc}	9.33±1.26^{ab}
		15	135	70.20±1.31^{bcd}	18.07±1.37^{gh}
45	-100	5	135	59.87±4.75^{b}	11.33±1.41^{abcde}
		10	135	59.07±2.98^{b}	11.20±1.84^{abcd}
		15	135	44.00±4.16^{a}	7.40±1.22^{a}
	-110	5	135	69.07±4.71^{bcd}	16.53±2.20^{cdefgh}
		10	135	68.33±3.19^{bcd}	13.13±1.50^{abcdefg}
		15	135	62.33±2.86^{bc}	11.67±1.16^{abcde}
	-120	5	135	60.93±3.46^{b}	7.93±1.01^{a}
		10	135	68.00±3.30^{bcd}	14.40±1.99^{bcdefg}
		15	135	65.80±0.98^{bcd}	12.20±0.66^{abcdefg}
60	-100	5	135	61.47±3.87^{b}	10.13±1.57^{ab}
		10	135	63.40±3.54^{bc}	12.87±2.48^{abcdefg}
		15	135	61.93±4.58^{bc}	11.00±2.84^{abc}
	-110	5	135	68.73±2.31^{bcd}	16.87±1.56^{cdefgh}
		10	135	66.40±2.92^{bcd}	17.13±1.64^{defgh}
		15	135	68.27±2.80^{bcd}	20.60±2.19^{h}
	-120	5	135	65.47±3.63^{bc}	12.67±1.58^{abcdefg}
		10	135	62.13±0.94^{ab}	9.53±1.25^{ab}
		15	135	68.00±1.17^{bcd}	17.60±0.84^{fgh}

N = no. of observation.
^{abcdefgh}Means with different superscripts within a column were significantly different (P<0.05).

Table 4.23: Velocity distribution (mean±SEM) of frozen-thawed sperm for different combinations of equilibration duration, vapour exposure temperature and vapour exposure duration

Equilibration duration (min)	Vapour temperature (˚C)	Vapour duration (min)	N	Rapid (%)	Medium (%)	Slow (%)	Static (%)
30	-100	5	135	16.07 ±1.62^{abcdefghi}	10.67 ±0.73^{cdefg}	38.73 ±1.71^{bcde}	34.40 ±2.55^{cd}
		10	135	17.20 ±1.66^{bcdefghi}	6.80 ±0.55^{ab}	37.40 ±2.58^{bcd}	38.47 ±4.06^{d}
		15	135	21.87 ±3.85^{hij}	10.33 ±1.20^{cdefg}	37.07 ±2.62^{bcd}	30.53 ±4.74^{abcd}
	-110	5	135	14.73 ±1.96^{abcdefgh}	10.27 ±1.58^{cdefg}	37.93 ±2.56^{bcde}	37.20 ±4.96^{cd}
		10	135	20.40 ±1.74^{efghij}	9.73 ±0.47^{bcdefg}	43.53 ±1.09^{bcde}	26.47 ±1.99^{abc}
		15	135	22.80 ±2.14^{ij}	11.87 ±0.86^{fg}	42.67 ±1.48^{bcde}	22.73 ±2.49^{ab}
	-120	5	135	15.07 ±3.43^{abcdefgh}	9.40 ±0.96^{abcdefg}	36.47 ±1.70^{bc}	39.07 ±4.35^{d}
		10	135	12.93 ±2.11^{abcd}	10.80 ±1.52^{defg}	40.40 ±2.74^{bcde}	35.87 ±4.36^{cd}
		15	135	23.00 ±1.59^{ij}	9.73 ±0.64^{bcdefg}	37.80 ±1.20^{bcde}	29.80 ±1.31^{abcd}

(continued)

(continued)

45	-100	5	135	14.80 ±1.89^{abcdefgh}	8.73 ±1.34^{abcdef}	36.33 ±2.58^{b}	40.07 ±4.77^{d}
		10	135	13.67 ±2.07^{abcdef}	7.67 ±0.50^{abcd}	37.80 ±2.73^{bcde}	40.93 ±2.98^{d}
		15	135	9.40 ±1.60^{a}	6.20 ±1.04^{a}	28.47 ±2.33^{a}	56.00 ±4.16^{e}
	-110	5	135	20.20 ±2.34^{defghij}	10.47 ±1.00^{cdefg}	38.07 ±2.35^{bcde}	30.93 ±4.71^{abcd}
		10	135	17.20 ±1.90^{bcdefghi}	12.00 ±0.80^{fg}	39.37 ±1.83^{bcde}	31.67 ±3.19^{abcd}
		15	135	14.67 ±1.47^{abcdefgh}	8.53 ±0.66^{abcde}	39.33 ±1.68^{bcde}	37.67 ±2.86^{cd}
	-120	5	135	10.60 ±1.15^{ab}	8.93 ±1.26^{abcdefg}	41.27 ±2.90^{bcde}	39.13 ±3.48^{d}
		10	135	19.13 ±2.72^{cdefghij}	11.13 ±0.53^{efg}	37.73 ±1.11^{bcde}	32.00 ±3.30^{abcd}
		15	135	11.40 ±0.98^{ab}	10.80 ±0.93^{defg}	38.80 ±1.62^{bcde}	39.00 ±2.33^{d}

(continued)

(continued)

60	-100	5	135	13.33 ±2.06^{abcde}	7.93 ±0.46^{abcde}	40.20 ±2.95^{bcde}	38.53 ±3.87^{d}
		10	135	16.93 ±3.03^{bcdefghi}	7.40 ±0.80^{abc}	39.00 ±1.96^{bcde}	36.60 ±3.54^{cd}
		15	135	14.20 ±3.19^{abcdefg}	6.20 ±0.84^{a}	41.40 ±4.05^{bcde}	38.13 ±4.59^{cd}
	-110	5	135	20.73 ±1.67^{fghij}	12.20 ±0.83^{g}	45.13 ±1.97^{e}	21.93 ±2.36^{a}
		10	135	21.40 ±1.67^{ghij}	9.13 ±0.41^{a}	36.13 ±2.07^{b}	33.60 ±2.92^{bcd}
		15	135	25.47 ±2.81^{j}	8.53 ±0.88^{abcdefg}	44.00 ±2.05^{cde}	22.40 ±2.69^{ab}
	-120	5	135	15.53 ±1.86^{abcdefgh}	10.07 ±1.53^{cdefg}	39.67 ±1.59^{bcde}	34.53 ±3.63^{cd}
		10	135	11.87 ±1.72^{abc}	10.53 ±1.28^{cdefg}	44.60 ±1.25^{de}	32.67 ±2.17^{abcd}
		15	135	23.20 ±0.90^{ij}	15.00 ±0.85^{h}	39.40 ±0.40^{bcde}	22.00 ±1.17^{a}

N = no. of observation.
^{abcdefghij}Means with different superscripts within a column were significantly different ($P<0.05$).

Table 4.24: Sperm motion characteristics (mean±SEM) of frozen-thawed sperm for different combinations of equilibration duration, vapour exposure temperature and vapour exposure duration

Equilibration duration (min)	Vapour temperature (˚C)	Vapour duration (min)	N	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
30	-100	5	135	35.46 ±1.36^{cdefgh}	30.63 ±1.19^{bcdefg}	52.54 ±1.39^{bcd}	3.18 ±0.12^{bcde}	30.31 ±0.74^{e}	85.47 ±0.53^{cd}	57.53 ±1.15^{d}
		10	135	42.93 ±1.31^{jk}	37.41 ±1.17^{ij}	59.07 ±1.57^{cde}	3.22 ±0.26^{bcde}	26.53 ±1.44^{abcde}	85.33 ±0.84^{cd}	61.60 ±0.75^{d}
		15	135	36.74 ±1.70^{defghi}	32.65 ±1.73^{defghi}	53.08 ±1.85^{bcd}	2.93 ±0.21^{abcde}	26.64 ±1.28^{abcde}	85.27 ±0.64^{cd}	59.87 ±1.30^{d}
	-110	5	135	34.69 ±1.87^{cdefg}	30.53 ±1.71^{bcdefg}	51.51 ±2.59^{bc}	2.79 ±0.22^{abc}	30.42 ±0.94^{e}	87.20 ±0.69^{cd}	56.53 ±2.72^{cd}
		10	135	37.10 ±1.52^{efghi}	33.29 ±1.14^{efghi}	53.99 ±2.14^{bcde}	2.83 ±0.16^{abc}	30.39 ±1.09^{e}	81.07 ±4.82^{cd}	56.67 ±2.66^{cd}
		15	135	39.02 ±1.90^{fghij}	33.99 ±1.92^{efghi}	57.27 ±1.96^{cde}	3.11 ±0.18^{abcde}	29.23 ±0.82^{de}	85.27 ±0.94^{cd}	58.27 ±1.93^{d}
	-120	5	135	28.25 ±2.15^{ab}	25.87 ±1.82^{ab}	39.88 ±3.76^{a}	2.69 ±0.26^{abc}	24.92 ±1.71^{abc}	63.93 ±9.19^{b}	45.33 ±4.65^{ab}
		10	135	35.13 ±1.60^{cdefg}	30.64 ±1.53^{bcdefg}	52.68 ±2.11^{bcd}	3.73 ±0.32^{de}	26.78 ±1.58^{abcde}	86.07 ±1.13^{cd}	57.53 ±1.87^{d}
		15	135	41.69 ±1.99^{ijk}	36.57 ±1.87^{hij}	58.33 ±1.72^{cde}	3.06 ±0.12^{abcde}	28.94 ±0.48^{de}	86.40 ±0.29^{cd}	61.07 ±0.94^{d}

(continued)

(continued)

45	-100	5	135	37.19 ±2.16^{efghi}	32.23 ±1.86^{defghi}	54.33 ±2.52^{bcde}	2.99 ±0.25^{abcde}	29.78 ±0.86^{e}	85.40 ±0.92^{cd}	58.60 ±1.41^{d}
		10	135	36.87 ±1.67^{defghi}	32.81 ±1.51^{defghi}	51.91 ±1.99^{bc}	2.97 ±0.29^{abcde}	28.73 ±0.73^{cde}	87.47 ±0.74^{cd}	61.73 ±1.63^{d}
		15	135	36.06 ±1.95^{cdefghi}	31.29 ±1.81^{cdefgh}	54.43 ±3.19^{bcde}	3.17 ±0.35^{bcde}	26.73 ±1.23^{abcde}	86.47 ±1.51^{cd}	58.80 ±2.35^{d}
	-110	5	135	35.46 ±1.30^{cdefgh}	30.80 ±1.20^{bcdefg}	52.11 ±1.69^{bc}	2.97 ±0.27^{abcde}	29.03 ±0.89^{de}	85.93 ±1.06^{cd}	59.60 ±1.69^{d}
		10	135	26.43 ±1.61^{a}	24.09 ±1.12^{ab}	36.37 ±3.51^{a}	2.25 ±0.22^{a}	23.63 ±1.94^{a}	52.07 ±9.69^{a}	40.13 ±4.72^{a}
		15	135	30.31 ±2.47^{abc}	27.67 ±2.18^{abcd}	41.09 ±4.23^{a}	2.70 ±0.28^{abc}	24.23 ±1.89^{ab}	58.00 ±9.66^{ab}	43.87 ±5.05^{ab}
	-120	5	135	31.14 ±2.11^{abcd}	26.83 ±1.70^{abc}	48.77 ±3.74^{b}	2.92 ±0.40^{abcde}	25.48 ±1.30^{abcd}	74.87 ±6.56^{c}	49.80 ±3.40^{bc}
		10	135	34.83 ±1.92^{cdefg}	30.21 ±1.76^{bcdef}	52.89 ±1.77^{bcd}	3.07 ±0.28^{abcde}	26.52 ±1.36^{bcde}	86.00 ±0.72^{cd}	57.13 ±1.21^{cd}
		15	135	34.36 ±0.48^{cdefg}	29.62 ±0.72^{bcdef}	52.30 ±0.56^{bc}	2.86 ±0.10^{abcd}	29.09 ±0.93^{abcde}	85.20 ±0.98^{cd}	56.80 ±1.31^{cd}

(continued)

(continued)

60	-100	5	135	36.65 ±1.89^{defghi}	32.11 ±1.65^{cdefgh}	55.29 ±1.89^{bcde}	3.45 ±0.33^{cde}	27.23 ±0.91^{de}	86.27 ±0.54^{cd}	57.33 ±1.58^{cd}
		10	135	38.77 ±2.33^{fghij}	33.45 ±2.28^{efghi}	56.86 ±2.04^{cde}	3.42 ±0.37^{bcde}	26.46 ±1.26^{abcde}	85.13 ±0.94^{cd}	58.47 ±2.08^{d}
		15	135	41.08 ±2.30^{hijk}	35.85 ±2.26^{ghij}	60.30 ±2.41^{de}	3.79 ±0.40^{e}	29.74 ±0.46^{abcde}	85.07 ±0.91^{cd}	57.67 ±2.07^{d}
	-110	5	135	35.41 ±1.00^{cdefgh}	30.83 ±1.03^{bcdefg}	51.95 ±1.08^{bc}	3.11 ±0.23^{abcde}	29.33 ±1.03^{e}	86.20 ±0.65^{cd}	58.87 ±1.69^{d}
		10	135	39.74 ±1.09^{ghijk}	34.93 ±1.01^{fghi}	55.64 ±1.60^{bcde}	2.81 ±0.18^{abc}	27.32 ±0.90^{de}	86.20 ±1.04^{cd}	61.93 ±1.70^{d}
		15	135	45.17 ±1.08^{j}	40.39 ±1.16^{j}	60.85 ±1.12^{e}	3.45 ±0.20^{cde}	28.58 ±0.59^{abcde}	87.67 ±0.83^{d}	64.13 ±1.34^{d}
	-120	5	135	32.34 ±1.68^{bcde}	28.73 ±1.56^{abcde}	48.61 ±1.98^{b}	2.54 ±0.16^{bc}	29.06 ±0.91^{cde}	87.60 ±0.77^{d}	59.00 ±1.71^{d}
		10	135	33.09 ±1.54^{bcdef}	29.19 ±1.31^{bcde}	51.21 ±1.87^{bc}	2.91 ±0.30^{a}	28.24 ±0.90^{de}	87.47 ±0.72^{cd}	57.00 ±0.65^{cd}
		15	135	36.30 ±1.20^{defghi}	31.28 ±1.05^{cdefgh}	54.96 ±1.45^{bcde}	2.94 ±0.16^{abcde}	27.81 ±0.23^{cde}	85.00 ±0.59^{cd}	56.80 ±1.07^{cd}

N = no. of observation.

^{abcdefghijk}Means with different superscripts within a column were significantly different (P<0.05).

4.5 OPTIMISATION OF COMBINATION OF EQUILIBRATION DURATION, VAPOUR EXPOSURE TEMPERATURE AND VAPOUR EXPOSURE DURATION FOR SPERM CRYOPRESERVATION IN THAI MAHSEER (*Tor tambroides*) FISH (EXPERIMENT 2)

This second experiment used three best combinations of equilibration duration, vapour exposure temperature and vapour exposure duration obtained from Experiment 1 which were combinations of 30 minutes, -110°C and 10 minutes; 30 minutes,-110°C and 15 minutes; and 30 minutes, -120°C and 15 minutes (i.e. combinations 1, 2 and 3, respectively). Table 4.25 shows the effects of using the best three combinations on total motility and progressive motility of frozen-thawed sperm. Combination 2 showed the highest values in both sperm motility characteristics (72.80±2.85% and 21.20±4.02%, respectively) compared to combination 1 (72.40±2.27% and 16.80±2.22%, respectively) and 3 (70.80±2.35% and 18.20±2.48%, respectively). There were no significant differences in all combinations involved for total motility and progressive motility (ranging from 70.80±2.35% to 72.80±2.85% and ranging from 16.80±2.22% to 21.20±4.02%, respectively).

For velocity distribution analysis in Table 4.26, the highest values in rapid and slow movements were obtained from combination 2 (26.80±5.54% and 34.40±4.18%, respectively) compared to combinations 1 (20.60±2.38% and 32.80±3.18%, respectively) and 3 (21.80±2.75% and 32.60±0.40%, respectively). However, the highest value in medium was demonstrated in combination 3 (9.60±2.14%) compared to combinations 1 and 2 (6.00±0.84% and 8.60±2.00%, respectively). Meanwhile, combination 1 (40.60±1.75%) gave the highest value of static velocity compared to combinations 2 and 3 (32.20±5.27% and 36.20±3.72%, respectively). Same conditions observed in this

experiment which attained no significant differences between all combinations in all velocity distributions (rapid, medium, slow and static) with a range from 20.60±2.38% to 26.80±5.54%, from 6.00±0.84% to 9.60±2.14%, from 32.60±0.40% to 34.40±4.18% and from 32.20±5.27% to 40.60±1.75%, respectively.

Table 4.27 demonstrates the sperm motion characteristics of frozen-thawed sperm using three best combinations which combination 2 showed the highest values in most characteristics such as VAP, VSL, VCL and STR (48.04±1.71µm/s, 42.20±1.54µm/s, 65.06±2.31µm/s and 65.80±0.86%, respectively) compared to combination 1 (45.40±1.46µm/s, 40.86±1.53µm/s, 63.22±1.36µm/s and 63.20±1.32%, respectively) and 3 (42.18±3.18µm/s, 37.82±3.01µm/s, 59.04±2.42µm/s and 63.20±2.52%, respectively). However, combination 1 yielded the highest values in ALH, BCF and LIN (3.48±0.10µm, 25.76±1.26Hz and 88.00±0.63%, respectively) compared to combination 2 (3.42±0.28µm, 21.72±2.48Hz and 87.40±0.60%, respectively) and 3 (2.90±0.14µm, 24.72±1.41Hz and 87.80±0.80%, respectively).

Table 4.25: Total motility and progressive motility (mean±SEM) for sperm cryopreservation of Thai mahseer sperm using the best three combinations of equilibration durations, vapour exposure temperatures and vapour exposure durations

Combination	N	Total motility (%)	Progressive motility (%)
1	15	72.40±2.27[a]	16.80±2.22[a]
2	15	72.80±2.85[a]	21.20±4.02[a]
3	15	70.80±2.35[a]	18.20±2.48[a]

Combination 1: 30 minutes, -110°C and 10 minutes.
Combination 2: 30 minutes, -110°C and 15 minutes.
Combination 3: 30 minutes, -120°C and 15 minutes.
N = no. of observation (straws).
[a]Means with same superscript a within a column were not significantly different (P>0.05).

Table 4.26: Velocity distribution (mean±SEM) for sperm cryopreservation of Thai mahseer sperm using the best three combinations of equilibration durations, vapour exposure temperatures and vapour exposure durations

Combination	N	Rapid (%)	Medium (%)	Slow (%)	Static (%)
1	15	20.60±2.38[a]	6.00±0.84[a]	32.80±3.18[a]	40.60±1.75[a]
2	15	26.80±5.54[a]	8.60±2.00[a]	34.40±4.18[a]	32.20±5.27[a]
3	15	21.80±2.75[a]	9.60±2.14[a]	32.60±0.40[a]	36.20±3.72[a]

Combination 1: 30 minutes, -110°C and 10 minutes.
Combination 2: 30 minutes, -110°C and 15 minutes.
Combination 3: 30 minutes, -120°C and 15 minutes.
N = no. of observation (straws).
[a]Means with same superscript a within a column were not significantly different (P>0.05).

Table 4.27: Sperm motion characteristics (mean±SEM) for sperm cryopreservation of Thai mahseer sperm using the best three combinations of equilibration durations, vapour exposure temperatures and vapour exposure durations

Combination	N	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
1	15	45.40 ±1.46[a]	40.86 ±1.53[a]	63.22 ±1.36[a]	3.48 ±0.10[a]	25.76 ±1.26[a]	88.00 ±0.63[a]	63.20 ±1.32[a]
2	15	48.04 ±1.71[a]	42.20 ±1.54[a]	65.06 ±2.31[a]	3.42 ±0.28[a]	21.72 ±2.48[a]	87.40 ±0.60[a]	65.80 ±0.86[a]
3	15	42.18 ±3.18[a]	37.82 ±3.01[a]	59.04 ±2.42[a]	2.90 ±0.14[a]	24.72 ±1.41[a]	87.80 ±0.80[a]	63.20 ±2.52[a]

Combination 1: 30 minutes, -110°C and 10 minutes.
Combination 2: 30 minutes, -110°C and 15 minutes.
Combination 3: 30 minutes, -120°C and 15 minutes.
N = no. of observation.
[a]Means with same superscript a within a column were not significantly different (P>0.05).

In Table 4.28, some positive correlations were obtained in sperm motility characteristics of frozen-thawed sperm using combination 1 as main factors. There were positive correlations between total motility and progressive motility, total motility and rapid, total motility and STR, progressive motility and rapid, progressive motility and STR, rapid and STR, VAP and VSL, VAP and VCL, and VAL and BCF. However, there were negative correlations between medium and LIN, and VCL and BCF.

For combination 2, there were positive correlations obtained as described in Table 4.29 such as between total motility and progressive motility, total motility and rapid, total motility and medium, progressive motility and rapid, progressive motility and medium, rapid and medium, slow and ALH, VAP and VSL, VAP and VCL, and BCF and STR. Meanwhile, only 1 negative correlation obtained, that is between rapid and static.

Positive correlations were observed in Table 4.30 that data obtained using combination 2 in sperm cryopreservation of Thai mahseer, i.e. between progressive motility and rapid, VAP and VSL, VAP and VCL, VSL and VCL, and VSL and LIN. There was no negative correlation obtained from this analysis.

Overall correlations for sperm motility characteristics of frozen-thawed sperm in cryopreservation of Thai mahseer using combinations 1, 2 and 3 were demonstrated in Table 4.31. Positive correlations were shown between total motility and progressive motility, total motility and rapid, progressive motility and rapid, static and BCF, VAP and VSL, VAP and VCL, VAP and LIN, VSL and VCL, VSL and LIN, VCL and ALH, BCF and STR, and STR and LIN. However, there were negative correlations obtained between progressive motility and static, progressive motility and BCF, rapid and static, rapid and BCF, medium and static, medium and VSL, medium and BCF, medium and STR, and medium and LIN.

Table 4.28: Correlation between frozen-thawed sperm motility characteristics using combination 1 in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.955*	.960**	-.474	-.675	.148	.655	.729	.462	.332	-.362	.940*	.711
Progressive Motility	1.000	.999**	-.430	-.708	.136	.789	.838	.630	.437	-.522	.925*	.669
Rapid		1.000	-.477	-.676	.099	.804	.854	.638	.404	-.518	.930*	.707
Medium			1.000	-.244	.615	-.695	-.721	-.553	.352	.294	-.472	-.951*
Slow				1.000	-.785	-.317	-.349	-.323	-.434	.483	-.521	-.010
Static					1.000	-.184	-.180	-.016	.073	-.315	-.090	-.490
VAP						1.000	.994**	.942*	.057	-.780	.627	.770
VSL							1.000	.907*	.085	-.735	.701	.816
VCL								1.000	-.118	-.932*	.366	.573
ALH									1.000	.201	.543	-.104
BCF										1.000	-.176	-.311
STR											1.000	.719
LIN												1.000

No. of observation = 15
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.29: Correlation between frozen-thawed sperm motility characteristics using combination 2 in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.895*	.886*	.904*	-.644	-.635	-.529	-.751	-.335	-.708	-.556	-.397	-.799
Progressive Motility	1.000	.985**	.897*	-.490	-.835	-.170	-.407	-.007	-.584	-.535	-.506	-.706
Rapid		1.000	.942*	-.419	-.886*	-.090	-.374	.102	-.471	-.668	-.641	-.769
Medium			1.000	-.296	-.876	-.160	-.456	.047	-.353	-.762	-.678	-.771
Slow				1.000	-.049	.735	.808	.597	.911*	.108	-.056	.632
Static					1.000	-.294	-.019	-.434	.050	.676	.737	.521
VAP						1.000	.923*	.957*	.795	-.152	-.422	.279
VSL							1.000	.773	.785	.232	-.043	.620
VCL								1.000	.731	-.423	-.660	-.006
ALH									1.000	-.090	-.248	.437
BCF										1.000	.955*	.838
STR											1.000	.717
LIN												1.000

No. of observation = 15
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.30: Correlation between frozen-thawed sperm motility characteristics using combination 3 in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.602	.463	-.491	.722	-.153	.182	.184	.078	.132	.162	.340	.322
Progressive Motility	1.000	.949*	-.091	.525	-.712	.365	.354	.170	.042	-.478	.232	.535
Rapid		1.000	.159	.255	-.865	.310	.277	.198	.302	-.722	-.073	.349
Medium			1.000	-.866	-.627	-.626	-.669	-.493	.517	-.668	-.831	-.750
Slow				1.000	.215	.532	.575	.294	-.518	.448	.875	.814
Static					1.000	.118	.164	.150	-.482	.873	.457	.122
VAP						1.000	.997**	.944*	-.138	-.104	.406	.857
VSL							1.000	.923*	-.210	-.042	.472	.886*
VCL								1.000	.119	-.180	.136	.642
ALH									1.000	-.611	-.863	-.534
BCF										1.000	.646	.121
STR											1.000	.774
LIN												1.000

No. of observation = 15
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.31: Overall correlation between frozen-thawed sperm motility characteristics using combination 1, 2 and 3 of in cryopreservation of Thai mahseer

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.793**	.746**	.082	-.491	-.331	.126	.097	.069	-.161	-.324	.236	.187
Progressive Motility	1.000	.975**	.389	-.404	-.709**	.263	.191	.198	-.231	-.574*	.030	.259
Rapid		1.000	.502	-.348	-.793**	.262	.157	.261	-.138	-.700**	-.164	.153
Medium			1.000	-.211	-.664**	-.447	-.570*	-.309	-.179	-.560*	-.663**	-.580*
Slow				1.000	-.174	.245	.236	.262	.459	.127	-.164	.226
Static					1.000	-.135	-.003	-.186	-.031	.670**	.479	-.014
VAP						1.000	.979**	.933**	.408	-.303	.171	.762**
VSL							1.000	.870**	.365	-.143	.332	.819**
VCL								1.000	.563*	-.439	-.111	.498
ALH									1.000	-.159	-.231	-.003
BCF										1.000	.560*	.016
STR											1.000	.610*
LIN												1.000

No. of observation = 45
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

4.6 EFFECTS OF THE LOW TEMPERATURE INCUBATOR AND THE NORMAL REFRIGERATOR ON THE FROZEN-THAWED SPERM CHARACTERISTICS WITH REFERENCE TO EQUILIBRATION DURATION (EXPERIMENT 3)

Table 4.32, Table 4.33 and Table 4.34 show the effects of both incubator and refrigerator on the frozen-thawed sperm motility characteristics analysed using IVOS for different equilibration durations (30, 45 and 60 minutes). Both incubator and refrigerator gave no significant differences for all equilibration durations (30, 45 and 60 minutes) in total motility (ranging from 97.07±0.74 to 97.60±0.50, from 87.80±1.74 to 88.40±1.59 and from 88.33±1.59 to 88.67±1.87, respectively) and progressive motility (ranging from 34.60±4.04 to 38.60±4.16, from 23.13±2.16 to 23.93±2.95 and from 21.13±1.83 to 21.47±2.48, respectively) values.

For velocity distribution (Table 4.35, Table 4.36 and Table 4.37), similar situations were observed which there were no significant differences for all velocity distributions, namely rapid, medium, slow and static values using low temperature incubator and normal refrigerator in all equilibration durations involved (30, 45 and 60 minutes) {(ranging from 50.73±5.03 to 54.40±5.17, from 32.13±3.00 to 32.60±3.66 and from 29.87±2.82 to 30.47±2.27, respectively), (ranging from 11.33±1.85 to 14.87±2.32, from 12.27±1.40 to 12.60±1.55 and from 11.67±1.20 to 12.00±1.57, respectively), (ranging from 30.93±2.93 to 14.87±2.32, from 42.87±1.81 to 43.40±2.14 and from 45.60±1.73 to 46.93±1.65, respectively) and (ranging from 2.47±0.49 to 2.93±0.74, from 11.60±1.59 to 12.20±1.74 and from 11.33±1.87 to 11.67±1.59, respectively), respectively}.

Meanwhile, similar situations were obtained in all sperm motion characteristics such as VAP, VSL, VCL, ALH, BCF, STR and LIN values in Table 4.38, Table 4.39 and

Table 4.40 which there were no significant differences between usage of low temperature incubator and normal refrigerator in all equilibration durations involved (30, 45 and 60 minutes) {(ranging from 48.77±5.91 to 56.90±5.24, from 40.89±2.04 to 41.81±2.48 and from 42.15±1.87 to 44.23±2.04, respectively), (ranging from 41.17±5.12 to 49.16±4.68, from 34.83±1.85 to 35.43±2.10 and from 35.95±0.71 to 37.43±1.84, respectively), (ranging from 66.65±6.28 to 74.67±4.95, from 58.75±1.73 to 58.81±2.42 and from 61.37±1.68 to 63.39±1.98, respectively), (ranging from 3.23±0.17 to 3.87±0.24, from 3.26±0.24 to 3.49±0.22 and from 3.54±0.20 to 3.74±0.26, respectively), (ranging from 23.90±1.36 to 25.07±1.93, from 27.36±1.05 to 27.60±1.09 and from 26.55±1.31 to 26.88±0.58, respectively), (ranging from 73.33±6.13 to 74.60±5.86, from 84.07±0.69 to 84.33±0.95 and from 82.93±0.63 to 83.67±0.93, respectively) and (ranging from 53.13±4.21 to 56.07±4.02, from 58.67±1.96 to 60.33±1.82 and from 58.40±1.51 to 58.40±2.16, respectively), respectively}.

Table 4.32: Total motility and progressive motility (mean±SEM) of frozen-thawed Thai mahseer sperm using modified Fish Ringer Extender (mFRE) after 30 minutes of equilibration phase for different incubator or refrigerator

Equilibration duration (min)	Incubator/refrigerator	N	Total motility (%)	Progressive motility (%)
30	Low temperature incubator	15	97.60±0.50[a]	34.60±4.04[a]
	Normal refrigerator	15	97.07±0.74[a]	38.60±4.16[a]

N = no. of observation.
[a]Means with same superscript a within a column were not significantly different (P>0.05).

Table 4.33: Total motility and progressive motility (mean±SEM) of frozen-thawed Thai mahseer sperm using modified Fish Ringer Extender (mFRE) after 45 minutes of equilibration phase for different incubator or refrigerator

Equilibration duration (min)	Incubator/refrigerator	N	Total motility (%)	Progressive motility (%)
45	Low temperature incubator	15	88.40±1.59[a]	23.93±2.95[a]
	Normal refrigerator	15	87.80±1.74[a]	23.13±2.16[a]

N = no. of observation.
[a]Means with same superscript a within a column were not significantly different (P>0.05).

Table 4.34: Total motility and progressive motility (mean±SEM) of frozen-thawed Thai mahseer sperm using modified Fish Ringer Extender (mFRE) after 60 minutes of equilibration phase for different incubator or refrigerator

Equilibration duration (min)	Incubator/refrigerator	N	Total motility (%)	Progressive motility (%)
60	Low temperature incubator	15	88.67±1.87[a]	21.47±2.48[a]
	Normal refrigerator	15	88.33±1.59[a]	21.13±1.83[a]

N = no. of observation.
[a]Means with same superscript a within a column were not significantly different (P>0.05)..

Table 4.35: Velocity distribution (mean±SEM) of frozen-thawed Thai mahseer sperm using modified Fish Ringer Extender (mFRE) after 30 minutes of equilibration phase for different incubator or refrigerator

Equilibration duration (min)	Incubator/refrigerator	N	Rapid (%)	Medium (%)	Slow (%)	Static (%)
30	Low temperature incubator	15	54.40±5.17[a]	11.33±1.85[a]	30.93±2.93[a]	2.93±0.74[a]
	Normal refrigerator	15	50.73±5.03[a]	14.87±2.32[a]	32.00±2.77[a]	2.47±0.49[a]

N = no. of observation.
[a]Means with same superscript a within a column were not significantly different (P>0.05).

Table 4.36: Velocity distribution (mean±SEM) of frozen-thawed Thai mahseer sperm using modified Fish Ringer Extender (mFRE) after 45 minutes of equilibration phase for different incubator or refrigerator

Equilibration duration (min)	Incubator/refrigerator	N	Rapid (%)	Medium (%)	Slow (%)	Static (%)
45	Low temperature incubator	15	32.60±3.66[a]	12.60±1.55[a]	42.87±1.81[a]	12.20±1.74[a]
	Normal refrigerator	15	32.13±3.00[a]	12.27±1.40[a]	43.40±2.14[a]	11.60±1.59[a]

N = no. of observation.
[a]Means with same superscript a within a column were not significantly different (P>0.05).

Table 4.37: Velocity distribution (mean±SEM) of frozen-thawed Thai mahseer sperm using modified Fish Ringer Extender (mFRE) after 60 minutes of equilibration phase for different incubator or refrigerator

Equilibration duration (min)	Incubator/refrigerator	N	Rapid (%)	Medium (%)	Slow (%)	Static (%)
60	Low temperature incubator	15	30.47±2.27[a]	12.00±1.57[a]	45.60±1.73[a]	11.67±1.59[a]
	Normal refrigerator	15	29.87±2.82[a]	11.67±1.20[a]	46.93±1.65[a]	11.33±1.87[a]

N = no. of observation.
[a]Means with same superscript a within a column were not significantly different (P>0.05).

Table 4.38: Sperm motion characteristics (mean±SEM) of Thai mahseer sperm for fresh semen with extender and after 30 minutes of equilibration phase for different incubator or refrigerator

Equilibration duration (min)	Incubator/refrigerator	N	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
30	Low temperature incubator	15	56.90±5.24[a]	49.16±4.68[a]	74.67±4.95[a]	3.87±0.24[a]	23.90±1.36[a]	74.60±5.86[a]	56.07±4.02[a]
	Normal refrigerator	15	48.77±5.91[a]	41.17±5.12[a]	66.65±6.28[a]	3.23±0.17[a]	25.07±1.93[a]	73.33±6.13[a]	53.13±4.21[a]

N = no. of observation.
[a]Means with same superscript a within a column were not significantly different (P>0.05).

Table 4.39: Sperm motion characteristics (mean±SEM) of Thai mahseer sperm for fresh semen with extender and after 45 minutes of equilibration phase for different incubator or refrigerator

Equilibration duration (min)	Incubator/refrigerator	N	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
45	Low temperature incubator	15	41.81±2.48[a]	35.43±2.10[a]	58.81±2.42[a]	3.26±0.24[a]	27.36±1.05[a]	84.33±0.95[a]	60.33±1.82[a]
	Normal refrigerator	15	40.89±2.04[a]	34.83±1.85[a]	58.75±1.73[a]	3.49±0.22[a]	27.60±1.09[a]	84.07±0.69[a]	58.67±1.96[a]

N = no. of observation.
[a]Means with same superscript a within a column were not significantly different (P>0.05).

Table 4.40: Sperm motion characteristics (mean±SEM) of Thai mahseer sperm for fresh semen with extender and after 60 minutes of equilibration phase for different incubator or refrigerator

Equilibration duration (min)	Incubator/refrigerator	N	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
60	Low temperature incubator	15	44.23±2.04[a]	37.43±1.84[a]	63.39±1.98[a]	3.54±0.20[a]	26.88±0.58[a]	82.93±0.63[a]	58.40±1.51[a]
	Normal refrigerator	15	42.15±1.87[a]	35.95±0.71[a]	61.37±1.68[a]	3.74±0.26[a]	26.55±1.31[a]	83.67±0.93[a]	58.40±2.16[a]

N = no. of observation.
[a]Means with same superscript a within a column were not significantly different (P>0.05).

The correlation between frozen-thawed sperm motility characteristics in cryopreservation of Thai mahseer using low temperature incubator for 30 minutes in equilibration phase was demonstrated in Table 4.41. There were positive correlations between total motility and progressive motility, total motility and rapid, total motility and VAP, total motility and VSL, total motility and VCL, progressive motility and rapid, progressive motility and VAP, progressive motility and VSL, progressive motility and VCL, progressive motility and LIN, rapid and VAP, rapid and VSL, rapid and VCL, rapid and LIN, medium and slow, medium and static, slow and static, slow and BCF, static and BCF, VAP and VSL, VAP and VCL, VAP and LIN, VSL and VCL, VSL and LIN, VCL and ALH, VCL and STR, VCL and LIN, BCF and STR, and STR and LIN. However, there were negative correlations such as between total motility and medium, total motility and slow, total motility and BCF, progressive motility and medium, progressive motility and slow, progressive motility and static, rapid and medium, rapid and slow, rapid and static, medium and VAP, medium and VSL, medium and VCL, medium and LIN, slow and VAP, slow and VSL, slow and VCL, slow and LIN, static and VAP, static and VSL, and static and VCL.

Meanwhile, Table 4.42 shows positive correlations between frozen-thawed sperm motility characteristics in cryopreservation of Thai mahseer using normal refrigerator for 30 minutes in equilibration phase, namely between total motility and progressive motility, total motility and rapid, progressive motility and rapid, progressive motility and VAP, progressive motility and VSL, rapid and VAP, rapid and VSL, rapid and VCL, medium and slow, medium and static, slow and static, VAP and VSL, VAP and VCL, VAP and LIN, VSL and VCL, VCL and LIN, and STR and LIN. Conversely, negative correlations was observed between total motility and medium, total motility and slow, total motility and

static, progressive motility and medium, progressive motility and slow, progressive motility and static, rapid and medium, rapid and slow, rapid and static, medium and VAP, medium and VSL, medium and VCL, slow and VAP, slow and VSL, and ALH and BCF.

In Table 4.43, there were positive correlations between frozen-thawed sperm characteristics obtained from sperm cryopreservation of Thai mahseer using low temperature incubator for 45 minutes in equilibration phase, namely between total motility and progressive motility, total motility and rapid, progressive motility and rapid, progressive motility and VAP, progressive motility and VSL, progressive motility and STR, progressive motility and LIN, VAP and VSL, VAP and VCL, VAP and STR, VAP and LIN, VSL and VCL, VSL and STR, VSL and LIN, and VCL and ALH. However, there were negative correlations between total motility and static, progressive motility and slow, progressive motility and static, rapid and slow, rapid and static, medium and VAP, medium and VSL, medium and STR, medium and LIN, VAP and BCF, VCL and BCF, ALH and STR, BCF and LIN, and STR and LIN.

Table 4.44 shows positive correlations between frozen-thawed sperm characteristics obtained from sperm cryopreservation of Thai mahseer using normal refrigerator for 45 minutes in equilibration phase, namely between total motility and progressive motility, total motility and rapid, total motility and medium, progressive motility and rapid, slow and static, slow and VAP, slow and VSL, slow and VCL, VAP and VSL, VAP and VCL, VAP and LIN, VSL and VCL, VSL and LIN, and STR and LIN. There were negative correlations between total motility and slow, total motility and static, progressive motility and slow, progressive motility and static, progressive motility and ALH, rapid and slow, rapid and static, rapid and ALH, medium and slow, medium and static, medium and VAP, medium and VSL, medium and VCL, and medium and LIN.

For 60 minutes of equilibration duration using low temperature incubator, there were of positive correlations observed in Table 4.45 such as between total motility an progressive motility, total motility and rapid, progressive motility and rapid, progressive motility and VAP, progressive motility and VSL, progressive motility and STR, progressive motility and LIN, rapid and VAP, rapid and VSL, rapid and LIN, rapid and VAP, rapid and VSL, rapid and LIN, slow and static, slow and ALH, static and ALH, VAP and VSL, VAP and VCL, VAP and LIN, VSL and VCL, VSL and LIN, and STR and LIN. Negative correlations were obtained between total motility and slow, total motility and static, total motility and ALH, progressive motility and slow, progressive motility and static, progressive motility and ALH, rapid and slow, rapid and static, rapid and ALH, rapid and slow, rapid and static, rapid and ALH, medium and VAP, medium and VSL, medium and VCL, medium and LIN, and ALH and LIN.

Meanwhile, the correlation between frozen-thawed sperm motility characteristics in cryopreservation of Thai mahseer using normal refrigerator for 60 minutes in equilibration phase was demonstrated in Table 4.46. There were positive correlations between total motility and rapid, progressive motility and rapid, progressive motility and VAP, progressive motility and VSL, progressive motility and LIN, rapid and VAP, rapid and VSL, rapid and VCL, slow and ALH, VAP and VSL, VAP and VCL, VAP and LIN, VSL and VCL, VSL and LIN, and STR and LIN. However, negative correlations were obtained between total motility and static, progressive motility and slow, rapid and slow, rapid and static, medium and VAP, medium and VSL, medium and VCL, medium and LIN, VAP and BCF, VSL and BCF, and VCL and BCF.

Table 4.47 shows overall correlations between frozen-thawed sperm characteristics obtained from sperm cryopreservation of Thai mahseer using low temperature incubator

for 30, 45 and 60 minutes in equilibration phase, namely between total motility and progressive motility, total motility and rapid, progressive motility and rapid, progressive motility and VAP, progressive motility and VSL, progressive motility and VCL, progressive motility and LIN, rapid and VAP, rapid and VSL, rapid and VCL, rapid and LIN, slow and static, slow and BCF, static and ALH, VAP and VSL, VAP and VCL, VAP and STR, VAP and LIN, VSL and VCL, VSL and LIN, VCL and ALH, VCL and STR, VCL and LIN, BCF and STR, and STR and LIN. Conversely, there were negative correlations between total motility and slow, total motility and static, total motility and ALH, progressive motility and medium, progressive motility and slow, progressive motility and static, progressive motility and ALH, progressive motility and BCF, rapid and medium, rapid and medium, rapid and slow, rapid and ALH, rapid and BCF, medium and VAP, medium and VSL, medium and VCL, medium and LIN, slow and VAP, slow and VSL, slow and VCL, slow and LIN, VAP and BCF, and VSL and BCF.

Table 4.48 shows overall correlation between frozen-thawed sperm characteristics obtained from sperm cryopreservation of Thai mahseer using normal refrigerator for 30, 45 and 60 minutes in equilibration phase. Positive correlations were obtained between total motility and progressive motility, total motility and rapid, total motility and VAP, total motility and VSL, total motility and VCL, progressive motility and rapid, progressive motility and VAP, progressive motility and VCL, rapid and VAP, rapid and VSL, rapid and VCL, slow and static, slow and BCF, static and BCF, VAP and VSL, VAP and VCL, VAP and LIN, VSL and VCL, VSL and LIN, VCL and ALH, VCL and LIN, and STR and LIN. There were also negative correlations between total motility and slow, total motility and static, total motility and BCF, progressive motility and medium, progressive motility and slow, progressive motility and static, progressive motility and BCF, rapid and medium,

rapid and slow, rapid and static, rapid and BCF, medium and VAP, medium and VSL, medium and VCL, medium and LIN, slow and VAP, slow and VSL, slow and VCL, static and VAP, static and VSL, static and VCL, VAP and BCF, VSL and BCF, VCL and BCF, and ALH and BCF.

Table 4.41: Correlation between frozen-thawed sperm motility characteristics of Thai mahseer after 30 minutes of equilibration duration using low temperature incubator

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.725**	.778**	-.549*	-.788**	-.991**	.626*	.622*	.539*	.296	-.637*	.049	.415
Progressive Motility	1.000	.990**	-.906**	-.914**	-.713**	.778**	.807**	.665**	.107	-.414	.272	.683**
Rapid		1.000	-.887**	-.939**	-.772**	.822**	.843**	.716**	.194	-.475	.269	.691**
Medium			1.000	.681**	.526*	-.811**	-.848**	-.682**	-.054	.272	-.247	-.656**
Slow				1.000	.799**	-.702**	-.710**	-.632*	-.255	.517*	-.273	-.632*
Static					1.000	-.611*	-.608*	-.524*	-.291	.661**	-.036	-.395
VAP						1.000	.996**	.958**	.485	-.298	.430	.788**
VSL							1.000	.937**	.430	-.324	.391	.766**
VCL								1.000	.626*	-.121	.632*	.872**
ALH									1.000	-.163	.408	.420
BCF										1.000	.541*	.173
STR											1.000	.860**
LIN												1.000

No. of observation = 15
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.42: Correlation between frozen-thawed sperm motility characteristics of Thai mahseer after 30 minutes of equilibration duration using normal refrigerator

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.882**	.873**	-.550*	-.905**	-1.000**	.484	.509	.392	-.434	-.230	.010	.368
Progressive Motility	1.000	.986**	-.806**	-.973**	-.882**	.585*	.600*	.487	-.507	-.203	.033	.439
Rapid		1.000	-.856**	-.967**	-.873**	.641*	.650**	.538*	-.507	-.197	.005	.433
Medium			1.000	.707**	.550*	-.695**	-.680**	-.619*	.492	-.012	-.089	-.483
Slow				1.000	.905**	-.546*	-.567*	-.440	.455	.298	.056	-.347
Static					1.000	-.484	-.509	-.392	.434	.230	-.010	-.368
VAP						1.000	.994**	.971**	.001	-.296	.168	.569*
VSL							1.000	.944**	-.018	-.323	.080	.499
VCL								1.000	.119	-.283	.353	.680**
ALH									1.000	-.596*	.051	-.110
BCF										1.000	.289	.122
STR											1.000	.875**
LIN												1.000

No. of observation = 15
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.43: Correlation between frozen-thawed sperm motility characteristics of Thai mahseer after 45 minutes of equilibration duration using low temperature incubator

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.584*	.749**	.311	-.411	-1.000**	-.028	-.049	-.036	-.131	.215	-.122	-.077
Progressive Motility	1.000	.954**	-.334	-.756**	-.584*	.579*	.638*	.145	-.455	-.288	.671**	.724**
Rapid		1.000	-.158	-.786**	-.749**	.472	.495	.176	-.326	-.238	.439	.514
Medium			1.000	-.114	-.311	-.703**	-.737**	-.369	.102	.459	-.602*	-.728**
Slow				1.000	.411	-.336	-.369	-.095	.325	.316	-.439	-.429
Static					1.000	.028	.049	.036	.131	-.215	.122	.077
VAP						1.000	.983**	.778**	.091	-.820**	.599*	.776**
VSL							1.000	.664**	-.051	-.769**	.721**	.863**
VCL								1.000	.633*	-.782**	.013	.229
ALH									1.000	-.354	-.552*	-.452
BCF										1.000	-.434	-.553*
STR											1.000	.950**
LIN												1.000

No. of observation = 15
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.44: Correlation between frozen-thawed sperm motility characteristics of Thai mahseer after 45 minutes of equilibration duration using normal refrigerator

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.706**	.764**	.525*	-.562*	-1.000**	-.325	-.313	-.387	-.490	-.154	.178	-.024
Progressive Motility	1.000	.938**	.002	-.569*	-.706**	-.025	.016	-.234	-.651**	.027	.432	.406
Rapid		1.000	.121	-.709**	-.764**	-.117	-.115	-.228	-.655**	.096	.119	.144
Medium			1.000	-.618*	-.525*	-.747**	-.742**	-.597*	.096	-.412	-.155	-.644**
Slow				1.000	.562*	.575*	.581*	.518*	.337	.110	.165	.406
Static					1.000	.325	.313	.387	.490	.154	-.178	.024
VAP						1.000	.989**	.921**	.196	-.130	.146	.693**
VSL							1.000	.868**	.163	-.152	.276	.767**
VCL								1.000	.410	-.196	-.177	.372
ALH									1.000	-.453	-.260	-.233
BCF										1.000	-.234	-.070
STR											1.000	.748**
LIN												1.000

No. of observation = 15
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.45: Correlation between frozen-thawed sperm motility characteristics of Thai mahseer after 60 minutes of equilibration duration using low temperature incubator

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.733**	.777**	.039	-.611*	-1.000**	.298	.280	.174	-.630*	.034	.132	.310
Progressive Motility	1.000	.965**	-.363	-.853**	-.733**	.613*	.635*	.310	-.747**	-.231	.573*	.782**
Rapid		1.000	-.380	-.866**	-.777**	.628*	.621*	.381	-.771**	-.137	.370	.686**
Medium			1.000	.017	-.039	-.739**	-.747**	-.515*	.396	-.110	-.183	-.677**
Slow				1.000	.611*	-.350	-.347	-.166	.569*	.315	-.402	-.482
Static					1.000	-.298	-.280	-.174	.630*	-.034	-.132	-.310
VAP						1.000	.991**	.869**	-.389	-.262	.279	.746**
VSL							1.000	.821**	-.394	-.267	.378	.805**
VCL								1.000	-.016	-.326	-.081	.339
ALH									1.000	-.121	-.314	-.652**
BCF										1.000	-.453	-.224
STR											1.000	.759**
LIN												1.000

No. of observation = 15
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.46: Correlation between frozen-thawed sperm motility characteristics of Thai mahseer after 60 minutes of equilibration duration using normal refrigerator

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.513	.642**	.255	-.310	-1.000**	.215	.144	.347	.104	-.334	-.276	-.195
Progressive Motility	1.000	.929**	-.410	-.698**	-.513	.566*	.612*	.433	-.379	-.371	.265	.580*
Rapid		1.000	-.332	-.748**	-.642**	.595*	.583*	.524*	-.350	-.279	-.073	.365
Medium			1.000	-.064	-.255	-.778**	-.819**	-.596*	.026	.209	-.155	-.742**
Slow				1.000	.310	-.107	-.124	-.016	.668**	-.015	-.040	-.173
Static					1.000	-.215	-.144	-.347	-.104	.334	.276	.195
VAP						1.000	.984**	.935**	.174	-.551*	-.098	.591*
VSL							1.000	.871**	.093	-.541*	.063	.711**
VCL								1.000	.428	-.623*	-.333	.301
ALH									1.000	-.471	-.335	-.338
BCF										1.000	-.188	-.330
STR											1.000	.690**
LIN												1.000

No. of observation = 15
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.47: Overall correlation between frozen-thawed sperm motility characteristics of Thai mahseer after 30, 45 and 60 minutes of equilibration duration using low temperature incubator

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.645**	.730**	.144	-.649**	-1.000**	.272	.252	.223	-.342*	-.107	-.211	-.048
Progressive Motility	1.000	.974**	-.496**	-.881**	-.643**	.698**	.722**	.529**	-.394**	-.368*	.086	.523**
Rapid		1.000	-.413**	-.920**	-.729**	.706**	.711**	.570**	-.345*	-.369*	.012	.418**
Medium			1.000	.157	-.148	-.679**	-.720**	-.530**	.064	.191	-.288	-.683**
Slow				1.000	.648**	-.564**	-.560**	-.467**	.298*	.416**	.020	-.296*
Static					1.000	-.269	-.249	-.219	.343*	.108	.212	.051
VAP						1.000	.994**	.938**	.085	-.383**	.299*	.676**
VSL							1.000	.904**	.036	-.401**	.283	.690**
VCL								1.000	.306*	-.250	.456**	.643**
ALH									1.000	-.169	.165	-.070
BCF										1.000	.376*	-.008
STR											1.000	.797**
LIN												1.000

No. of observation = 45
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.48: Overall correlation between frozen-thawed sperm motility characteristics of Thai mahseer after 30, 45 and 60 minutes of equilibration duration using normal refrigerator

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.701**	.751**	.090	-.661**	-1.000**	.357*	.355*	.363*	.014	-.359*	-.208	-.069
Progressive Motility	1.000	.978**	-.464**	-.899**	-.701**	.621**	.641**	.531**	-.230	-.319*	-.132	.283
Rapid		1.000	-.439**	-.918**	-.751**	.650**	.657**	.579**	-.200	-.303*	-.190	.207
Medium			1.000	.181	-.090	-.646**	-.640**	-.549**	.192	-.052	-.050	-.513**
Slow				1.000	.661**	-.462**	-.480**	-.399**	.227	.313*	.218	-.074
Static					1.000	-.357*	-.355*	-.363*	-.014	.359*	.208	.069
VAP						1.000	.993**	.966**	.186	-.394**	-.023	.444**
VSL							1.000	.937**	.157	-.408**	-.076	.417**
VCL								1.000	.333*	-.414**	.077	.432**
ALH									1.000	-.547**	-.100	-.215
BCF										1.000	.238	.042
STR											1.000	.807**
LIN												1.000

No. of observation = 45
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

4.7 AN ATTEMPT TO TRANSFER TECHNOLOGY FROM LABORATORY TO THE FIELD CONDITION FOR THE SPERM CRYOPRESERVATION OF THAI MAHSEER (*Tor tambroides*) (EXPERIMENT 4)

Results of two different conditions were compared in order to determine possibility of transfering the Thai mahseer sperm cryopreservation technology to under field condition. The same best combination of factors obtained from Experiment 2 was used in this experiment. The results that are shown in Table 4.49 were designated as control for this experiment. Freezing process under laboratory ambient produced significantly higher values in total motility, progressive motility, rapid, medium, slow and ALH (75.03±1.49%, 19.47±1.46%, 24.80±1.83%, 10.23±0.74%, 38.53±1.52% and 3.26±0.12 µm, respectively) compared to freezing process under field condition (35.81±1.69%, 8.96±0.64%, 10.36±0.75%, 4.76±0.37%, 23.11±1.36% and 2.95±0.10 µm, respectively). However, in static velocity value, the freezing process under field condition (61.69±2.07%) yielded significantly higher compared to the freezing process under laboratory ambient (27.47±2.05%). The most interesting facts from this attempt was that there were no significant differences in VAP, VSL, VCL, BCF, STR and LIN values between the freezing under laboratory ambient and field condition with a range from 41.54±0.91 µm/s to 43.53±1.33 µm/s, from 37.24±0.90 µm/s to 38.10±1.28 µm/s, from 57.30±0.95 µm/s to 61.16±1.35 µm/s, from 24.96±0.62 Hz to 25.48±1.04 Hz, from 86.33±0.53% to 87.94±0.44% and from 62.03±1.20% to 62.93±0.69%, respectively.

Table 4.49: Sperm motility characteristics (mean±SEM) between performing sperm cryopreservation under laboratory ambient and field condition on of Thai mahseer frozen-thawed sperm

Condition	Field	Laboratory
N	85	30
Total motility (%)	35.81±1.69[a]	75.03±1.49[b]
Progressive motility (%)	8.96±0.64[a]	19.47±1.46[b]
Rapid (%)	10.36±0.75[a]	24.80±1.83[b]
Medium (%)	4.76±0.37[a]	10.23±0.74[b]
Slow (%)	23.11±1.36[a]	38.53±1.52[b]
Static (%)	61.69±2.07[b]	27.47±2.05[a]
VAP (µm/s)	41.54±0.91[a]	43.53±1.33[a]
VSL (µm/s)	37.24±0.90[a]	38.10±1.28[a]
VCL (µm/s)	57.30±0.95[a]	61.16±1.35[a]
ALH (µm)	2.95±0.10[a]	3.26±0.12[b]
BCF (Hz)	24.96±0.62[a]	25.48±1.04[a]
STR (%)	87.94±0.44[a]	86.33±0.53[a]
LIN (%)	62.93±0.69[a]	62.03±1.20[a]

N = no. of observation (straws).
[ab]Means with different superscripts within a row were significantly different ($P<0.05$).

Table 4.50 shows the correlation of frozen-thawed sperm in sperm cryopreservation of Thai mahseer under laboratory ambient using 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration. There were positive correlations between total motility and progressive motility, total

motility and rapid, total motility and medium, total motility and slow, progressive motility and medium, progressive motility and slow, progressive motility and VAP, progressive motility and VSL, progressive motility and VCL, rapid and medium, rapid and slow, rapid and VAP, rapid and VSL, rapid and VCL, medium and slow, slow and ALH, static and STR, VAP and VSL, VAP and VCL, VAP and ALH, VAP and LIN, VSL and VCL, VSL and ALH, VSL and STR, VSL and LIN, VCL and ALH, VCL and LIN, and STR and LIN. However, there were negative correlations between total motility and static, total motility and STR, progressive motility and static, progressive motility and BCF, progressive motility and LIN, rapid and static, rapid and BCF, medium and static, medium and VAP, medium and VSL, medium and VCL, medium and LIN, slow and static, slow and STR, VAP and BCF, VSL and BCF, VCL and BCF, ALH and BCF, ALH and STR, and BCF and LIN.

Meanwhile, there were of positive correlations in frozen-thawed sperm characteristics of sperm cryopresevation of Thai mahseer under field condition using the same best factors as described in Table 4.51, namely between total motility and progressive motility, total motility and rapid, total motility and medium, total motility and slow, progressive motility and rapid, progressive motility and medium, progressive motility and slow, progressive motility and VAP, progressive motility and VSL, progressive motility and VCL, medium and slow, slow and ALH, static and STR, VAP and VSL, VAP and VCL, VAP and ALH, VAP and LIN, VSL and VCL, VSL and ALH, VSL and STR, VSL and LIN, VCL and ALH, VCL and LIN, and STR and LIN. There were negative correlations between total motility and static, total motility and STR, progressive motility and static, progressive motility and BCF, medium and static, medium and VAP, medium and VSL, medium and VCL, medium and LIN, slow and static, slow and STR,

VAP and BCF, VSL and BCF, VCL and BCF, ALH and BCF, ALH and STR, and BCF and LIN.

Table 4.50: Correlations between frozen-thawed sperm motility characteristics in sperm cryopreservation of Thai mahseer under laboratory ambient using 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.786**	.828**	.753**	.783**	-.945**	.084	.003	.128	.103	-.057	-.198*	-.043
Progressive Motility	1.000	.976**	.589**	.475**	-.792**	.287**	.247**	.262**	-.080	-.234*	.055	.250**
Rapid		1.000	.620**	.528**	-.834**	.296**	.235*	.315**	-.015	-.240**	-.046	.165
Medium			1.000	.487**	-.727**	-.230*	-.299**	-.191*	-.163	.007	-.149	-.198*
Slow				1.000	-.888**	.109	.027	.152	.329**	-.078	-.299**	-.112
Static					1.000	-.138	-.053	-.175	-.144	.133	.218*	.037
VAP						1.000	.953**	.882**	.448**	-.540**	.163	.493**
VSL							1.000	.855**	.381**	-.558**	.339**	.629**
VCL								1.000	.564**	-.526**	-.081	.195*
ALH									1.000	-.205*	-.308**	-.148
BCF										1.000	-.086	-.358**
STR											1.000	.796**
LIN												1.000

No. of observation = 85
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.51: Correlations between frozen-thawed sperm motility characteristics in sperm cryopreservation of Thai mahseer under field condition using 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration

	Progressive Motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total Motility	.786**	.828**	.753**	.783**	-.945**	.084	.003	.128	.103	-.057	-.198*	-.043
Progressive Motility	1.000	.976**	.589**	.475**	-.792**	.287**	.247**	.262**	-.080	-.234*	.055	.250**
Rapid		1.000	.620**	.528**	-.834**	.296**	.235*	.315**	-.015	-.240**	-.046	.165
Medium			1.000	.487**	-.727**	-.230*	-.299**	-.191*	-.163	.007	-.149	-.198*
Slow				1.000	-.888**	.109	.027	.152	.329**	-.078	-.299**	-.112
Static					1.000	-.138	-.053	-.175	-.144	.133	.218*	.037
VAP						1.000	.953**	.882**	.448**	-.540**	.163	.493**
VSL							1.000	.855**	.381**	-.558**	.339**	.629**
VCL								1.000	.564**	-.526**	-.081	.195*
ALH									1.000	-.205*	-.308**	-.148
BCF										1.000	-.086	-.358**
STR											1.000	.796**
LIN												1.000

No. of observation = 30
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Chapter 5
DISCUSSION

Chapter 5

5.0 DISCUSSION

5.1 EFFECTS OF EQUILIBRATION DURATION, VAPOUR EXPOSURE TEMPERATURE AND VAPOUR EXPOSURE ON FROZEN-THAWED SPERM MOTILITY CHARACTERISTICS IN THAI MAHSEER (*Tor tambroides*)

Cooling and freezing phases were described as traumatic period in sperm cryopreservation process that could gave various degrees of cryoinjury, depending on species of fish (Maldjian *et al*., 2004). Equilibration duration is the interval period between the milt-diluent mixing and entering the vapour exposure phase (Routray *et al*., 2007). Generally, 10 to 20 minutes of equilibration duration was applied for fish sperm cryopreservation (Billard and Zhang, 2001). Some findings stated that fish sperm size is too small which makes easier for cryoprotectants agents to penetrate into the cell and therefore, no need of longer equilibration duration (Legendre and Billard, 1980; Leung and Jamieson, 1991). Meanwhile, longer duration was required for sperm cryopreservation of *Tor tambroides* which showed optimal duration is important in order to allow the penetration of the DMSO into the cell while minimising toxicity (Huang *et al*., 2004b). However, Stoss and Holtz (1983) suggested the longer duration of sperm exposure to cryoprotectant may cause the lower frozen-thawed sperm motility.

There were no significant differences in sperm motility in *Tor putitora* using different equilibration durations (45 to 175 minutes) (Ponniah *et al*., 1999b). The same results were obtained in frozen-thawed motility of sperm cryopreservation of *Oreochoromis niloticus* which showed no significant difference in using 30 and 90 minutes equilibration duration (Rana, 1990). High frozen-thawed sperm motility percentage (92 to 98%) in *Tor khudree* was reported by Basavaraja *et al*. (2002) using combination of

modified FRS, 5 and 10% DMSO and equilibrated for 10, 20 and 30 minutes. In *Xiphophoprus helleri* and *Xiphophoprus couchianus*, sperm equilibrated less than 30 minutes demonstrated the highest frozen-thawed sperm motility, but no consistent difference was resulted within 10 to 120 minutes of equilibration duration (Huang *et al*., 2004a,b). While the best equilibration duration for zebrafish was 10 minutes (Yang *et al*., 2007).

In this study, three different equilibration durations were used to optimise the equilibration duration. Equilibration duration of 30 minutes showed the highest value in total motility (67.31±1.27%) compared to 45 and 60 minutes (61.93±1.31% and 65.09±1.02%, respectively). The motility of frozen-thawed sperm increased when using 60 minutes (65.09±1.02%) as compared to 45 minutes (61.93±1.31%), which was contrary to expectation. A possible explanation for this finding might be that a high individual variation of milt quality that was caused by individual fish (Rana, 1995). In carps, variability of individual fish and seasonal fish occurs and might affect the frozen-thawed sperm motility and fertilisation rate after cryopreservation (Billard *et al*., 1995b; Lubzens *et al*., 1997; Linhart *et al*., 1995; Linhart *et al*., 2000). Christ *et al*. (1996) stated the variability in individual and seasonal period did occur in carps. This statement was supported by Parks and Graham (1992) who stated that different levels of lipid composition of the sperm plasma membrane caused significant variation in the sperm characteristics. Similar finding was also observed by Routray *et al*. (2007) who stated that variability of quality of sperm could be due to variation in chemical composition and molarity of the sperm interspecifically as well as individually.

According to Asmad *et al*. (2008), longer duration of equilibration duration (60 minutes) gave the highest sperm motility compared to shorter duration (30 and 45 minutes)

in sperm cryopreservation of red tilapia using TYACE extender and glycerol as cryoprotectant. However, there was no significant different in cryopreservation of *Tor putitora* with 45 to 175 minutes of equilibration duration (Ponniah *et al*., 1999b). As considering the time consuming aspect, it can be said from this study that 30 minutes was the best duration for sperm cryopreservation of Thai mahseer compared to 45 and 60 minutes.

Similar pattern was observed in correlations among sperm motility characteristics for the three equilibration durations (30, 45 and 60 minutes) except differences in correlation of progressive motility and rapid at 30 and 45 minutes; progressive motility and slow, rapid and slow as well as VSL and BCF at 45 minutes and rapid and LIN at 60 minutes. Further observation demonstrated that patterns of 30 and 60 minutes were more similar compare with 30 and 45 minutes and 45 and 60 minutes. This may explained why the total motility of frozen-thawed sperm in 30 and 60 minutes were not significantly different.

The freezing phase consist two important factors which is vapour exposure temperature and vapour exposure duration. The effect of vapour exposure temperature in this study showed -110°C is the best temperature for sperm cryopreservation of Thai mahseer which obtained 68.53±1.13% and 15.75±0.61% in total motility and progressive motility, respectively. Viveiros *et al*. (2000) and Vuthiphandchai *et al*. (2009) reported that sperm samples have to reach the optimal end point dehydradation of the sperm before been plunged into the liquid nitrogen to minimise the cyroinjury. From this study, it demonstrated that -110°C gave better protection to the sperm compared to -100°C. This result was correlated with Hammerstedt *et al*. (1990) and Christensen *et al*. (2005) that stated that more ice formation and cell dehydration occurs in slower freezing rate.

However, the frozen-thawed sperm motility and progressive motility became lower at -120°C compared to -110°C. Possible explanation for this situation is due to shorter exposure did not provided enough duration for the optimal dehydration in the cell and the cell was equilibrated with the unfrozen fraction, at least in part that caused by the intracellular ice formation (Viveiros *et al*., 2000). Meanwhile, too long exposure could cause cryoinjury due to solution effects (Mazur, 1977) and/or from a continued osmotic shrinkage of the cells (Viveiros *et al*., 2000). Liu *et al*. (2000) stated that the permeating cryoprotectant agent concentration and the slow cooling rates plays ample roles in reaching the optimal level of dehydration of the cells in order to survive during freezing phase.

Meanwhile, no significant different was found in total motility between the three vapour exposure durations which ranging from 63.87±1.31% to 65.25±1.25%. However, 15 minutes (14.83±0.71%) demonstrated significantly differences compared to 5 and 10 minutes (12.31±0.58% and 12.82±0.58%, respectively) in progressive motility.

Viable frozen-thawed sperm of sea bass were obtained using either 10 to 24°C/minute (Sansone *et al*., 2002) or 60 to 65°C/minute (Fauvel *et al*., 1998). However, many researchers such as Rana and Gilmour, (1996), Linhart *et al*. (2000), Sansone *et al*. (2002) and Lang *et al*. (2003) suggested some species of fishes have their own optimal combination of freezing rates to yield the highest frozen-thawed motility. For example, the frozen-thawed sperm of C*yprinus carpio* could attained to their highest motility using 5°C/minute from 2 to -7°C and then 25°C/minute from -7 to -70°C (Cognie *et al*., 1989). Decreasing hatching rates was observed when the cells were plunged into liquid nitrogen below -40°C (at -2°C/minute), -50°C (at -5°C/minute), or -55°C (at -10°C/minute) and recommended the shorter duration of slow cooling to increase the semen survivability at temperatures below from these values (Vivieros *et al*., 2001).

Correlations of sperm motility characteristics among the three vapour exposure temperatures showed all temperatures have similar patterns of positive significant correlations except at -100°C which obtained significant correlation between total motility and VCL as well as progressive motility and STR. However, at -110°C, it showed significant correlation between VAP and BCF.

There was similar pattern of frozen-thawed sperm characteristics obtained in sperm cryopreservation using 5 and 10 minutes of vapour exposure duration and slightly different with freezing using 15 minutes. Additional positive correlations obtained in freezing using 10 minutes compared 5 and 15 minutes were between progressive motility and slow, rapid and slow, VAP and BCF as well as VSL and BCF. However, there were positive correlations between total motility and ALH in 5 minutes and total motility and LIN as well as progressive motility and STR in 15 minutes.

5.2 EFFECTS OF COMBINATIONS OF EQUILIBRATION DURATION, VAPOUR EXPOSURE TEMPERATURE AND VAPOUR EXPOSURE DURATION ON FROZEN-THAWED SPERM MOTILITY CHARACTERISTICS IN THAI MAHSEER (*Tor tambroides*)

Mazur *et al.* (1974a,b) emphasised the importance of variables selection involved in the sperm cryopreservation procedure, which include concentration and type of cryoprotectant agents, dilution ratio between extender and semen, equilibration duration and optimal temperature of exposure, cooling and warming rates. The results of this study have shown that the combination of equilibration duration, vapour exposure temperature and vapour exposure duration for sperm cryopreservation of Thai mahseer that gave the highest frozen-thawed sperm characteristics was 30 minutes, -110°C and 15 minutes, respectively,

with average total sperm motility of 77.27±2.49%. However, the highest value of progressive motility (20.60±2.19%) was observed in combination of 60 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration. There was no significant different in progressive motility between the combination of 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration and combination of 60 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration which ranging from 18.07±1.37% to 20.60±2.19%.

In cryopreservation of *Tor khudree* sperm using modified FRS, Basavaraja *et al*. (2002) yielded the high frozen-thawed sperm motility rates ranged from 92.00 to 98.00% which equilibrated for 10, 20 or 30 minutes, while 15% DMSO at three equilibration periods significantly reduced ($P<0.05$) frozen-thawed motility of sperm stored up to 70 days. The best combination of factors involved in sperm cryopreservation of red snapper fish was using Fish Ringer Extender (10% DMSO), 10 minutes of equilibration duration and cooling rates of 5 or 10°C/minute (Vuthiphandchai *et al*., 2009). Huang *et al*. (2004a) performed the sperm cryopreservation of *Xiphophorus couchianus* using 14% as cryoprotectant a dilution ratio of sperm to HBSS–glycerol of 1:10 or 1:20, equilibrated for 10 to 20 minutes, cooled at 25°C/minute from 5 to -80°C before plunging into liquid nitrogen, and thawed at 40°C in a water bath for 7 seconds which yielded as high as 78.00%.

5.3 OPTIMISATION OF COMBINATION OF EQUILIBRATION DURATION, VAPOUR EXPOSURE TEMPERATURE AND VAPOUR EXPOSURE DURATION FOR SPERM CRYOPRESERVATION IN THAI MAHSEER (*Tor tambroides*) FISH

Combination 2 (30 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour temperature) proved to be favourable combination of factors for sperm cryopreservation of mahseer sperm. This combination has obtained 72.80±2.85% and 21.20±4.02% in total motility and progressive motility, respectively. In general, successful in semen cryopreservation process apparently depends on composition of the extender, suitable cryoprotectants and the optimum freezing and thawing rates (Hammerstedt *et al*., 1900; Curry *et al*., 1994). However, there were no significant differences in all sperm motility characteristics among the three combinations that gave high frozen-thawed sperm motility characteristics.

The optimal duration of major carp fish sperm in equilibration phase was generally around 30 to 45 minutes (Routray *et al*., 2007). The optimal survival rates of fingerlings produced using frozen-thawed sperm in *Tor khudree* was obtained with 30 minutes of equilibration duration (Basavaraja *et al*., 2002). The optimal rates for freezing process are varying widely in sperm fish and depend on the fish species (Steyn, 1993; Sansone *et al*., 2002). In general, the optimal rates of freezing phase in fish sperm vary from 5 to 45°C/minute for cooling from 5 to -80°C (Rana and Gilmour, 1996; Linhart *et. al*., 2000). They demonstrated the highest motility obtained when frozen at cooling rates of 20 to 30°C/minute in sperm cryopreservation of *Xiphophorus couchianus*. The optimal cooling rate of striped bass sperm reported by Thirumala *et al*. (2006) was ranged from 14 to 20°C/minute. In sperm cryopreservation of black grouper, 20 to 154°C/minute was the optimal cooling rate (Gwo *et al*., 1993).

It is generally assumed that motility and fertility are well-correlated (Jobling *et* al., 2000; Lahnsteiner *et al*., 2000). A positive correlation between percentage of sperm velocity and fertilisation yield of frozen-thawed sperm was found by Linhart *et al*. (2000).

Dreanno *et al.* (1999) performed the sperm cryopreservation of turbot which stated that instead of sperm velocity, the sperm motility percentage determines the success of fertilisation process. However, some findings have shown that the changes in sperm at the end of spermatogenesis period, may decreases the sperm motility as well as fertilisation process (Munkittrick and Moccia, 1987). Lubzens *et al.* (1997) reported that sperm motility and fertilisation were not positively correlated.

Combination 2 demonstrated the highest values in rapid velocity, VAP, VSL, VCL and STR compared with combinations 1 and 3. These results were in agreement with the results of other researchers who have shown that shown positive correlation between fertilisation/hatching rate and specific sperm motility characteristics such as VSL and VCL (Rurangwa *et al*., 2001). Percentages of motility and sperm velocity have been positively correlated to reproductive success in fish (Lahnsteiner *et al*., 1998; Cosson *et al*., 2000; Kime *et al*., 2001). Sperm motility characteristics generated using computer-assisted sperm analysis (CASA) such as motility percentage, flagellum beat frequency and velocity distribution could be a reference for evaluating the viability of sperm cells after cryopreservation process as well the successful of fertilisation test (Cosson *et al*., 2000).

Correlations of frozen-thawed sperm motility characteristics among the three combinations of factors studied have been shown generally to have similar trend, except for some sperm motility characteristics. For examples, positive correlations were observed between total motility and STR, progressive motility and STR as well as rapid and STR in combination 1, while between total motility and medium, progressive motility and medium, rapid and medium as well as slow and ALH in combination 2.

5.4 EFFECTS OF THE LOW TEMPERATURE INCUBATOR AND THE NORMAL REFRIGERATOR ON THE FROZEN-THAWED SPERM CHARACTERISTICS WITH REFERENCE TO EQUILIBRATION DURATION

As a preparation for Experiment 4 which is transferring the technology from the ambient condition to under field condition, we have suggested and tested the usage of normal refrigerator in sperm cryopreservation of Thai mahseer. The refrigerator was lighter and cheaper as well as easy and convenient to handle compared to low temperature incubator. The challenge in this experiment was to maintain the equilibration temperature around 4°C to allow the sperm went through the equilibration phase convincingly and as a preparation before entering the vapour exposure phase.

There were no significant difference among all sperm motility characteristics between using of low temperature incubator and normal refrigerator in 3 different equilibration durations. It was evidenced that normal refrigerator was convinient and suitable for the application of sperm cryopreservation under field condition. The frozen-thawed sperm motility characteristics obtained from the results of this pioneer research was encouraging in spite of various challenges faced during the process of carrying out the field experiment. It is believed that this is the first report of sperm cryopreservation using modified Fish Ringer Extender in Thai mahseer species. Even though, there was slight fluctuation in the temperature during equilibration phase, the frozen-thawed sperm motility characteristics have been shown relatively similar to that of low temperature incubator. Therefore, with fine tuning of the factors involved during the freezing process, it may be possible to apply this sperm freezing protocol routinely under field condition. If this becomes a reality, then it may revolutionise sperm freezing protocol and open the window of opportunity for other fish species under field condition.

To illustrate the simple innovation from this research, previous researchers such as Taitson *et al*. (2008) and Viveiros *et al*. (2009) used nitrogen vapour vessel to perform the freezing under field condition in neotropical fish and curimba fish, respectively. The vessel is more expensive compared to polystyrene box that used in this study. Even though, both studies demonstrated better results (62.00±14.00% and 95.00±4.00%, respectively) in frozen-thawed sperm motility compared to results yielded in this study (35.81±1.69%), nevertheless, with refine studies in the future, comparable results or better could be obtained using our present protocol.

Generally, the sperm cryopreservation of Thai mahseer using low temperature incubator and normal refrigerator gave similar pattern of frozen-thawed sperm motility characteristics. However, there were positive corelations between progressive motility and LIN, rapid and LIN, static and ALH, VAP and STR, VCL and STR as well as BCF and STR in freezing using low temperature incubator. Meanwhile, freezing using normal refrigerator had obtained positive correlation between total motility and VAP, total motility and VSL, total motility and VCL as well as static and BCF. Therefore, these results supported the usage of normal refrigerator for Thai mahseer sperm cryopreservation under field condition, producing relatively comparable results.

5.5 ATTEMPTS TO TRANSFER OF TECHNOLOGY FROM LABORATORY TO THE FIELD CONDITION FOR THE SPERM CRYOPRESERVATION OF THAI MAHSEER (*Tor tambroides*) UNDER FIELD CONDITION

More studies have been performed in order to apply the fish sperm cryopreservation in commercial-scale usage (Tiersch and Mazik, 2000; Lang *et al*., 2003). A great technology needs to be simplified as easy as possible and therefore, it will convince people especially

conservationists, farmers and entrepreneurs to apply it widely. Process of sperm cryopreservation was more practically done under the field condition and elucidated the limitation of expensive and larger equipment used in laboratory ambient (Taitson *et al*., 2008). Thus, in this experiment, it was a first attempt to perform the sperm cryopreservation of Thai mahseer under field condition. The other target of this experiment was to simplify the usage of liquid nitrogen and to facilitate with suitable and simple equipment. For example, low temperature incubator was replaced with normal refrigerator as considering the refrigerator was lighter, cheaper and easier to handle compared to the incubator. As been showed in Experiment 3, the normal refrigerator proved to be relevant and suitable in sperm cryopreservation for application under field condition. The other replacement equipment compared to sperm cryopreservation procedure under laboratory ambient was the smaller special rack and polystyrene box for vapour exposure phase. The smaller polystyrene box provides a good advantage in usage of liquid nitrogen as it only requires small amount of liquid nitrogen compared to amount to be used under current standard ambient condition in our laboratory.

The first report on sperm cryopreservation in silver carp under farming condition was demonstrated by Alvarez *et al*. (2003) who obtained the highest frozen-thawed motility using DMSO (10%) as its cryoprotectant. According to Taitson *et al*. (2008), they successfully cryopreserved the sperm of neotropical fish using dry-shipper with DMSO and 25.7-30.8°C/minute as its cryoprotectant and cooling rate. This finding showed that they managed to attain 62.00±14.00% of frozen-thawed sperm motility. Attempt has made by Zhang *et al*. (2003) to cryopreserve the flounder sperm with simpler and practical methodology under field condition who obtained 79.27±4.00% of frozen-thawed sperm motility. Meanwhile, Viveiros *et al*. (2009) developed a simple and successful sperm

cryopreservation procedure under field condition for curimba fish using nitrogen vapour vessel which attained 95.00±4.00%. These positive findings are corroborating with the results shown in this study.

From these attempts, the sperm cryopreservation of Thai mahseer was successfully performed under the field condition which yielded satisfactory results (35.81±1.69% of frozen-thawed sperm total motility), considering this was the first attempt to perform such procedure. Despite there were relatively lower results in total motility, progressive motility, rapid, medium, slow and ALH compared to freezing process under field condition than the laboratory setup, the objective of this experiment had been achieved successfully. Generally, similar patterns were shown in the sperm motility characteristics of frozen-thawed both under laboratory ambient and field condition, except under ambient condition, there were some positive significant correlations between rapid and medium, rapid and slow, rapid and VAP, rapid and VSL and rapid and VCL. It showed the quality and fertilisation ability of frozen-thawed sperm under the laboratory ambient were better than under the field condition. Viability measurements and progressive motion of fresh and frozen sperm (VSL, VAP and VCL) correlated well with the fertility in African catfish (Rurangwa *et al*., 2001).

The most interesting finding was the successful of carried out the method for the first time under field condition and some new facility invention, that is smaller special rack and smaller polystyrene box for vapour exposure phase which is critical during the freezing process. This invention has alleviated the problem of expensive equipment and its heavy handling as well indirectly has minimised the usage of liquid nitrogen. With the early sign of positive and encouraging results, further improvement and refinement of this

techniques, it is hoped that it can be applied under field condition practically and routinely in future.

5.6 GENERAL DISCUSSION

Thai mahseer is a heritage species of economic as well as diversity importance in South East Asia countries such as Thailand, Malaysia and Indonesia. However, this fish species is endangered and much efforts have been planned to prevent it from extinction. Sperm cryopreservation has been shown to be a useful alternative to preserve fish germplasm and to prevent fish species from extinction (Robles *et al*., 2003). It also could ensure the continuous production of gametes as it plays an ample affect in utilisation of hatchery facilities or experimentation (Lubzens *et al*., 1997). The superior gametes would be protected well in liquid nitrogen tank which reduced the loss of valuable gametes (Lubzens *et al*., 1997). Proper labeling of straws containing the frozen sperm would allow the identification of superior males fishes, and upon thawing, the sperm could produce high fertilisation and hatching rates. In addition, production of genetically superior fish could be improved through gamete cryopreservation and artificial fertilisation which have significant commercial bearing (Christensen and Tiersch, 2005). In the long term, the initiation of new genetic lines through application of cryopreserved gametes would provide a system towards diseases protection (Lubzens *et al*., 1997). Also, transportation of the frozen sperm becomes easier, cheaper and safer compared to transferring of the live broodstock (Liebo, 1984; Lubzens *et al*., 1997).

The process of sperm cryopreservation including several major period such as decreased temperature, cellular dehydration, freezing and thawing phases (Medeiros *et al*., 2002). There are plenty of risks for sperm to be injured as it has to go through all the

phases and requires optimal condition to ensure maximal sperm survival and to maintain their genetic materials intact. Every phase has its own level of cellular injury and accumulated injuries could be evaluated in frozen-thawed sperm characteristics (Medeiros *et al*., 2002). Exposures to cryoprotectants, cooling, freezing and thawing will cause inside and outside sperm cell drastic changes (Guruprasad *et al*., 2007). It has been shown that some level of sperm damage happened in each sperm cryopreservation process (Watson, 2000). Therefore, aspects such as sperm biophysical properties effect to adaptation in cooling phase, changes of sperm packaging systems and the consistency and accuracy in freezing of large numbers of samples were become among vital studies for the past few years (Roca *et al*., 2006). Yoshida (2000) suggested improvement in cryopreservation of semen could be obtained with the achievable of significant findings in the gamete physiology and the biochemical processes occurring during semen collection, processing and freezing-thawing.

From this study, the effects of equilibration duration, vapour exposure temperature and vapour exposure duration on frozen-thawed sperm motility characteristics were determined. The results showed that Thai mahseer sperm may be frozen in liquid nitrogen under ambient condition which retained good level of frozen-thawed sperm motility characteristics. Meanwhile, attempt to perform the procedure under field condition showed some promise which retained satisfactory results of frozen-thawed sperm motility characteristics.

The semen collection of Thai mahseer could be performed using abdomen stripping and does not require sacrificing the fish. It was very useful technique to be used as considering the extinction situation of this species as well as easier and more economical

way compared to humanised killed technique. The fishes also may be used frequently for next replication experiment as well reducing the sperm quality variability in each fish.

Every freezing process must have it own extender as it has huge roles in retaining the functional capability and fertilising ability of sperm by controlling the pH, osmolality, ion concentration and in sources of energy during cooling, freezing and thawing (Holt, 2000; Vishwanath and Shannon, 2000). Suitable extender has been shown to prevent the activation of sperm motility via controlling the osmolality of the extender (Yang and Tiersch, 2008). The survivability of sperm for long-term storage really depends on the extender as it provides energy, protection to cryoinjury and sustaining the appropriate environment to sperm (Purdy, 2006). In the past 30 years, various extenders with different kinds of chemical composition and complexity have been successfully used for the gametes cryopreservation (Scott and Baynes, 1980; McAndrew *et al*., 1993). In freshwater fish sperm cryopreservation, various ion concentrations, osmolality and pH have been tested and managed to freeze the sperm (Linhart *et al*., 2000; Ji *et al*., 2004).

Not all extenders could functionally well in every species of fishes such as Kurokura-1 extender for D-15 extender for grass carp (Chen *et al*., 1992), silver carp (Chen *et al*., 1992), mandarin fish and Ringer extender for most of the freshwater fishes (Li *et al*., 1994). Previous reports stated the suitability of modified Fish Ringer Solution (mFRS) plus dimethyl sulfoxide (DMSO) as its extender for freezing of mahseer (*Tor khudree*) sperm process (Basavaraja and Hegde, 1998; 2004; Basavaraja *et al*., 2002). Moreover, this extender was found to be suitable for the cryopreservation of sperm of *Oreochromis mossambicus* (Harvey, 1983), *Oreochromis niloticus* (Rana *et al*., 1990) and yellow catfish sperm (Pan *et al*., 2008). In this study, the diluent was called as ‘modified

Fish Ringer Extender' after the mixing of milt, mFRS and cryoprotectant agent in order to standardise the sperm cryopreservation protocol in our laboratory.

The dilution ratio between milt and extender requires matching ratio with each freezing process. For example, the ratio of 1:3-9 (milt:extender) was suggested the best range of ratio to obtained the highest results in sperm cryopreservation of salmonid fish (Scott and Baynes, 1980; Lahnsteiner *et al*., 1995). While in sperm cryopreservation of *Tor khudree*, the suitable range of ratio was 1:10-20 (Basavaraja and Hedge, 2004). Asmad *et al*. (2008), using Tris Citric Acid Yolk Extender (TCAYE), reported the optimal ratio for sperm cryopreservation of red tilapia was 1:9. In this study, the ratio of 1:10 was selected as the milt dilution ratio using modified Fish Ringer Extender.

In preparing extender, one of the important ingredients is cryoprotectant agent which plays huge roles in sperm cryopreservation process. Generally, it will provide protection which will avoid the formation of intracellular ice crystal and excessive dehydration to sperm during freezing and thawing (Yang and Tiersch, 2008). The suitability of each cryoprotectant is varying in different species of fish.

Generally, in marine fish, 10% and 20% of DMSO were selected to be the concentration of the cryoprotectant for fish sperm cryopreservation (Gwo, 2000). In mahseer, DMSO, glycerol, and methanol were usually been used as cryoprotectant agent which obtained moderately good results in frozen-thawed sperm motility and fertilisation test (Ponniah *et al*., 1992; Ponniah *et al*., 1999a,b). Basavaraja and Hedge (2004) successfully performed the sperm cryopreservation of *Tor khudree* using modified Fish Ringer Solution which showed that DMSO was the best cryoprotectant compared to methanol and propylene glycol. No frozen-thawed sperm motility was obtained in those cryopreserved without DMSO (Basavaraja *et al*., 2002). However, there was no significant

difference in motility and hatching rates between the usage of DMSO and glycerol (Ponniah *et al*., 1999a,b). Earlier study by Ponniah *et al*. (1992), frozen-thawed sperm in cryopreservation of *Tor khudree* using glycerol showed higher sperm motility (80%) compared to DMSO (50%).

The usage of 10% DMSO yielded better results in frozen-thawed sperm quality (Basavaraja and Hedge, 2003; Vuthiphandchai *et al*., 2009). In this study, 10% DMSO was chosen as the cryoprotectant agent, and its ratio to modified Fish Ringer Solution (mFRS). The usage of DMSO facilitate the frozen-thawed sperm evaluation process which could obtain various types of sperm motility characteristics such as total motility, progressive motility, sperm velocity distribution, VAP, VSL and VCL.

Moreover, other researchers have used DMSO as cryoprotectant agent to perform the freezing process such as in mahseer (Ponniah *et* al., 1999a; Basavaraja and Hedge, 2004), striped bass (He and Woods III, 2004), black grouper (Palmer *et al*., 1993), turbot (Dreanno *et al*., 1997; Chen *et al*., 2004; Thirumala *et al*., 2006), common carp (Linhart *et al*., 2000), black drum (Wayman *et al*., 1997), tropical bagrid catfish (Muchlisin *et al*., 2004), European catfish (Linhart *et al*., 2005), Atlantic croaker (Gwo *et al*., 1991), mandarin fish (Ding *et al*., 2009), sea bass (Fauvel *et al*., 1998), yellow flounder (Richardson *et al*., 1999) and gilthead sea bream (Chambeyron and Zohar, 1990).

Basic steps involved in slow freezing procedure started with slow cooling phase, continued with rapid cooling phase and straw plunging into liquid nitrogen as final stage (Viveiros *et al*., 2000). They elaborated the process needed the optimal intermediate temperature to allow prevention of ice crystals formation via optimal dehydration in the cell before being plunged into liquid nitrogen. They stated that the temperature below the

solution freezing point could initiate the spontaneous and induced ice nucleation formation.

Ion concentrations (Na^+, K^+, Ca^{2+}, etc.), osmolality and pH were vital in activation of sperm after releasing from testis; the sperm activation involving the phase of cell membrane depolarisation, affecting sperm tail capacity for flagellar motility and finally the stimulation of sperm motility (Morisawa and Suzuki, 1980; Morisawa *et al*., 1983). Rurangwa *et al*. (2004), suggesting that a suitable activation or activation medium has to be applied in thawing phase in order to obtain optimal results in hatcheries. However, in general, no specific best medium was established for each sperm cryopreservation of fish.

Sperm motility of frozen-thawed results was crucial measurement in evaluating the successful of each freezing process (Dreanno *et al*., 1999). Percentage of motility and sperm velocity has been correlated to reproductive success in fish (Lahnsteiner *et al*., 1998; Kime *et al*., 2001; Rurangwa *et al*., 2001). Viability measurements and progressive motion of fresh and frozen sperm (VSL, VAP and VCL) correlated well with the fertility in African catfish (Rurangwa *et al*., 2001). Our study also showed positive results which produced good level of frozen-thawed sperm motility characteristics.

5.7 CONSTRAINTS AND SUGGESTIONS FOR FUTURE IMPROVEMENT

In this study, several problems were encountered before obtaining positive results of frozen-thawed sperm motility characteristics. The first problem was the limited resources of Thai mahseer fish due to the approaching towards the limit of endangered species. Fortunately, we managed to buy from a local company that reared the Thai mahseer and only sold it for research purposes. Therefore, sufficient number of fish was available to enable us to carried out this project. Another problem faced by the author was

to obtain optimal quality of sperm during collection due to the stress of the male as well as due to faecal and/or urine contamination. Therefore, it was convenient to the author to use two males for semen collection as a precautionary measure, whereby if one collection was spoilt than another collection could be used.

Hormonal treatment may increase the sperm motility of Atlantic halibut sperm compared to non-hormonal treated sperm (Vermeirssen *et al*., 2000). Various reports in several fishes stated the increased sperm fluidity after hormonal stimulation of GnRH (Vermeirssen *et al*., 1998; Shangguan and Crim, 1999; Vermeirssen *et al*., 2000). In this study, hormone stimulation using Ovaprim only used one concentration (0.5 ml/kg) for the whole experiments. Therefore, study of ovaprim concentration should be carried out in future in order to determine the optimal concentration of Ovaprim to be used.

Variability of fresh sperm quality among individual fish may influence of maturation of fish testis. The maturity of fish normally was correlated with the age and body weight of the fish. Other factors that may affect the fresh sperm quality are age of the testis (Rana, 1995), degree of sexual stimulation, frequency of ejaculation (Zbinden *et al*., 2001), age and methods of semen collection Hafez and Hafez (2000). Research needs to be performed in future to determine the range of optimal fish age and body weight in order to obtain the optimal results in frozen-thawed sperm characteristics. In this study, a range of 400-1000 g of Thai mahseer fish was used for the Experiments 1, 2 and 3. In Experiment 4, body weight of the fish used ranging from 435 to 1125 g. Findings of the optimal maturation age should help in improving the excellent frozen-thawed sperm quality as well increasing the successful percentage of fertilisation process.

The lipid-sperm agglutination may occur during the mixing the semen with the unsuitable extender used. Therefore, the mFRE extender was selected as it could mix well

with the semen of the Thai mahseer. Several factors such as osmotic pressure, temperature, pH, ionic composition (Na^+, K^+, Ca^{2+} and Mg^{2+}) and dilution rate need to be studied as it influence the fresh sperm motility (Chowdhury and Joy, 2007). During the frozen-thawed sperm evaluation using IVOS, the reading must be taken as soon as possible after the sperm activation with water due to short duration of sperm movement and the motility of frozen-thawed sperm decreased gradually after 15-180 seconds. To make it worse, it might affect the fertiliseability when perform the *in vitro* fertilisation using the frozen-thawed sperm in unsuitable extender. .

In freezing process under field condition, the challenge was the fluctuation of the normal refrigerator and environment temperatures that may influence the quality of frozen-thawed sperm characteristics during equilibration phase. In this study, we used the thermo-couple to make sure the normal refrigerator temperature maintained within a range of 3.5-4.5ºC. New invention and improving technique is require in order to improve the frozen-thawed sperm motility characteristics such as better technique in maintaining the normal refrigerator temperature for equilibration phase.

There is dearth of information regarding the physiology of Thai mahseer sperm particularly with reference to freezing mechanism. Therefore, this project was iniatially designed using 'trial and error' approach by combining possible various factors such as different levels of equilibration duration as well as temperature and duration of vapour exposure. Routray *et al*. (2007) suggested the important of research in basic characteristics of the sperm cell in developing a sperm cryopreservation protocol such as seminal plasma composition, sperm morphology and sperm density as it differ depending on the species of the fish. Membrane lipids are often described as having a role in cell cryopreservation, mainly because of their influence on the more or less heterogeneous phase transition

withstood by the plasma membrane during cooling (Quinn, 1985). It was previously shown that plasma membrane lipids in trout sperm could be manipulated by changing the rearing temperature of the broodstock (Labbe and Maisse, 1996) and by feeding different dietary lipids. Therefore, the study of the membrane structure of Thai mahseer sperm needs to be performs in future to understand more about the structure that may relate to sperm survivability during freezing and thawing process.

In future, the sperm cryopreservation of Thai mahseer should be performed using different extenders in order to determine the optimal extender to be used due to different types and concentrations of chemical consituents. Besides mFRE, various kinds of extenders have been used in fish sperm cryopreservation such as BSMSIS, TYACE, Kurokura-1, D-15 extender, Ginsburg extender and others. A significant effect in frozen-thawed sperm motility and hatching rate were obtained between sperm cryopreservation of *Tor putitora* using six different extenders (Ponniah *et al*., 1999b).

The toxicity test against the cryoprotectant agents (CPA) needs to be carried out to determine the effect of each cryoprotectant agent and its concentration as well. Types of CPA and its concentration could determine the successful of each freezing and fertilisation process. Generally, different optimal cryoprotectant was used in different types of fish as it will prove via experimentation (Full *et al*., 2004). Most of the experimental work on cryopreservation of African catfish semen has focused on trials to identify the optimal extender for conservation (Urbanyi *et al*., 1999; Rurangwa *et al*., 2001), the cryoprotective agent (Steyn and van Vuren, 1987; Horvath and Urbanyi, 2000; Viveiros *et al*., 2000) and the cooling rates (Steyn and van Vuren, 1987; Viveiros *et al*., 2000).

There were some researchers stated that duration of storage plays important in determining the longest duration of storage can be performs as longer storage may produce

gradual decreased values of frozen-thawed sperm characteristics. Frozen-thawed sperm motility might be reduced with improper storage duration via irregular sensitivity to the cell (Billard and Cosson, 1992). Longer storage of frozen sperm may suffer more cryoinjuries and decrease the frozen-thawed sperm motility (Basavaraja *et al*., 2004). This area needs to be study in future as considering the lacking of report in effect of duration on frozen-thawed sperm motility especially in *Tor* species.

One important phase involved in sperm cryopreservation was thawing phase which the duration and temperature of water should be optimise. It needs a suitable condition for this phase to ensure the prevention of ice crystal formation. The initiation of most external fertilising sperm motility is induced after the sperm have been released into the water and metabolically activated (Rurangwa *et al*., 2004; Alavi and Cosson, 2005). However, most freshwater fish sperm move actively after the activation for less than 30 seconds and within 2 minutes, the sperm motility reducing gradually (Morisawa and Suzuki, 1980; Perchec *et al*., 1993; Billard *et al*., 1995a; Kime *et al*., 2001)

In this study, we managed to evaluate the quality of frozen-thawed sperm using their percentage of motility, velocity distribution and other sperm characteristics. However, the better analysis or measurement of frozen-thawed sperm is via *in vitro* fertilisation test which the results are more beneficial and reliable. Some of researchers unfortunately found that fertilisation could not successfully occur using good motility of frozen-thawed sperm (Lubzens *et al*., 1997). Thus, IVF test should be performed in near future to study the potential of fingerlings production using frozen-thawed sperm of Thai mahseer. For consequent studies, it should determine the viability, survivability and developmental ability of larvae produced from cryopreserved sperm via IVF. These findings would play

crucial roles in conservation programmes of this species as well the commercialisation plans in future.

Riley *et al*. (2004) suggested the excellent commercialisation mechanism must have the efficient and effective methodology of freezing in order to achieve the optimal benefits. Some of the matters that need to be focused are size of the straws, freezing procedure, storage process, ratio of sperm and egg used in IVF and the suitable medium for the development of produced fingerlings. Place of hatchery operation requires time-saving and cost effective facilities (Riley *et al*., 2004).

Chapter 6
CONCLUSIONS

Chapter 6

6.0 CONCLUSIONS

e) Thai mahseer sperm was successfully frozen using modified Fish Ringer Extender with special emphasis on equilibration duration, vapour exposure temperature and vapour exposure duration.

f) Duration of 30 minutes equilibration gave higher total motility and progressive motility than 45 and 60 minutes durations for Thai mahseer sperm cryopreservation protocol.

g) Among three temperatures used for vapour exposure phase in this study, -110°C was considered to be the most suitable temperature compared to -100°C and -120°C which obtained the highest values in total motility and progressive motility.

h) Meanwhile, 15 minutes of vapour exposure duration produced the highest values of total motility and progressive motility compared to 5 and 10 minutes durations.

i) Combination of 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 10 minutes of vapour exposure duration; combination of 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration; and combination of 30 minutes of equilibration duration, -120°C of vapour exposure temperature and 15 minutes of vapour exposure duration were considered as three best combinations in sperm cryopreservation of Thai mahseer produced the significantly highest values in total motility and progressive motility and were used in subsequent experiment.

j) Combination of 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration showed the highest values in total motility and progressive motility compared to other two combinations used.

k) Both incubator/refrigerator attained the same effect during the equilibration phase which showed no significant differences using all equilibration durations (30, 45 and 60 minutes).

l) As for comparison, the freezing process under laboratory ambient produced significantly higher values in total motility, progressive motility, rapid, medium, slow and ALH compared to freezing process under field condition. However, the most interesting facts from this attempt, there were no significantly different in VAP, VSL, VCL, BCF, STR and LIN values between the freezing under laboratory ambient and field condition.

m) In summary, the results demonstrate that the Thai Mahseer sperm could be cryopreserved using modified Fish Ringer Extender with good frozen-thawed sperm motility characteristics both under laboratory ambient as well as field condition.

n) To our latest knowledge, this is the first successful report of Thai mahseer sperm cryopreservation in Malaysia, both under laboratory and field conditions using modified Fish Ringer Extender.