EFFECTS OF DIFFERENT EXTENDERS, CRYOPROTECTANTS, EQUILIBRATION AND VAPOUR EXPOSURE ON FREEZABILITY OF AFRICAN CATFISH (*Clarias gariepinus*) SPERM

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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

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ABBREVIATIONS

ABEL	Animal Biotechnology-Embryo Laboratory
ALH	Amplitude of lateral head displacement
ANOVA	Analysis of variance
BCF	Beat-cross frequency
CPA	Cryoprotectant agent
DMRT	Duncan's Multiple Range Test
DMSO	Dimethyl-sulfoxide
FRE	Fish-Ringer Extender
ISB	Institute Biological Sciences
LIN	Linearity
LN_2	Liquid nitrogen
SEM	Standard error mean
SPSS	Statistical Package for Social Science
TCAYE	Tris-Citric Acid Yolk Extender
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
STR	Straightness

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ORIGINAL LITERARY WORK DECLARATIONS

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ABSTRACT

The aim of this study was to develop an optimal freezing protocol for African catfish (*Clarias gariepinus*) sperm with special reference to type of extender and cryoprotectant, molarity, equilibration duration, vapour temperature and vapour exposure duration. Using Tris-Citric Acid Yolk Extender (TCAYE), a 3x3x3x3 factorial experiment was carried out consisting of 3 molarities of glycerol (0.5, 1.0 and 2.0 M), 3 equilibration durations (120, 140 and 160 minutes), 3 vapour temperatures (-80, -90 and -100^{o}C) and 3 vapour exposure durations (5, 10 and 15 minutes). In addition, using Fish-Ringer Extender (FRE), a 3x3x3 factorial experiment was also conducted involving 3 equilibration durations (120, 140 and 160 minutes), 3 vapour temperatures (-80, -90 and -100^{o}C) and 3 vapour exposure durations (5, 10 and 15 minutes). The molarity of cryoprotectant in FRE extender was fixed at 10% DMSO. Briefly, the straws containing the sperm were placed in refrigerator at 4^{o}C with the fixed equilibration duration after which exposed to liquid nitrogen vapour at the fixed vapour temperature with the fixed vapour exposure duration. Subsequently, the straws were directly plunged into liquid nitrogen. The frozen sperm were thawed at 30^{o}C for 30 seconds to evaluate the sperm motility characteristics using the automated semen analyzer (IVOS; Hamilton Thorne, USA). The effects of factors and parameters measured were analysed using Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT). In Experiment 1, large body weight (BW) of African catfish gave the highest fresh sperm total motility (82.40±4.59%) followed by medium BW (51.64±9.82%) and small BW (40.40±12.16%), whereby small BW fish were significantly different in total motility compared with the other two groups studied. In Experiment 2, glycerol with molarity of 0.5 M showed significantly the highest value of frozen-thawed sperm total motility (32.27±2.05%) as compared to 1.0 M (24.50±1.81%) and 2.0 M (2.63±0.29%). At 140 minutes equilibration duration, the value of total motility (31.69±2.19%) was

significantly higher as compared to 120 minutes (25.26±1.76%). There were no significant differences (P>0.05) in value of total motility for -80, -90 and -100^{o}C which were ranged from 25.95±2.34% to 29.41±1.69%. The value of total motility did not show any significant differences (P>0.05) among the three vapour exposure durations (5, 10 and 15 minutes), which were ranged from 27.63±2.02% to 28.45±2.14%. In Experiment 3, there were no significant differences (P>0.05) in values of total motility at 120 minutes (76.65±2.27%) and 160 minutes equilibrations (76.01±2.04%), but these durations gave comparatively higher values of total motility than 140 minutes (66.90±2.60%). The values of total motility for vapour temperatures of -90^{o}C (74.07±2.02%) and -100^{o}C (74.95±1.88%) did not show any significant differences (P>0.05), but they were significantly different with -80^{o}C, which gave comparatively lower values (64.59±5.08%). There were no significant differences (P>0.05) in values of total motility for 5, 10 and 15 minutes which were ranged from 72.67±2.27% to 73.99±2.34%. In Experiment 4, there were no significant differences (P>0.05) for values of total motility between 1.0 M (24.50±1.81%) and 2.0 M of glycerol in TCAYE (26.74±2.14%), but they were comparatively lower than 0.5 M of glycerol that showed higher significant value (32.27±2.05%). On the other hand, combination of DMSO (10%) in FRE extender showed the highest significant value of total motility (73.52±1.35%) as compared to the three molarities of glycerol in TCAYE extender. In summary, the best combination to obtain the highest frozen-thawed sperm motility characteristics for TCAYE extender was 0.5 M of glycerol, 140 minutes equilibration duration, -90^{o}C vapour temperature and 5 to 15 minutes vapour exposure duration, whereas for FRE extender was 120 minutes equilibration duration, -100^{o}C vapour temperature and 5 to 15 minutes vapour exposure duration. In conclusion, results obtained in this study showed that 10% DMSO with FRE extender produced higher frozen-thawed sperm total motility than TCAYE extender.

Future studies are needed through refinement in factors involved during freezing process that influence sperm survival before it can be used routinely in the reproduction of African catfish (*Clarias gariepinus*).

ABSTRAK

Matlamat kajian ini adalah untuk membangunkan protokol penyejukbekuan sperma yang optimum bagi keli Afrika (*Clarias gariepinus*) dengan merujuk khusus kepada jenis ekstender dan krioprotektan, molariti, tempoh pengimbangan, suhu pengewapan serta tempoh pendedahan kepada wap nitrogen cecair. Dengan menggunakan ekstender TCAYE, eksperimen berbentuk faktorial 3x3x3x3 dijalankan yang terdiri daripada 3 molariti gliserol (0.5, 1.0 dan 2.0 M), 3 tempoh pengimbangan (120, 140 dan 160 minit), 3 suhu pengewapan nitrogen (-80, -90 dan -100^{o}C) dan 3 tempoh pendedahan kepada wap nitrogen cecair (5, 10 dan 15 minit). Di samping itu, eksperimen berbentuk faktorial juga dijalankan ke atas ekstender FRE yang melibatkan 3x3x3, terdiri daripada 3 tempoh pengimbangan (120, 140 dan 160 minit), 3 suhu pengewapan nitrogen (-80, -90 dan -100^{o}C) dan 3 tempoh pendedahan kepada wap nitrogen cecair (5, 10 dan 15 minit). Molariti krioprotektan dalam ekstender FRE ditetapkan pada 10% DMSO. Secara ringkas, straw yang mengandungi sperma diletakkan ke dalam peti sejuk pada suhu 4^{o}C dalam tempoh pengimbangan yang telah ditetapkan dan seterusnya didedahkan kepada wap nitrogen cecair pada suhu pengewapan dan tempoh pendedahan yang telah ditetapkan. Berikutnya, straw dijunamkan secara langsung ke dalam nitrogen cecair. Sperma yang telah mengalami proses penyejukbekuan dinyahsejukbekukan pada suhu 30^{o}C selama 30 saat untuk menganalisis ciri-ciri motiliti sperma menggunakan penganalisis semen automatik (IVOS; Hamilton Thorne, USA). Kesan faktor-faktor dan parameter-parameter yang diukur dianalisis dengan menggunakan Analisis Varians (ANOVA), diikuti dengan "*Duncan Multiple Range Test"* (DMRT). Dalam Eksperimen 1, didapati bahawa berat badan ikan keli Afrika yang besar menunjukkan peratusan kadar motiliti sperma segar yang paling tinggi (82.40±4.59%), ini diikuti oleh ikan yang memiliki berat badan yang sederhana (51.64±9.82%) dan berat badan ikan yang kecil (40.40±12.16%), yang mana

ikan berberat badan yang kecil menunjukkan perbezaan yang signifikan dalam peratusan kadar motiliti berbanding kedua-dua kumpulan yang dikaji. Dalam Eksperimen 2, gliserol dengan molariti 0.5 M menunjukkan peratusan kadar motiliti sperma sejukbeku-nyahsejukbeku signifikan yang paling tinggi (32.27±2.05%) berbanding 1.0 M (24.50±1.81%) dan 2.0 M (2.63±0.29%). Dalam tempoh 140 minit pengimbangan, peratusan kadar motiliti menunjukkan nilai signifikan yang tinggi (31.69±2.19%) berbanding 120 minit (25.26±1.76%). Tiada perbezaan yang signifikan (P>0.05) didapati bagi peratusan kadar motiliti pada suhu -80, -90, -100^{o}C yang berjulat daripada 25.95±2.34% sehingga 29.41±1.69%. Peratusan kadar motiliti bagi masa pendedahan kepada wap nitrogen cecair (5, 10 dan 15 minit) juga tidak memberikan perbezaan yang signifikan (P>0.05) yang berjulat daripada 27.63±2.02% sehingga 28.45±2.14%. Dalam Eksperimen 3, tiada perbezaan yang signifikan (P>0.05) bagi peratusan kadar motiliti pada 120 minit (76.65±2.27%) dan 160 minit (76.01±2.04%) tempoh pengimbangan, akan tetapi kedua-duanya menunjukkan peratusan kadar motiliti yang tinggi berbanding 140 minit (66.90±2.60%). Peratusan motiliti sperma bagi suhu pengewapan -90^{o}C (74.07±2.02%) dan -100^{o}C (74.95±1.88%) tidak menunjukkan perbezaan yang signifikan (P>0.05), akan tetapi kedua-duanya adalah berbeza dengan signifikan pada suhu -80^{o}C, yang menunjukkan nilai yang paling rendah (64.59±5.08%). Tiada perbezaan yang signifikan (P>0.05) bagi peratusan kadar motiliti sperma dalam masa 5, 10 dan 15 minit pendedahan ke atas wap nitrogen cecair yang berjulat 72.67±2.27% sehingga 73.99±2.34%. Dalam Eksperimen 4, tiada perbezaan yang siginifikan (P>0.05) antara 1.0 M (24.50±1.81%) dengan 2.0 M gliserol (26.74±2.14%) yang terkandung di dalam ekstender TCAYE, akan tetapi kedua-duanya menunjukkan peratusan kadar motiliti sperma yang rendah berbanding 0.5 M gliserol yang mencatatkan peratusan yang tinggi (32.27±2.05%). Selain itu, kombinasi 10% DMSO bersama ekstender FRE memberikan peratusan kadar motiliti yang

paling tinggi (73.52±1.35%) berbanding ketiga-tiga molariti gliserol di dalam ekstender TCAYE. Secara rumusannya, kombinasi yang terbaik bagi menghasilkan ciri-ciri motiliti sperma yang paling tinggi untuk ekstender TCAYE adalah 0.5 M of gliserol, 140 minit tempoh pengimbangan, -90^{o}C suhu pengewapan dan selama 5 hingga 15 minit tempoh pendedahan ke atas wap nitrogen cecair, manakala bagi ekstender FRE adalah 120 minit tempoh pengimbangan, -100^{o}C suhu pengewapan dan tempoh 5 hingga 15 minit tempoh pendedahan ke atas wap nitrogen cecair. Kesimpulannya, keputusan yang diperoleh dalam kajian ini menunjukkan bahawa 10% DMSO bersama ekstender FRE pada amnya mengekalkan ciri-ciri sperma sejukbeku-nyahsejukbeku yang normal berbanding ekstender TCAYE. Kajian selanjutnya perlu diteruskan pada masa akan datang melalui penelitian yang lebih mendalam dalam faktor-faktor yang terlibat semasa proses penyejukbekuan yang mempengaruhi keterushidupan sperma sebelum ianya dapat digunakan secara rutin dalam pembiakan keli Afrika (*Clarias gariepinus*).

Chapter 1

INTRODUCTION

Chapter 1

1.0 INTRODUCTION

The genus of *Clarias* is present in both Asian and African continents (Teugels, 1996). Among the Asian continents that are native to this species are India, Sri Lanka, Pakistan, Myanmar, Malaysia, Singapore, Philippines, Borneo, Java and Thailand (Talwar and Jhingran, 1991). *Clarias gariepinus* (locally called African catfish) is widely considered as an excellent food fish in Asian countries, and interest in farming of this catfish is growing. Owing to its taste and low fat content, the fish is very popular as a heart-patients' dish and fetches a high price in the market. The fish are found in all types of freshwater but more abundant in swampy waters. It can live out of water for some time as it has an accessory respiratory organ. Due to the fast growth rate, hardiness, efficient feed utilisation and ability to survive in poorly oxygenated waters; these features make this fish as potential candidate for aquaculture.

A variety of species of the genus *Clarias* and their hybrids are cultured, for reasons of their high growth rate, disease resistance and amenability to high density culture, related to their air-breathing habits (Huisman and Richter, 1987; Haylor, 1993). Among the species studied are *Clarias macrocephalus* (Areerat, 1987), *Clarias batrachus* (Zheng *et al*., 1988; Singh and Singh, 1992), *Clarias fuscus* (Zheng *et al*., 1988; Anderson and Fast, 1991) and *Clarias isheriensis* (Fagbenro and Sydenham, 1990), the African species (*Clarias gariepinus*) has been subject to particularly intensive research in notably South Africa (Hecht *et al*., 1988) and the Netherlands (Huisman and Richter, 1987).

Clarias gariepinus presents a definite interest for aquaculture because its gametogenesis is continuous once sexual maturity is reached. Therefore, this ensures the availability of gametes throughout the year and constant supply of fish. To collect the semen, the males have to be killed and the testes dissected out as the semen cannot easily

be obtained by stripping. Unlike the female, hand stripping is impractical for the male. Several reports have indicated that the stripping of semen from catfishes is difficult because the testes are located deep within the body cavity and are covered by other organs, for examples, gut and stomach. Therefore, during stripping, most of the pressure applied to the abdomen is on the other organs. In addition, the ripe milt gathers along the convex lobular edge of the testes rather than passing through the sperm ducts. This condition has been described for two African catfish species, namely *Clarias gariepinus* (Hogendoorn, 1979) and *Heterobranchus longifilis* (Oteme *et al*., 1996), Channel catfish, *Ictalurus punctatus* (Legendre, 1986) and Asian green catfish, *Mystus nemurus* (Christianus *et al*., 1998). Moreover, thick interstitial tissue surrounds the spermatogenic-cell area in parts of the testis and seminal vesicle (also called glandular testis), possibly blocking sperm flow during abdominal massage (Tan-Fermin *et al*., 1999). Furthermore, the sperm ducts are surrounded by up to 50 finger-like seminal vesicle extensions (Fishelson *et al*., 1994) that may prevent sperm flow when pressure is applied to the abdomen (Richter, 1976).

One key constraint to the culture of *Clarias gariepinus* is the limited quality fingerlings as seed material. The collection of stocking material from the wild is not sustainable. Induced spawning may be a dependable alternative for obtaining high quality seed material. Application of sperm cryopreservation has become an indispensable alternative in fish selection and synchronisation of gamete availability of this species. Many advantages can be obtained with the use of cryopreserved sperm, these include: (a) synchronisation of gamete availability of both sexes: ovulation is only noticed when sperm production declines in cross fertilisation of different strains and autumn spawning herring (*Clupea harengus* L.; Blaxter, 1953), (b) use of the total volume of available semen : this is useful for sperm economy in species where semen is difficult to obtain, Japanese eel, (*Anguilla japonica*; Ohta and Izawa, 1996), but also in species where only low volume of

semen can be stripped in captivity yellowtail flounder, (*Pleuronectes ferrugines* L.; Clearwater and Crim, 1995) or turbot, (*Psetta maxima* L.; Suquet *et al.*, 1994), (c) simplifying broodstock maintenance: off-season spawning can be induced in most cultured-fish species, by the manipulation of photoperiod and temperature cycles (Bromage, 1995), (d) transport of gamete: useful when male and female gametes are collected in different locations; this enables also the introduction of genes from the wild into hatchery stocks, (e) avoiding aging sperm: the senescence of sperm during the course of the spawning season has been reported for many fish species and results in decrease of milt quality (Rana, 1995). Cryopreservation allows the collection of sperm when it has the highest quality and (f) conserving genetic variability in domesticated populations: the use of a limited breeders leads to a reduction of heterozygosity. Gene banks of cryopreserved semen can also be used to maintain genetic diversity of fish populations that are endangered and protected against inbreeding.

Development of sperm cryopreservation protocols for African catfish is challenging due to the seminal composition of this fish which consists of lipid. In relation to this, the choice of extender is important to ensure easy solubilisation and absorption into the sperm cells. In the present research, two extenders were used Tris-Citric Acid Yolk Extender (TCAYE) and Fish-Ringer Extender (FRE). TCAYE has been used for goat sperm cryopreservation at the ISB Mini (Livestock) Farm, the University of Malaya. Later, the same extender was first introduced in the sperm cryopreservation in red tilapia but with some modification. Using TCAYE extender for tilapia fish sperm cryopreservation, it has been shown that post-thawed motility was higher at equilibration duration of 45 minutes (58.80 ± 6.48%), 1 M molarity of glycerol in extender (56.33 ± 3.48%), and -85^{o}C (37.50 ± 8.30%) of vapour temperature in sperm cryopreservation (Fung, 2006; Lim, 2006; Ting,

2006). Freshwater FRE extender is another type of diluents used in this study and it has frequently being used in sperm cryopreservation of freshwater fish (Kurokura *et al*., 1984).

Another challenge in the development of sperm cryopreservation in African catfish is limited volume of semen and rapid decline in sperm motility as well as defects of sperm in the cryopreservation process after collection. Even though the occurrence of limited sperm volume is rare, this problem can be solved through the identification of maturity age of catfish. As the age of catfish achieved its maturity, the fish produces high amount of semen to fertilise eggs during spawning. Apart from that, induction of gonadotrophin-releasing hormone (GnRH) can be used to increase spermatogenesis in catfish as an alternative.

Reproductive hormones have been used throughout this research to assist the milt collection by stimulating reproductive processes and inducing ovulation/spermiation. Among various reproductive hormones that are commonly used for inducing or maintaining spermatogenesis in many fish species are pituitary gland (PG), human chorionic gonadotrophin (hCG) and GnRHa (Munafi *et al*., 2006). PG and hCG have been used to induce spermiation in mullet (*Mugil cephalus;* Shehadeh *et al*., 1973), Japanese eel (*Anguilla japonica*; Miura *et al*., 1991) and bream (*Abramis brama*; Kucharczyk *et al*., 1997). Carp-PG treatment induced increasing volume and sperm cells in *Mystus nemurus* (Christianus *et al.*, 1996). A single hCG injection induced a 13-fold increase in stripped sperm volume in *Pangasius bocourti* (Cacot *et al*., 2003). Both PG and hCG treatments induced testis hydration and facilitated semen collection from testis of African catfish (Hecht *et al*., 1982). Treatment with GnRHa has also proven effective in enhancing milt production in fish (reviewed by Zohar and Mylonas, 2001).

The suitability of extenders and cryoprotectants are important factors in cryopreservation because it differs among fish species. Cryoprotectants are playing

important role in long-term cryopreservation. Cryoprotectants are needed to protect the sperm cell from a cold or heat-shock treatments and prevent cell dehydration (Chao *et al*., 2001; Muchlisin, 2005). The cryoprotectants act through providing a cryoprotection to the labile enzyme, for examples, catalase and stabilizing protein in unfrozen and aqueous solution. They can also prevent ice formation during pre-freezing but the same levels can be lethal to unfrozen cell (Chao, 1991). Examples of cryoprotectants used in sperm cryopreservation in fish are dimethyl acetamide (DMA) has been used for Rainbow trout sperm (Mc Niven *et al*., 1993) and Dimethyl sulphoxide (DMSO) was found to be suitable for muskellunge sperm (Ciereszko *et al*., 1999), rainbow trout (Ciereszko *et al*., 1996a), penaeid shrimp (Alfaro *et al*., 2001) and Arctic charr (Richardson *et al*., 2000). In addition, methanol was reported suitable for Japanese bitterling (Ohta *et al*., 2001), African catfish (Viveiros *et al*., 2000), European catfish testicular sperm (Ogier de Baulny *et al*., 1999) and salmonid sperm (Lahnsteiner *et al*., 1997) with glycerol effective for ejaculated sperm of European catfish (Ogier de Baulny *et al*., 1999).

Exposure to cryoprotectant prior to freezing is another important parameter in cryopreservation of sperm from many species. Its effect will vary depending on the cryoprotectant, duration of exposure and concentration (Morris *et al*., 1981). Increased exposure to cryoprotectants can improve the cryoprotective effect, but can also result in increased toxicity to the sperm cells (Jamieson *et al*., 1991; Christensen *et al*., 1996). Besides advantages, cryoprotectants have disadvantage as it can induce protein denaturation at higher temperature and cause cellular toxicity at cellular systems. It has long been recognised that exposure to cryoprotectants can cause damage to the cells and tissues during equilibration prior to freezing due to their toxicity (Fahy, 1986). The apparent toxicity of cryoprotectants is dependent on type and concentration of cryoprotectants, the equilibration duration and the temperature during loading (Chao,

2001). Several cryoprotectants have been reported to be toxic on sperm and embryos of the fish. For instance, sodium citrate is known to be harmful to the structural and integrity of some fish sperm (Gwo and Arnold, 1992), while propylene glycol and ethylene glycol are reported to be more toxic than DMSO in oyster embryos (Chao *et al*., 1994). However, high levels of DMSO can be toxic to fish sperm compared with other commonly used cryoprotectants such as methanol, ethanol and glycerol (Simione, 1998).

Cooling rate can affect the rate of osmosis, diffusion and formation of ice crystals within a cell (Morris *et al*., 1981). Thawing temperature and duration are also critical factors in the survival of cryopreserved sperm cells (Morris *et al*., 1981).

This research was conducted to answer a few of the following questions such as: a) How body weight affects the quality of fresh semen (pre-freezing)?, b) Could TCAYE extender or FRE extender maintain good sperm quality after freezing?, c) What is the optimum molarity of glycerol?, d) How equilibration duration effects the post-thawed cryopreserved sperm?, e) Do the vapour temperature and exposure duration play a role in sperm freezability? and f) What is the best combination of factors to obtain optimum sperm survivability after cryopreservation?

The first successful attempt to cryopreserve African catfish sperm, *Clarias garipienus* was achieved by Steyn *et al*. (1985). The techniques used were improved later by Steyn and Van Vuren (1987), who studied the optimal cryo-diluents and freezing rates to be used. Cryopreservation was then used as support for genetic studies on *Clarias garipienus* (Van der Bank and Steyn, 1992; Van der Walt *et al*., 1993).

The objectives of the present study were:

a) To develop suitable technique for sperm freezing in African catfish (*Clarias gariepinus*).

b) To determine sperm motility characteristics in fresh semen based on individual fish body weight.

c) To evaluate the effectiveness of different molarities of glycerol in TCAYE extender on sperm survival after freezing.

d) To obtain the optimal freezing rate with special focus on equilibration temperature and duration, vapour temperature and exposure vapour duration on the sperm viability in African catfish (*Clarias gariepinus*).

e) To evaluate the effectiveness of using FRE extender on sperm motility characteristics in cryopreservation of African catfish (*Clarias gariepinus*).

f) To compare the effects of different types of extender and cryoprotectant on sperm motility characteristics after freezing.

g) To correlate among sperm motility characteristics according to different levels of factors involved during sperm freezing process.

Chapter 2

REVIEW OF LITERATURE

Chapter 2

2.0 REVIEW OF LITERATURE

2.1 THE TESTES

2.1.1 Morphology

The testes of teleost fishes show greater morphological variation than in other vertebrates (Lofts, 1968; Dodd, 1972; de Vlaming, 1974; Callard *et al.*, 1978). In most cases, testes of teleost fishes are a pair of elongated structures composed of branching seminiferous tubules embedded in the stroma. The testis consists of thin-walled tubules or lobules that contain germ cells (the spermatogonia) which are endodermal in origin. Germ cells divide in clusters enclosed by a cyst. Primary spermatogonia (the stem cells) which are present throughout the year, divide mitotically to give rise to secondary spermatogonia which get transformed into primary spermatocytes. They divide by meiosis and give rise to spermatids from which sperm are formed. The seminiferous tubules are packed with sperm in the pre-spawning and spawning periods.

Billard *et al*. (1982) defined the testes of clariid African catfish as the lobular type. In adult males of African catfish, *Heteronbronchus longifilis,* the testes appear as two elongated lobes (Figure 2.1), the size and weight vary greatly from one fish to another independent of the weight of the individual considered. They lie dorsally and to the rear of the abdominal cavity along with their associated seminal vesicles which are more or less developed and branched (Oteme *et al*., 1996).

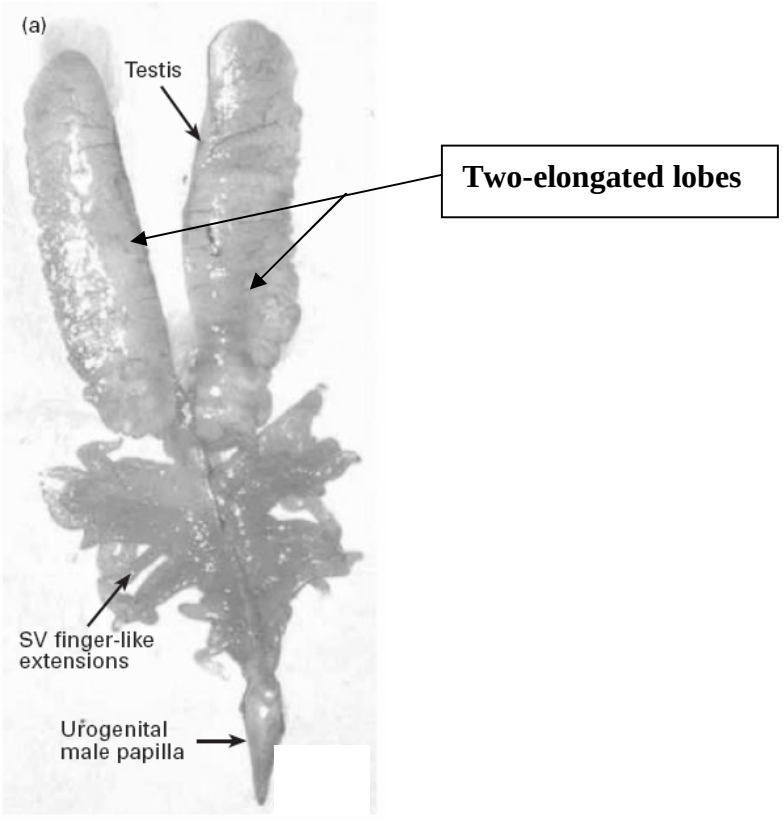

Figure 2.1: Reproductive organs of a male African catfish, *Clarias gariepinus* at 14 months after hatching (Viveiros *et al.*, 2001).

2.1.2 Cellular Source of Steroid Hormone

Lofts *et al.* (1972) and Guraya (1976, 1979) have summarised the literature on cellular sources of testicular steroids in teleost fishes. Leydig cells or interstitial cells, the large polygonal cells usually located within the interlobular spaces, produce androgen (Guraya, 1976; Hoar and Nagahama, 1978). Van den Hurk *et al.* (1978), who have made an ultrastructural and enzyme cytochemical study of the testis in rainbow trout, reported that the Leydig cells are the main source of steroids and the steroidogenic activity is at a peak when the testes are mature and new spermatogonia are being formed. Sertoli cells also have enzymes involved in steroidogenesis when males are in spermiation. Further, stromal

cells around the vas deferens epithelium are also steroidogenic when sperm are stored in the lumen of vas deferens (Guraya, 1976).

2.1.3 Sex Accessory Glands

Seminal vesicles, the sex accessory glandular structures, attached to the testes have been reported in various species of catfishes and gobiid fishes (Sundararaj, 1958; Dodd, 1960), teleosts belonging to Gobiidae, Siluridae and Blennidae (Van Tienhoven, 1983; Van den Hurk *et al*., 1987; Patzner, 1991; Singh and Joy, 1999).They show seasonal variations in secretory activity correlated with those of the testes (Nayyar and Sundararaj, 1970).

A synergism among androgen, prolactin and growth hormone has been demonstrated in the regulation of secretory activity in the seminal vesicles of catfish, *Heteropneustes fossills* (Sundararaj and Nayyar, 1969; Nayyar and Sundararaj, 1969, 1970) and the gobiid fish, *Gillichthys mirabilis* (de Vlaming and Sundararaj, 1972). The secretion contains mucoproteins and mucopolysaccharides acid. The presence of secretory seminal vesicles enhances the fertilising capacity of males (Sundararaj and Nayyar, 1969; Nayyar and Sundararaj, 1970).

In catfish, the reproductive system consists of one to many pairs of seminal vesicle lobes, which arise posterior to the testes laterally on the sperm duct. In parallel with the maturity of testis, the seminal vesicle epithelial cells secrete a mucopolysaccharide-protein-lipid-rich fluid (Seminal Vesicle fluid, SVF), whose content increases gradually and reaches the peak level in the spawning phase (Nayyar and Sundaraj, 1970; Van den Hurk *et al*., 1987; Singh and Joy, 1999).

Different functions have been reported for the SV/SVF such as production of sialomucins which help to attach eggs to the sea grass, concentrate sperm and promote

fertilisation as in grass goby (Lahnsteiner *et al*., 1992), secretion of nutrients, steroids, enzymes, and ions, enhancing sperm quality and fertilisation of eggs and pheromonal functions as in gobies and catfish (Van den Hurk *et al*., 1987; Van den Hurk and Resink, 1992; Lahnsteiner *et al*., 1992; Singh and Joy, 1998, 1999).

2.1.4 Gonad Development and Maturation Scale.

Sexual activity of African catfish is cyclic in natural conditions (Clay, 1979). Female fish has a sexual resting time which lasts for 4 to 6 months. The period when running ripe fish remains dominant is approximately one month. Similarly, all matured male fish have a well-developed testis and can produce viable sperm in the main season. Later ratio of male capable to produce sperm goes down and in off season all the testes are in inactive stage. Size of inactive testis is small and looks brown-reddish or translucent (Figure 2.2). It is impossible to distinguish between active and inactive males by external (visual) examination. As a result, selection of males available for reproduction in out of season period is difficult.

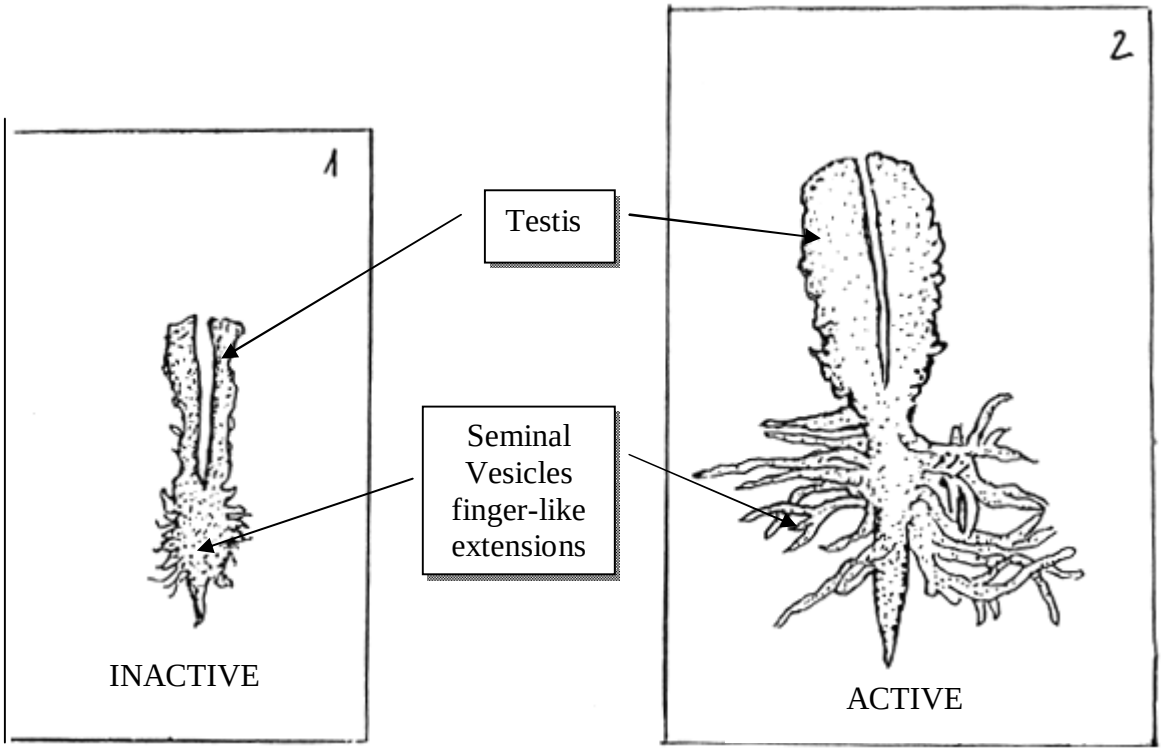

Figure 2.2: Testis of African catfish in inactive (1) and active period (2) (Janssen, 1987)

2.1.5 Seminal Vesicle and Testis Secretions

Analysis of testicular fluid (produced by Sertoli cells and epithelial cells of testicular main ducts) and seminal fluid (produced by the spermatic duct epithelium) of teleosts demonstrates considerable intra- and inter-species variability in the physical and chemical composition (Stoss, 1983; Kruger *et al*., 1984; Linhart *et al*., 1991; Suquet *et al*., 1993; Lahnsteiner *et al*., 1995, 1996; Billard *et al*., 1996; Wang and Crim, 1997).

Knowledge of seminal vesicle fluid (SVF) biochemical composition and function in catfish can introduce methods that enhance milt quality and fertilisation efficiency in artificial breeding. Table 2.1 is a compilation of data of physical and chemical characteristics of the plasma. Chowdhury *et al*. (2001) reported that on a visual comparison, the colour of the fluids varied from colourless (Seminal Vesicle Plasma, SVP) to yellowish white (Testicular Plasma, TP). SVP is more viscous than TP. Specific gravity

and osmolality are significantly higher for SVP than TP. The pH of SVP is near the neutral range, but TP is slightly acidic.

SVP has higher concentrations of Na^{+}, Ca^{2+}, Mg^{2+} and Cu^{2+}, proteins and hexosamines than TP, while the concentrations of K^{+}, Zn^{2+}, glucose, fructose, lipids and glycosidases are not significantly different from TP.

Table 2.1: Physical and chemical composition of seminal vesicles plasma (SVP) and testis plasma (TP) of *Heteropneustes fossilis* in early spawning phase (July) (Chowdhury *et al*., 2001).

Component	Seminal vesicles plasma (SVP)	Testis plasma (TP)
Specific gravity	1.06	1.02
Viscosity (centipoise)	4.61	1.90
pH	6.99	6.46
Osmolarity (mOsmol kg^{-1})	277.40	151.80
Cations (mmol l^{-1})		
Na^{+}	135.00	80.90
K^{+}	15.30	17.70
Ca^{2+}	19.80	10.71
Mg^{2+}	1.81	0.51
Cu^{2+}	0.18	0.04
Zn^{2+}	0.10	0.06
Proteins (g l^{-1})	11.50	4.90
Hexosamines (mg l^{-1})	694.68	273.17
Monosaccharides (mg l^{-1})		
Glucose	76.18	85.70
Fructose	333.33	283.36
Lipids (mg l^{-1})		
PL	112.84	201.72
FC	109.99	126.00

EC	170.72	174.96
Glycosidases (μ mol mg^{-1} protein hr^{-1})		
β-glucuronidase	7.20	7.56
β-glucosaminidase	83.00	85.39

2.2 CHARACTERISTICS OF SPERM QUALITY IN FISH

2.2.1 General Characteristics of Fish Sperm

Sperm are stored in seminal plasma fluid in the genital tract and in contrast with mammals; most externally fertilising teleosts have sperm that are immotile on ejaculation. Sperm only become motile and metabolically active after released into the water.

2.2.2 Sperm Motility Characteristics

Fish sperm show species differences in the initiation (Morisawa, 1985; Cosson *et al*., 1995), duration (Billard, 1978; Billard and Cosson, 1992) and pattern of motility (Boitano and Omoto, 1992; Ravinder *et al*., 1997). The difference in K^+ ion concentration (in salmonids) or osmotic pressure (in cyprinids, clariids and other families) between the seminal plasma and water, trigger the initiation of movement (Morisawa *et al.*, 1983; Billard, 1986).

In most freshwater species, sperm usually moves for less than 2 minutes and in many cases is only highly active for less than 30 seconds (Morisawa and Suzuki, 1980; Perchec *et al*., 1993; Billard *et al*., 1995; Kime *et al*., 2001). Some fish species such as the spotted wolffish (*Anarhichas minor*) and the 3- and the 15-spined sticklebacks (*Gasterrosteus aculeatus*, *Spinachia spinachia*), which are characterised by release of eggs in a sticky gelatinous mass, have sperm which remains motile for a far longer period after release (Elofsson *et al*., 2003; Kime and Tveiten, 2002). In these species, the sperm has

characteristics which differ markedly from that of the majority of other teleost species, the sperm is motile on stripping, remains so for 1 to 2 days and becomes immotile in contact with seawater. Similar characteristics have also been found in some marine sculpins (Koya *et al*., 1993), and the ocean pout, *Macrozoarces americanus*, a species which has internal fertilisation (Yao and Crim, 1995). The sperm of the ocean pout remain motile in seminal fluid or in specially designed milt diluent for up to 5 days at 4^{o}C without losing much activity (Yao *et al*., 1999).

Osmotic pressure seems to be the major controlling factor in cyprinids (Morisawa *et al*., 1983; Billard *et al*., 1995; Redondo-Mu¨ ller *et al*., 1991) and partly controls the motility in paddlefish (*Polyodon spathula*) sperm (Linhart *et al*., 1995). The circular trajectories of trout sperm, which become tighter with time elapsed after activation, is induced by the influx of Ca^{2+} ions (Cosson *et al*., 1989). The very short window of sperm motility (Kime *et al*., 2001) found most teleost fish (e.g. < 30 seconds in salmonids) has a critical influence on successful fertilisation, since the sperm must find and enter the micropyle during this limited period. For large eggs with diameters around 5 mm such as those of salmonids, the time of motility (< 30 seconds) allows sperm to swim less than halfway (3 to 4.9 mm) round the egg (Perchec *et al.*, 1993).

2.3 SPERM MOTILITY ANALYSIS IN FISH

2.3.1 Sperm Trackers

Computer-assisted sperm trackers comprise essentially a microscope coupled to a CCD camera which conveys a signal to a monitor, VCR recorder and computer (Boyer *et al*., 1989). Sperm movement is usually recorded onto videotape which is later analysed by the computer software. Various parameters can be measured using CASA as described in Table 2.2.

From studies using such sperm tracking systems conducted on African catfish, carp, goldfish, roach, Eurasian perch, trout, lake sturgeon, 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*., 1996a; Kime *et al*., 2001; Rurangwa *et al*., 2001, 2002; Jobling *et al*., 2002).

If the trajectory is a straight line, then VCL and VSL are identical. The angular path velocity (VAP, the velocity along a derived smoothed path) is generally of little use in most fish since, unlike mammalian sperm, the tracks are general smooth curves, so that VAP and VCL are identical.

Table 2.2: Sperm motility characteristics calculated by the computer-assisted sperm trackers (Wilson-Leedy *et al*., 2007).

Parameter	Description
Percent motility	Percent of tracked sperm identified by the plugin as exhibiting motility during the 1 second period of analysis.
Velocity curvilinear (VCL)	The total point to point distance traveled by the sperm over the time period analyzed averaged to a per second value.
Velocity average path (VAP)	Velocity over an average path, generated by a roaming average of sperm position from one-sixth of the video's frame rate, such that each point is generated by averaging the coordinates of a set number of locations on the VCL path.
Velocity straight line (VSL)	The maximum distance moved on the VAP path by the sperm from the first VAP point during the video segment analyzed, calculated to a per second value based on the number of frames for which VAP points were calculated.

Linearity (LIN)	A measure of path curvature determined by dividing VSL by VAP.
Straightness (STR)	A measure of path curvature determined by dividing VAP by VCL.

2.4 FACTORS AFFECTING SPERM QUALITY IN FISH

A considerable amount of literature has been published on factors affecting sperm quality in fish. The most critical factors are rearing photoperiod and temperature, nutrition, water and food contamination, stress, age of broodstock and breeding season, diseases of broodstock and hormonal induction and spermiation which are reviewed in the following section. The summary of main factors that can influence gamete quality in fish and main parameters that can be recorded fully characterised gamete quality (Bobe and Labbe, 2009) are shown in Figure 2.3.

2.4.1 Rearing Photoperiod and Temperature

Photoperiod manipulation is employed in aquaculture to accelerate or delay gonadal recrudescence so that fish spawn at a convenient time of the year for the aquaculturist (Nash, 1999).

In sunshine bass (*Morone chrysops* X *M. saxatilis*) exposed to shifted photothermal cycles (6 to 12 months), sperm concentration, duration of motility and seminal fluid pH differed among males on the different cycles, but these differences produced no changes in fertilities (Tate and Helfrich, 1998).

In wolffish (*A. minor*), however, there was no difference in volume of ejaculate or sperm concentration between males kept under two different light cycles (18D/6L and 6D/18L) (Pavlov *et al*., 1997) and in goldfish, photoperiod manipulation did not affect

sperm production (Iigo and Aida, 1995). Labbe´ and Maisse (1996) found that the ability of rainbow trout (*Onchorhynchus mykiss*) sperm to withstand cryopreservation was improved by rearing at high temperature during gametogenesis followed by transfer to colder water.

2.4.2 Nutrition

Broodstock nutrition is an important factor susceptible to affect only fecundity and gametogenesis but also gamete quality, and existing work has been extensively reviewed (Kjorsvik *et al*., 1990; Brooks *et al*., 1997; Izquierdo *et al*., 2001). Improvement in broodstock nutrition and feeding greatly improves gamete quality and seed production (Izquierdo *et al*., 2001).

Polyunsaturated fatty acid (PUFAs)-enrichment of commercial diets enhances reproductive performance of male sea bass (*Dicentrarchus labrax*) (Astuarino *et al*., 2001). Sea bass fed commercial pelleted diet enriched with fish oil had a longer spermiation period, higher milt volumes and sperm concentration and higher survival of embryos and larvae after fertilisation when compared to those fed a non-enriched wet diet. In rainbow trout, dietary lipids alter the composition but not the fluidity of the sperm plasma membrane and increase their fertilisation capacity (Labbe´ *et al*., 1995).

The importance of dietary ascorbic acid (Vitamin C) on male fish fertility has been demonstrated in rainbow trout (Ciereszko *et al*., 1996b). 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 (Ciereszko and Dabrowski, 1995).

Feeding rainbow trout with gossypol (a naturally occurring compound in cotton seeds) did not affect sperm motility and fertilising ability although testosterone (T) and 11-

ketotestosterone (11-KT) levels were elevated in some experimental groups (Dabrowski *et al*., 2000, 2001). However, in male lamprey (*Petromyzon marinus*) injected with gossypol acetic acid, sperm motility was reduced (Rinchard *et al*., 2000) and *in vitro* sperm assays confirmed its toxicity to perch sperm (Ciereszko and Dabrowski, 2000). More importantly, gossypol was recently found to be transferred to the eggs in trout which may lead to reduced reproductive performance in female rainbow trout although it had no effect on fish growth and mortality (Blom *et al*., 2001).

2.4.3 Water and Food Contamination

Exposure to environmental toxicants or hormones can affect reproduction in general, leading to decreased sperm quality. For instance, juvenile black porgy (*Acanthopagrus schlegeli*) fed a diet containing 4 mg / kg oestradiol-17β had suppressed spermiation after 7 months of exposure (Chang *et al*., 1995).

Oestrogenic substances such as 17a-ethynyloestradiol and genistein are sufficiently potent to produce sex-reversed male fish and masculinisation (Kwon *et al*., 2000). In genistein-fed rainbow trout, sperm motility and concentration were decreased in a dose-dependent manner at spawning (Bennetau-Pelissero *et al*., 2001).

2.4.4 Stress

The quality of fish gametes depends on the appropriate hormonal environment during development but this may be disturbed by stress (Kime and Nash, 1999).

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 (testosterone and 11-ketotestosterone) (Kubokawa *et al*., 1999).

Stress may also act by inducing changes in plasma osmolarity which in turn can affect sperm quality in fish. As an example, white bass *M. chrysops* transported for 5 hours in freshwater had reduced seminal fluid osmolalities and motility at activation (10 to 25% motile cells in 38% of sperm samples) (Allyn *et al*., 2001). In striped and white bass, male brood stock captured from the wild during the spawning season and moved to captivity produce milt with non-motile sperm (Berlinsky *et al*., 1997).

In rainbow trout, repeated acute stress during reproductive development prior to spawning significantly delayed ovulation and reduced egg size, and significantly decreased sperm counts and most importantly significantly decreased survival rates for progeny from stressed fish compared to that from unstressed controls (Campbell *et al*., 1992). Since many of the handling and transportation procedures used in aquaculture can be potentially stressful, quantitative evaluation of the effects of such procedures on sperm quality could facilitate changes in the conditions employed so that stress is minimised and sperm quality is not affected.

2.4.5 Age of Broodstock and Breeding Season

The age of broodstock has a significant influence on the sperm quality and may affect the success of storing sperm (Vuthiphandchai and Zohar, 1999).

In captive-reared striped bass (*Morone saxatilis*), 3-year-old fish had higher sperm quality than the 1- or 12-month-old fish, based on higher sperm production and increased sperm longevity during short-term storage. However, the fertilising capacity of virgin and repeat spawners was comparable in Atlantic cod, *G. morhua* (Trippel and Neilson, 1992).

In fish species with an annual reproductive cycle, the quality of sperm varies across the spawning season and the mating frequency. In the three-spined stickleback, *G. 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). In the common carp (*Cyprinus carpio*), computer-assisted semen analyzer (CASA) has shown that sperm production and quality can be lower at the beginning and end of the breeding season (Christ *et al*., 1996). Similar decreases in sperm quality were also observed in turbot, *P. maxima* (Suquet *et al*., 1998).

2.4.6 Diseases of Broodstock

Diseases can affect the sperm quality in many fish. For instance, the cestode, *Ligula intestinalis* (L.), a common parasite of cyprinid fishes, may affect fish gamete production by preventing gonad development. Infectious Pancreatic Necrosis (IPN) virus has been reported to attach to sperm cells of farmed rainbow trout (Rodriguez *et al*., 1993) which could affect sperm quality, although no confirmatory or experimental data is available yet.

2.4.7 Hormonal Induction of Spermiation

Many farmed fish species do not spawn readily in captivity and hormonal treatments are necessary to either induce ovulation /spermiation or to synchronise gamete release of the two sexes at a time convenient for the fish farm (Zohar and Mylonas, 2001).

However, this practice is known to increase the fluidity of the milt (low concentration of sperm) in plaice, *Pleuronectes platessa* (Vermeirssen *et al*., 1998), winter flounder, *P. americanus* (Shangguan and Crim, 1999) and Atlantic halibut, *H. hippoglossus* (Vermeirssen *et al*., 2000).

Artificial induction of spermiation can also affect the responsiveness of male fish. In European catfish (*Silurus glanis*), the total number of sperm collected was significantly higher when carp pituitary extract was injected than when GnRH analogue implants were used to artificially induce spermiation (Linhart and Billard, 1994). In common carp (*C.*

carpio), oral and intraperitoneal administration of salmon gonadotrophin hormone-releasing hormone analogue (sGnRH-a) and Pimozide (Pim) induced gonadotrophin II (GtH II) release and milt production significantly (Roelants *et al.*, 2000).

Sperm production, milt volume, sperm motility and seminal plasma pH were increased by GnRHa treatment in yellowtail flounder, *P. ferrugineus* (Clearwater and Crim, 1998). In captive white bass (*M. chrysops*) treated with GnRHa during the spermiation period, GtH II levels and milt production increased (Mylonas *et al*., 1997). Increased milt volume and prolonged spermiation were also observed in sea bass (*D. labrax*) administered GnRHa (Sorbera *et al*., 1996). GnRHa-microspheres increased significantly sperm production in Atlantic salmon, *S. salar* and stripped bass, *M. saxatilis* (Mylonas *et al*., 1995).

In male goldfish, the oocyte maturation-inducing steroid 17,20h-dihydroxy-4-pregnen- 3-one (17,20hP) also functions by release into the water as a pheromone that increases male serum GtH-II concentration, milt volume, duration of sperm motility, proportion of motile sperm and sexual activity and paternity in multi-male spawnings (Zheng *et al*., 1997). Mature male goldfish placed with either a receptive female or stimulus pairs of spawning goldfish had sperm volumes greater than those of males kept in all-male groups (Kyle *et al*., 1985).

Similar stimulation of spermiation in males by ovulating females was noticed in carp in earthen ponds (Billard *et al*., 1989). The increase in milt production in pair-spawners may be due to both neurally and hormonally mediated events that ensure milt availability for imminent spawning activity. In natural populations of a coral reef fish, the bluehead wrasse (*Thalassoma bifasciatum*), males with the higher spawning frequency produced fewer sperm per mating indicating a trade off between spawning frequency and sperm volume, and an ability to vary the amount of sperm produced (Warner *et al*., 1995).

In carp, the osmolality of seminal plasma and the capacity of sperm to move are highly variable after hormonal injection (Redondo-Mu¨ ller *et al*., 1991). The time lapse between hypophysation and the moment at which the initial quality of sperm begins to decline may vary according to species. As an example, in Siberian sturgeon, *A. baeri*, a delay of 36 hours after stimulation before milt collection clearly provided the most motile sperm as compared with shorter (24 hours) or longer (48, 60 hours) delays (Williot *et al*., 2000). Time schedules for hormonal injection should therefore take this into account. Assessment of sperm quality could therefore be used to optimise the hormonal dosage, and its timing, or the proportions of males in the holding tanks.

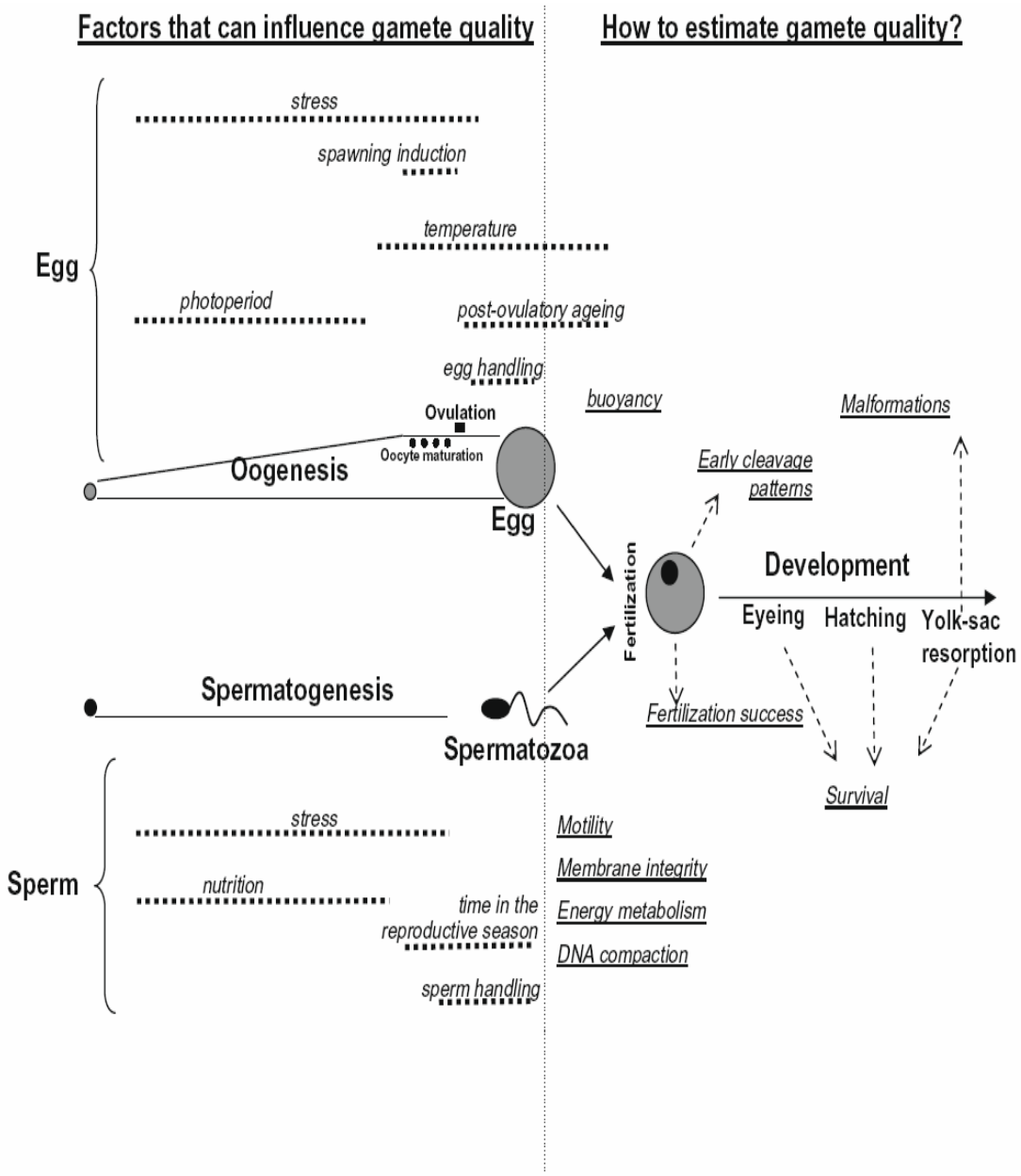

Figure 2.3: Summary of main factors that can influence gamete quality in fish and main parameters that can be recorded fully characterised gamete quality (Bobe and Labbe, 2009).

2.5 DEVELOPMENT OF SPERM CRYOPRESERVATION PROTOCOLS

Investigation of the extender, the cryoprotectant as well as the cooling and thawing conditions are critical factors in order to establish a cryopreservation protocol as all parameters may interact with each other.

2.5.1 Extender

Extender is a cryopreservation diluent, which purpose is to supply the sperm cells with sources of energy, protect the cells from temperature-related damage, and maintain a suitable environment for the sperm to survive while cryopreserved. The extender used for cryopreservation of semen contains cryoprotectants agents (such as glycerol and egg yolk), substances to maintain the osmolarity, energy source (such as glucose and fructose), and enzymes as well as antibiotics that are essential for maintaining the viability of the sperm during cooling, freezing and thawing (Holt, 2000; Vishwanath and Shannon, 2000).

An ideal sperm cryopreservation medium consists of a non-penetrating cryoprotectant (for example milk and egg yolk), a penetrating cryoprotectant (for examples glycerol, ethylene glycol or dimethyl sulfoxide), a buffer (for example, Tris or Test), one or more sugars (for examples, glucose, lactose or sucrose), salts (for examples, sodium citrate or citric acid) and antibiotics (for examples, Penicillin or Streptomycin) (Evans and Maxwell, 1987). The compositions of the most successful extenders used in marine and freshwater fish are reported in Table 2.4 and Table 2.5.

Nutrients and buffer are the two ingredients that an ideal extender should have as nutrients acts as an energy source and buffer prevents harmful changes of pH by maintaining a physical osmotic pressure and concentration of electrolytes which can inhibit bacterial growth and protects the cells from cold shock during the freezing and thawing processes (Concannon and Battista, 1989).

Aberrations of the normal sperm morphology have been observed due to cryopreservation. The plasma membrane is one of the main structures affected by cryopreservation. Damage to plasma membranes has been observed when sperm were exposed to cryoprotectants before freezing (Lahnsteiner *et al*., 1992; Tadei *et al*., 2001) with a greater percentage of sperm losing their integrity and normal function of their plasma membranes during the freezing or thawing processes.

Another important factor to be considered in cryopreservation is dilution ratios of sperm in extender. It ranges from 1:1 to 1:20 (volume of semen: volume of diluent). Lower survival of frozen-thawed sperm was recorded for dilution ratios larger than 1:20 in Atlantic croaker (Gwo *et al*., 1991) and larger than 1:50 in seabream (Chambeyron and Zohar, 1990). The motility duration of black grouper sperm decreased from 40 to 2 minutes when increasing the semen dilution ratio from 1:10 to 1:100 (Gwo, 1993). Increasing the dilution rate from 1:1 up to 1:9 did not modify the percentage of motile frozen-thawed turbot sperm (Dreanno *et al*., 1997). It is suggested that seminal plasma proteins protect sperm viability and higher dilution ratios than 1:10 may reduce this effect. This was observed in freshwater fish species (Billard, 1983) and in turbot (Chauvaud *et al*., 1995).

Table 2.3: Composition of the extenders successfully used for freezing sperm of marine fish species.

Species	Extender composition	References
Plaice	NaCl	Pullin (1972)
Grey mullet	Ringer solution for marine fish	Chao *et al*. (1975)
Cod	Sucrose, reduced glutathione, $KHCO_3$	Mounib (1978)
Grouper	NaCl, $NaHCO_3$, fructose, lecithin,mannitol	Withler and Lim (1982)
Atlantic halibut	NaCl-Glycine-$NaHCO_3$	Bolla *et al*. (1987)
Baramundi	Ringer solution for freshwater fish	Leung (1987)
Atlantic croaker	NaCl, Glucose or sucrose	Gwo *et al*. (1991)
Black grouper	NaCl	Gwo (1993)
Mullet	Ringer for marine fish	Joseph and Rao (1993)
Puffer	Glucose	Gwo *et al*. (1993)
Pacific herring	Ringer for marine fish	Pillai *et al*. (1994)
Ocean pout	Medium mimicking seminal fluid	Yao *et al*. (1995)
Sea bream	NaCl	Barbato *et al*. (1996)
Hirame	Ringer for freshwater fish	Tabata and Mizuta (1997)
Turbot	Sucrose, reduced glutathione, $KHCO_3$	Dreanno *et al*. (1997)
Sea bass	Sucrose, reduced glutathione, $KHCO_3$	Fauvel *et al*. (1998)

Table 2.4: Composition of extenders successfully used for freezing sperm in freshwater fish species.

Species	Extender composition	References
R.quelen	V2e (NaCl, $NaHCO_3$, KCl, glucose, egg yolk)	Fogli da Silveira *et al*. (1985)
Sharptooth catfish (*Clarias gariepinus*)	Ginzburg fish ringer	Viveiros *et al*. (2000)
L.macrocephalus	Glucose + Egg yolk	Ribeiro and Godinho (2003)
P.corruscans	Glucose + milk powder	Carolsfeld *et al*. (2003)
B.insignis	Glucose + Egg yolk	Shimoda (2004)
Tropical bagrid catfish (*Mystus nemurus*)	Ringer	Muchlisin *et al*. (2004)
European catfish (*Silurus glanis*)	Immobilising solution (NaCl, Tris-HCl)	Linhart *et al*. (2005)
Piracanjuba (*Brycon orbignyanus*)	Beltsville Thawing Solution (BTS^{TM}) and Merck III ($MIII^{TM}$)	Maria *et al*. (2006a)
B.orthotaemia	Glucose + Egg yolk	Melo and Godinho (2006)
B.natterreri	BTS^{TM} or 0.9% NaCl	Oliveira *et al*. (2007)
Yellow catfish (*Pelteobagrus fulvidraco*)	Ringer extender	Pan *et al*. (2008)

2.5.2 Cryoprotectant

The multiple roles of cryoprotectant during the cooling process were viewed by (Jamieson, 1991). Cryoprotectants are classified as permeating and non-permeating, according to their ability to pass through the cell membrane.

Permeating cryoprotectants such as ethylene, propylene glycol, glycerol, DMSO and methanol were tested for cryopreservation of sperm of marine fish. The effects of cryoprotectants and cryopreservation procedures on the cellular structure of sperm have been intensively studied. DMSO generally gave the best results and its success can be explained by the fast penetration into sperm and by its interaction with the phospholipids of the sperm membrane (Ogier de Baulny *et al*., 1996). Flow cytometric analysis revealed a high percentage of turbot sperm presenting no cryo-injuries of the plasma membrane and mitochondria in the presence of DMSO (Ogier de Baulny, 1997). However, DMSO is toxic at high concentrations, for instance the motility duration of frozen-thawed barramundi (*Lates calcarifer*, Bloch) sperm was reduced when DMSO concentration was higher than 5% (Leung, 1987) and also in the black grouper; sperm motility was decreased at a concentration of 30% (Gwo, 1993).

Further studies found methanol to be the best among five tested cryoprotectants for channel catfish sperm, and reported the first use of thawed sperm for production of channel catfish by artificial fertilisation (Tiersch *et al*., 1994). Increased exposure to cryoprotectants can improve cryoprotective effect, but can also result in increased toxicity to the sperm cells (Jamieson, 1991; Christensen and Tiersch, 1997).

In freshwater fish, non-penetrating cryoprotectants such as proteins (BSA) or lipoproteins (egg yolk) have been commonly used to prevent damages to the plasma membrane (Scott and Baynes, 1980). Cabrita *et al*., (1998) suggested that they increase the membrane resistance to osmotic stress and the motility rate of frozen-thawed rainbow trout

sperm. Egg yolk has been identified as having beneficial effects on the cooling and freezing of the male gamete (Bwanga, 1991). Many studies have considered the effect of egg yolk or the addition of given lipid moieties on sperm during cryopreservation. Table 2.6 demonstrates literature review on semen cryopreservation of African catfish and related species.

Table 2.5: Literature review on semen cryopreservation of African catfish and related species.

Catfish species	Extender	Cryoprotectant	Container	Freezing rates	Hatching Rates (%)	References
Clarias gariepinus	Glucose (5 %)	Glycerol (5 %)	Straw or bio-freeze vial	-7^{o}C / min to -65^{o}C; LN_2	Not measured	Steyn *et al*. (1985)
	Glucose (5%)	Glycerol (11 %)	1 ml cryo tube	-11^{o}C / min to -70^{o}C; LN_2	Frozen : 51% Control : 51%	Steyn *et al*. (1987)
	Glucose (4%)	Glycerol (9%)	1 ml cryo tube	-5 and -11^{o}C / min to -70^{o}C; LN_2	Not measured	Steyn (1993)
	Glucose (4%)	Glycerol (9%)	1 ml cryo tube	5 ^{o}C / min to -70^{o}C; LN_2	Not measured	Van der Walt *et al.* (1993)
	Fructose (333 mmol L^{-1})	DMSO (10%)	250 µL straw	-11^{o}C / min to -80^{o}C; LN_2	Not measured	Urbanyi *et al*. (1999)
Clarias batrachus	NaCl (0.6%)	Glycerol (10%)	1.5 ml tube	Directly to -70^{o}C; stored at -70^{o}C	75% of control	Padhi *et al*. (1995)
Heterobranchus longifilis	Mounib solution	DMSO (5%) + glycerol (5%) + egg yolk (10%)	5 ml straw	20 min at 3 cm above LN_2 level; LN_2	Frozen : 79% Control : 81%	Oteme *et al*. (1996)

2.5.3 Equilibration Duration

Equilibration duration is a duration for the sperm and extender to mix and to allow reduction of temperature slowly (slow cooling) in order to prepare for the freezing processes. Cold shock during freezing can be avoided by having equilibration duration.

Because sperm are small, the penetration of cryoprotectants is rapid (Jamieson, 1991), and no equilibration period is required. Therefore, the toxic effect of DMSO can be minimised. Increasing the equilibration period from 5 to 60 minutes and the DMSO concentration from 10 to 30% lowered the post-thaw motility of yellowfin seabream sperm (Gwo, 1994). In seabream, the fertilising ability of frozen-thawed sperm decreased in DMSO extender when the equilibration period exceeded 2 minutes (Billard, 1978). A similar effect was observed after one hour in grey mullet (Chao *et al*., 1975).

Equilibration duration of 10 to 60 minutes had no effect on the fertility of frozen-thawed sperm of hirame (Tabata and Mizuta, 1997). In rainbow trout, Ogier de Baulny (1997) observed that DMSO needed 10 minutes to penetrate into sperm although the fertilistion capacity of frozen-thawed sperm was the same with or without equilibration period. This observation suggests that the protective role of DMSO does not depend on its penetration into sperm.

With glycerol as a cryoprotectant, the motility rate of frozen-thawed sperm of bluefin tuna (*Thunnus thynnus* L.) was increased at long equilibration periods (30 minutes) compared to short ones (10 minutes), but the opposite was recorded for DMSO (Doi *et al*., 1982). Since penetration of glycerol is slow, equilibration duration may be necessary.

2.5.4 Cooling Rate

Cooling rate is the rate of gradually decreasing a temperature during the cryopreservation process. Cooling rate is an important variable in cryopreservation, and single-stage and

multiple-stage procedures have been developed (Leung and Jamieson, 1991; Rall, 1993). The cooling rate is determined by the height of the tray or the depth at which canisters are placed.

Cooling rate is varied among species. For fish sperm, optimal rates reported vary from 5 to 45^{o}C / min for cooling from 5 to 80^{o}C, but some species show high post-thawed motility with a combination of different cooling rates (Rana and Gilmour, 1996; Sansone *et al*., 2002).

Best cryopreservation of sperm in African sharptooth catfish, *Clarias gariepinus*, were obtained using a two-step cooling regime, including a cooling rate of 5^{o}C / min (Steyn, 1993), whereas, in channel catfish, a cooling rate of 45^{o}C / min yielded higher post-thaw motility than did 3^{o}C / min (Christensen and Tiersch, 2005). A cooling rate from 5 to 11^{o}C / min was specified as optimal in cryopreservation of European catfish (*Silurus glanis*) (Linhart *et al*., 2005). The final temperature and its duration just before plunging the frozen sperm into liquid nitrogen were very important.

A critical temperature zone (between -15 and -30^{o}C) is responsible for exerting most of the damage to sperm and if cooling rates were not optimal, all the cells might be damaged by -80^{o}C (Polge, 1957). Besides that, cooling rate also can affect the rate of osmosis, diffusion, and formation of ice crystals within a cell (Morris, 1981).

2.5.5 Thawing Rate

Rapid thawing is necessary to avoid recrystallisation. Thawing rates used in marine fish are shown in Table 2.7 and are lower than those reported for freshwater fish (30 to 80^{o}C: Rana, 1995), as examples in Brazilian freshwater fish in Table 2.8. In channel catfish, a thawing temperature of 50^{o}C within durations of 5 or 10 seconds or a temperature of 40^{o}C with duration of 10 seconds performed the best (Christensen and Tiersch, 2005).

Thawed sperm must be rapidly used within 60 minutes after thawing, the percentage of motile turbot sperm stored on crushed ice decreased for 35% (Dreanno *et al*., 1997). This indicates that cryopreservation induced damages in sperm. Diluting frozen-thawed sperm of this species in a medium mimicking the seminal fluid improved their short term storage capacity. Also, in halibut, short term storage ability of thawed sperm was lower than for fresh sperm (Billard, Cosson and Crim, 1993).

Table 2.6: Thawing rates used in marine fish species.

Species	Thawing rate (^{o}C min^{-1})	References
Cod	38	Mounib (1978)
Bluefin tuna	40	Doi *et al*. (1982)
Grouper	25	Withler and Lim (1982)
Atlantic halibut	10-40	Bolla *et al*. (1987)
Barramundi	30	Leung (1987)
Yellowfin bream	20	Thorogood and Blackshaw (1992)
Puffer	25	Gwo *et al*. (1993)
Ocean pout	1	Yao *et al*. (1995)
Yellowtail flounder	30	Richardson *et al*. (1995)
Sea bream	26	Barbato *et al*. (1996)
Hirame	20	Tabata and Mizuta (1997)
Turbot	30	Dreanno *et al*. (1997)
Sea bass	35	Fauvel *et al*. (1998)

Table 2.7: Thawing rates used in Brazilian freshwater fish species (Viveiros and Godinho, 2009)

Species	Container	Freezing	Thawing	References
B.orbignyanus	0.5 ml straw 0.5 ml straw 0.5 ml straw 0.5 ml straw	Dry-shipper Dry-shipper Dry-shipper Dry-shipper	37-50^{o}C for 10 s 60^{o}C for 5 s 50^{o}C for 5 s 60^{o}C for 8 s	Bedore (1999) Murgas *et al.* (2001) Murgas *et al*. (2003) Maria *et al*. (2006a, b); Viveiros *et al*. (2007)
L.macrocephalus	0.5 ml straw	Dry-shipper	30oC for 6 s	Ribeiro and Godinho (2003)
B.insignis	0.5 ml straw	Dry-shipper	30^{o}C for 7 s	Shimoda (2004)
B.orthotaemia	0.5 ml straw	Dry-shipper	35^{o}C for 7-10 s	Melo and Godinho (2006)
B.amazonicus	0.5 ml straw 0.5 or 4 ml straw 0.5, 1.8, 2.5 ml straw 4 ml straw	1 cm above LN_2 surface 1 cm above LN_2 surface Dry-shipper Dry-shipper	35^{o}C for 60 s 36^{o}C for 10-30 s 35^{o}C 36^{o}C for 10 s	Cruz-Casallas *et al.* (2006) Ninhaus-Silveira *et a*l. (2006b) Velasco-Santamaria *et al*. (2006)
B.nattereri	0.25 or 0.5 ml straw	Dry-shipper	60^{o}C or 50^{o}C for 8 s	Oliveira *et al*. (2007)
L.obtusidens	0.5 ml straw 0.5 ml straw 0.5 ml straw	Dry-shipper Dry-shipper Dry-shipper	60oC for 8 s 33^{o}C for 14 s 35-37^{o}C for 10 s	Viveiros *et al.* (2007) ; Koch *et al.* (2007) Carvalho (2007) Taitso *et al.* (2007)

2.6 SIGNIFICANT MILESTONES OF FISH SPERM CRYOPRESERVATION

Table 2.9: Timelines for significant findings in fish sperm cryopreservation

Year	Author	Significant finding
1995	Monkonpunya *et al.*	Cryopreservation of Mekong giant catfish sperm (*Pangasius gigas*) – Best results were obtained when sperm were cryopreserved in 9% DMSO in either extender [bicarbonate buffer (BCB) or calcium-free Hanks' balanced salt solution (C-F HBSS)] in 5 ml cryotubes and frozen at -12^{o}C/ min. The percentage of fertilisation was 65 to 66% (actual) and 73 to 74% (control).
1999	Ogier de Baulny *et al.*	Best protection condition for sperm cryopreservation in European catfish, *Silurus glanis* was dimethylacetamide (10 and 15%) in sucrose solution. The percentage of cells with an intact membrane (90%), and the protection of the activity of the mitochondria was (47%). Addition of dimethylacetamide (DMA) increased ATP content of the sperm.
2000	Lahnsteiner *et al.*	Cryopreservation of sperm in cyprinid fishes –The optimal sperm equilibration period in extender was ≤ 5 min. The frozen-thawed sperm obtained 35 to 65% and 5 to 25% locally motile sperm depending on the quality of the fresh semen.
	Viveiros *et al.*	Sperm cryopreservation of African catfish, *Clarias gariepinus* – 5 to 25% DMSO and methanol tested as cryoprotectants, by diluting in Ginzburg fish ringer. Highest hatching rates obtained by sperm frozen in 10% methanol and post-thaw diluted to 1:200.
2001	Babiak *et al.*	Multifactorial effect of extender constituents on sperm resistance in rainbow trout against injuries.
	Rurangwa *et al.*	Mounib's extender provided the best cryoprotection to the sperm for all post-thawed sperm quality measurements and at all freezing durations in African catfish (*Clarias gariepinus*).

	Taddei *et al.*	Cryopreserved sperm of *Diplodus puntazzo* – subdivides the cryopreservation procedure into three phases, fresh, prefreezing (samples equilibrated in cryosolutions) and post-thawed stages, and examines the ultrastructural anomalies and motility profiles of sperm in each stage with different cryodiluents. In Cryosolution A (0.17 M NaCl + 15% DMSO), during the prefreezing phase, the plasmalemma of 61% of the cells was absent or damaged as compared with 24% in the fresh sample. In cryosolution B (0.1 M sodium citrate + 10% DMSO), the number of cells lacking the head plasmatic membrane increased from the prefreezing to the post-thawed stages (32 to 52%).
	Viveiros *et al.*	Tested two step freezing protocols for the African catfish sperm (*Clarias gariepinus*) with difference cooling rates (-2, -5 and -10^{o}C/min) and different temperatures at plunging into LN_2. Slow cooling rates of -2 to -5^{o}C/min, hatching rates can be maximized by plunging as soon as T_{semen} reaches -38^{o}C. A simple and efficient protocol can be obtained by cooling at a rate of -5 to -10^{o}C/min combined with a 5 min holding period in the freezer at -40^{o}C.
2002	Basavaraja *et al.*	First report on the successful production of viable fry of Decan mahseer (*Tor khudree*) from cryopreserved sperm. *T.khudree* can be successfully cryopreserved using fish Ringer and 5 to 15% DMSO at an equilibration time of 10 to 90 min.
	Lahnsteiner *et al.*	The cryopreservation of sperm of the burbot, *Lota lota* (Gadidae, Teleostei) – The highest motility rate (46.6±8.0%, fresh semen control 86.5±8.2%) and fertility (78.1±2.7% embryo survival in hatching stage, fresh semen control 82.2±2.9%) when 10% methanol, 1.5% glucose and 7% hen egg were used as cryoprotectants.
2003	Lahnsteiner *et al.*	Investigate various fertilisation techniques and media, straw volumes as well as optimal semen volume for cryopreservation of cyprinid – The highest fertilisation rates obtained with sperm to egg ratios of (1.3 to 2.5) x 10^6 : 1 and were 77 to 92% of fresh semen control.

2004	Huang *et al.* (a)	First evidence of conservation genetic resources in live-bearing fishes (*Xiphophorus helleri*). Osmolality of HBSS without cryoprotectant in which the highest motility (67%) was 320±3 mOsm/kg. When cryopreserved with 10% DMSO, the highest motilities within 10 min after thawing were 240 to 300 mOsm/kg. Sperm suspended in HBSS at 320 mOsm/kg with a dilution factor 100 maintained motility for 24 hours at room temperature and persisted for 10 days when stored at 4^{o}C.
	Huang *et al.* (b)	Sperm cryopreservation of a live-bearing fish, the platyfish (*Xiphophorus couchianus*) – The highest average sperm motility (78±3%) at 10 minutes after thawing was obtained in HBSS at 300 mOsm/kg with 14% glycerol, diluted at a ratio of sperm to HBSS-glycerol (1:20), equilibrated for 10 minutes, cooled at 25^{o}C/minutes from 5 to -80^{o}C before plunging into LN_2, and thawed at 40^{o}C in waterbath for 7 seconds.
2005	Cabrita *et al.*	In rainbow trout, the averages of fragmented DNA and olive tail moment after cryopreservation (11.19 to 30.29% tail DNA and 13.4 to 53.48% Olive tail moment in fresh and cryopreserved sperm, respectively). In gilthead sea there was no significant differences in the percentage of tail DNA between the control samples and sperm diluted 1:6 and cryopreserved (28.23 and 31.3% DNA, respectively).
	Christensen *et al.*	Cryopreservation of channel catfish (*Ictalurus punctatus*) – A cooling rate of 45^{o}C/minutes resulted in lower motility reduction (33±9%) than a rate of 3^{o}C/minutes (83±13%). A thawing temperature of 50^{o}C resulted in lower motility reduction (25±14%) than 30^{o}C (51±21%) or 40^{o}C (59±11%). A thawing duration of 10 seconds resulted in lower motility reduction (38±12%) than a duration of 5 seconds (52±12%). A 5% methanol resulted in lower motility reduction (43±17%) than 10% methanol (67±14%).

	Gwo *et al.*	Development of cryopreservation procedures for semen of Pacific bluefin tuna, *Thunnus orientalis* – sperm suspended in glycerol showed a gradual increase in motility during the first 20 seconds after thawing and motility was reduced later after 480 seconds.
	Horvath *et al.*	Cryopreservation of two North-American sturgeon species (*Acipenser brevirostrum* and *Scaphyrinchus albus*) – the highest post-thaw motility was found using 5% DMSO (26±13%) while the use of 5% methanol resulted in the highest rates for fertilisation at the 4-cell stage (40±15%), neurulation (38±13%) and hatching (32±12%).
	Miskolczi *et al.*	Examination of larval malformations in African catfish, (*Clarias gariepinus*), following fertilisation with cryopreserved sperm – Some of the malformed larvae hatched from eggs fertilised with cryopreserved sperm were haploids. Haploids occurred only when 0.25 or 0.5 ml straws were used for freezing.
	Thirumala *et al.*	The optimal rate of cooling for *X. helleri* sperm cells in the presence of CPAs ranged from 20 to 35^{o}C/minutes.
2006	Dong *et al.*	Cryopreservation of the green swordtail (*Xiphophorus helleri*) – No adverse effects on sperm motility of fresh and cryopreserved samples with centrifugation at 1000x*g* for 10 minutes at 4^{o}C the presence of glucose in HBSS yielded higher and longer motility for fresh and thawed samples. Addition of 20% FBS prior to freezing increased the post-thawed motility.
	Maria *et al.* (a)	Piracanjuba (*Brycon orbignyanus*) semen diluted (1:10 total volume) in NaCl 200 mM or in Saad solution (NaCl 200 mM, Tris 30 mM) maintained motility above 35% for as long as 7 days, at 4^{o}C. Motility of only 7% was observed on undiluted semen after 3 days at 4^{o}C. Methylglycol was the most effective cryoprotector compared to DMSO and methanol.

	Melo *et al.*	Successful developed protocol of sperm cryopreservation of matrinxa (*Brycon orthotaenia*). Obtained the highest post-thaw motility rate and duration of sperm motility (70.5±11.4% and 62.2±8.5 seconds, respectively) using DMSO and thawed sperm were activated with 119 mM $NaHCO_3$.
	Routray *et al*.	Cryopreservation of dead fish sperm of Indian major carp (*Labeo rohita*) – The best stored condition was shown by sperm collected 8 hours of fish death and maintained at 0^oC which gave 30% larval survival.
	Yang *et al.*	Sperm was not motile when the osmolality was lower than 116 or higher than 425 mOsmol/ kg. High motility (~55%) was obtained in sperm after thawing when cryopreserved with 10 to 15% glycerol, and dilution of thawed sperm in fresh HBSS (1:4; v:v) was found to be decreased the motility significantly.
2007	Dong *et al.*	Sperm agglutination in oysters – standardise sperm concentration through research of sperm agglutination in pacific oysters (*Crassostrea gigas*). First detailed report addressing the sperm agglutination phenomenon of thawed samples from any aquatic organism.
	Horvath *et al.*	Cryopreservation of common carp (*Cyprinus carpio*) sperm – test the suitability of using 1.2 and 5 ml straws and investigate the ploidy of malformed larvae among the hatched progeny. The highest hatch rate for 1.2 ml straws was (69±16%) at freezing time of 4 minutes, and (39±27%) for 5 ml straws at 5 minutes.
	Yang *et al*.	Sperm cryopreservation in zebrafish (*Danio rerio*) – The highest motility (35±23%) and fertility (13±8%) in thawed sperm obtained with the combination of 8% methanol and cooling rate of 10^oC/minutes.
2008	Daly *et al*.	Cryopreservation of sperm from Murray cod (*Maccullochella peelii peelii*) - First successful cryopreservation of sperm from Murray cod. A cryopreservation diluent composed of 300 mOsm kg-1 D-sorbitol (DS) solution with 10% methanol produced the best post-thawed motility (51.4±3.4%), followed by Tris-Sucrose-Potassium (TSK) solution

		with 10% methanol and Modified Kurokuras medium with 10% methanol (27.5±4.8%). Fertilisation trials using sperm frozen in DS solution with 10% methanol produced a hatching rate of 11.0% (63.1±18.23% of a control of fresh sperm hatching rate) and 6.4% (58.5±32.50% of fresh sperm hatching rate) using sperm frozen with TSK with 10% methanol.
	Francois *et al.*	The presence of antifreeze proteins in the seminal fluid of the Atlantic wolfish (*Anarhichas lupus*), facilitate the process of cryoconservation in association with increased post-thawed motility and fertilisation rates.
	Pan *et al.*	Development of cryopreservation for maintaining yellow catfish, *Pelteobagrus fulvidraco,* sperm - Ringer extender and 10% methanol was the best combination maintained the highest post-thaw motility (65.00±5.00%), fertilisation (90.47±3.67%) and hatching rate (88±4%).The fertilisation and hatching rate was similar to those of fresh sperm (97.55±2.74% and 92±5%).
	Shuyan *et al.*	First report on Mandarin fish (*Siniperca chuatsi*) sperm can be successfully fertilised eggs after long-term cryopreservation.
	Tian *et al*.	Cryopreservation of spotted halibut (*Verasper variegatus*) sperm –cryopreserved spotted halibut semen with extender TS-2 and 13.3% DMSO or 13.3% PG produced fertilisation rate (34.52±10.92%) and hatching rate (25.53 ±11.80%).
	Viveiros *et al.*	Cryopreservation of curimba (*Prochilodus lineatus*) semen - successful cryopreserved in 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%.
	Xiao *et al.*	At concentration of 15% propylene glycol (PG) and 30 minutes exposure, the hatching rate of the embryos was (93.3±7.0%). However, in DMSO, EG, glycerol and methanol, the hatching rate was 82.7±10.4, 22.0±5.7, 0.0±0.0, and 0.0±0.0%, respectively.

2009	Jing *et al.*	In zebrafish sperm, higher percent motility is obtained when collected through dissecting without crushing (89±3%) or abdominal massage (90±4%) than dissecting with crushing (65±13%).The total number of motile sperm was higher for dissecting without crushing (147.0±102.3x10^5/male) than abdominal massage (7.1±11.93x10^5/male).
	Martinez-Paramo *et al*.	Cryobanking as tool for conservation of biodiversity- No reduction in sperm viability after freezing (87.0±3.32% to 77.9±3.59% and 77.6±6.53% to 11.5±2.50% in the Esla and Duerna basins, respectively.
	Matteo *et al.*	Cropreservation of the Mediterranean mussel (*Mytilus galloprovincialis*) sperm – study on the effects of cryoprotectants, cooling rate and freezing. Thawing results showed *M. galloprovincialis* sperm are very sensitive to rapid pre-freezing and freezing protocols and only a slow procedure assured good motility and fertilisation.
	Muchlisin *et al*.	Cropreservation of baung sperm (*Mystus nemurus*)- study on the effect of cryoprotectants on abnormality and motility of baung using transmission and scanning electron microscopy. The effects of cryoprotectants on the sperm abnormality were significant.
	Vuthiphandchai *et al*.	Cryopreservation of red snapper (*Lutjanus argentimaculatus*) sperm – first reported attempt for sperm cryopreservation of *L. argentimaculatus* sperm, equilibrated in 10% DMSO and cooled at a rate of 10^oC/ minutes to final temperature of -80^oC had the highest motility (91.1±2.2%) and viability after thawing (92.7±2.3%).

Chapter 3

MATERIALS AND METHODS

Chapter 3

3.0 MATERIALS AND METHODS

3.1 INTRODUCTION

The present work was carried out in July 2008 to July 2009 at the Institute of Biological Sciences Mini (Livestock) Farm, the University of Malaya, Kuala Lumpur with the aim to develop suitable technique for sperm cryopreservation in African catfish (*Clarias gariepinus*). Effects of extender, cryoprotectant, molarity of cryoprotectant, equilibration duration, vapour temperature and vapour exposure duration on frozen-thawed sperm characteristics of African catfish (*Clarias gariepinus*) were determined using the automated semen analyzer (IVOS; Hamilton-Thorne, USA).

3.2 EXPERIMENTAL FISH AND MAINTENANCE

Eighty-two adult male African catfish, *Clarias gariepinus*, broodstocks established for this research were bought from a local fish farm in Rembau, Negeri Sembilan. The broodstocks that were healthy and sexually mature aged from 1 to 2 years old with body weight in a range of 1 to 2 kg were chosen for the experimental purposes. Upon arrival, the broodstocks were acclimatised in a fibreglass tank in the fish house at the Institute of Biological Sciences Mini (Livestock) Farm, the University of Malaya (Figure 3.1). Routine management of fish was scheduled accordingly to avoid stress during experimental period. This includes periodical exchange the water with fresh clean water to ensure easy absorption of oxygen and disease-preventive measure. The tap water was dechlorinated before being supplied to the fish. The net was used to cover on top of the tanks to ensure the fish did not jump out from the tanks. The broodstocks were hand-fed with “commercial finisher layer mash” twice a day, *ad libitum* and daily monitored.

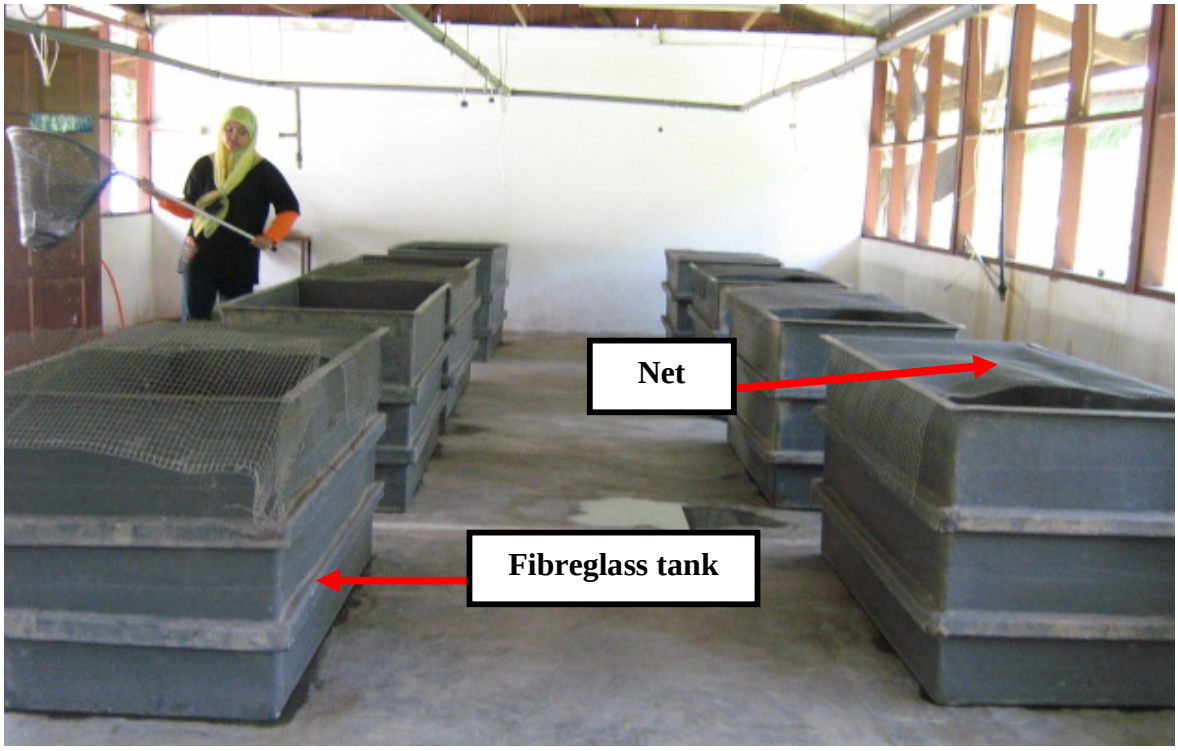

Figure 3.1: Acclimatisation of African catfish broodstocks in a fibreglass tanks at the Institute of Biological Sciences Mini (Livestock) Farm, the University of Malaya.

3.3 INDUCTION OF SPERMATOGENESIS

Body weight of selected catfish was weighed to quantify the dosage of hormone per body weight for each individual fish. Subsequently, Ovaprim (0.5 ml/kg body weight; Syndel, Vancouver, Canada) was injected intramuscularly into the dorsal muscle of catfish (Figure 3.2). Prior to this procedure, the head of the catfish was covered by a wet towel in order to keep it quiet and calm during injection. Most of the fish kept still if their eyes were covered. After receiving the hormone treatment, these males were isolated for overnight in a separate tank to avoid aggressive interaction with other males and to maximise care during the experimental period (Figure 3.3).

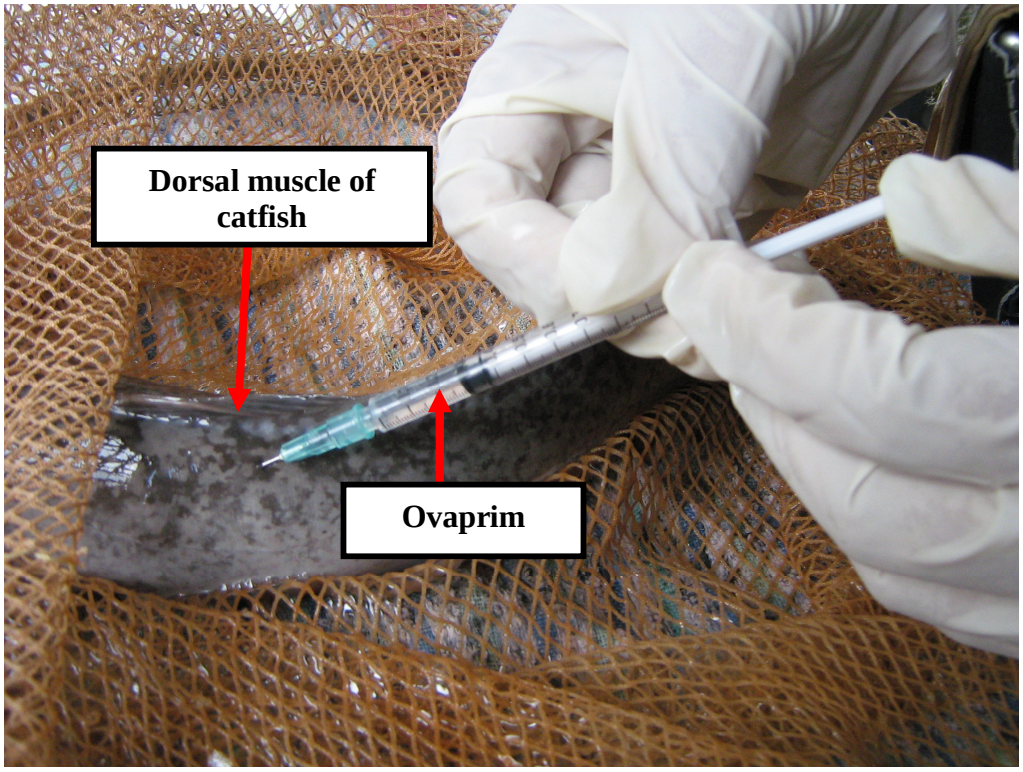

Figure 3.2: Hormonal injection into the dorsal muscle of catfish.

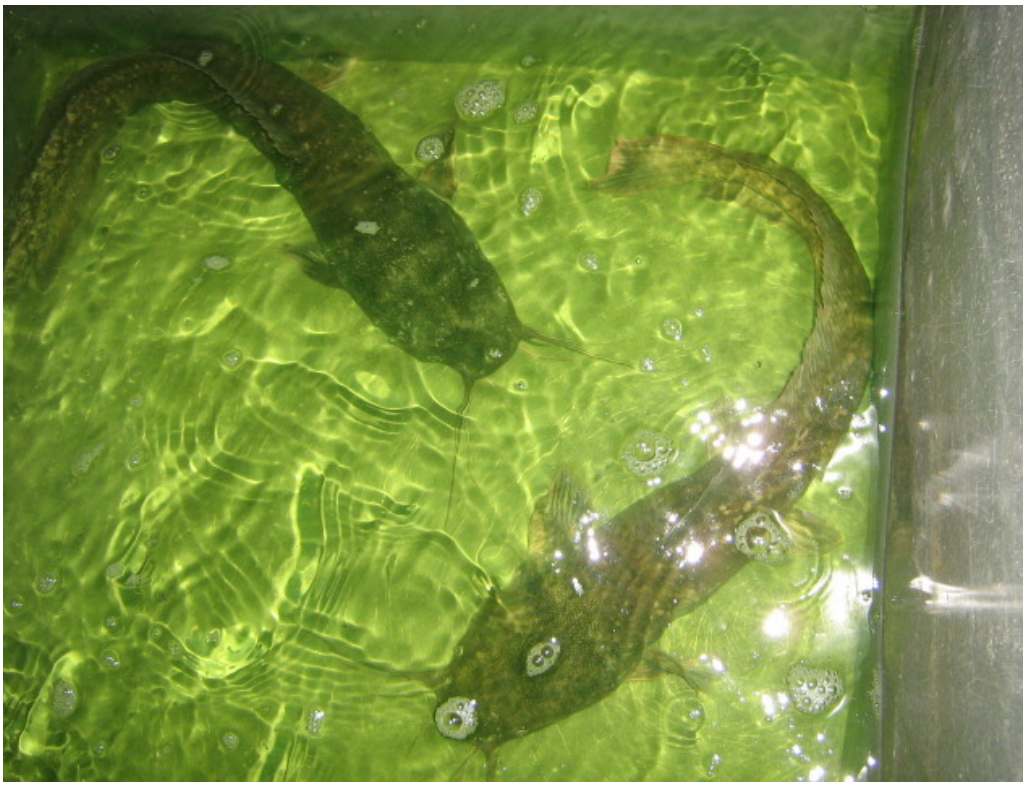

Figure 3.3: Two-injected African catfish were separated from other fish.

3.4 COLLECTION OF MILT

According to the literature, the males of the African catfish cannot be stripped and consequently the sperm can only be obtained by sacrificing a male. Each time, two males were sacrificed and the body surface thoroughly dried after which the testis was dissected out (Figure 3.4). Then, the milt was rapidly perforated out from the testis using a needle. The whitish-like semen was extruded out from the holes and the semen was collected using Eppendorf tube. This procedure has to be done carefully because the testis of catfish comprises of many capillaries, if the sperm and blood mix together leading to sperm dying consequently reduced the sperm motility.

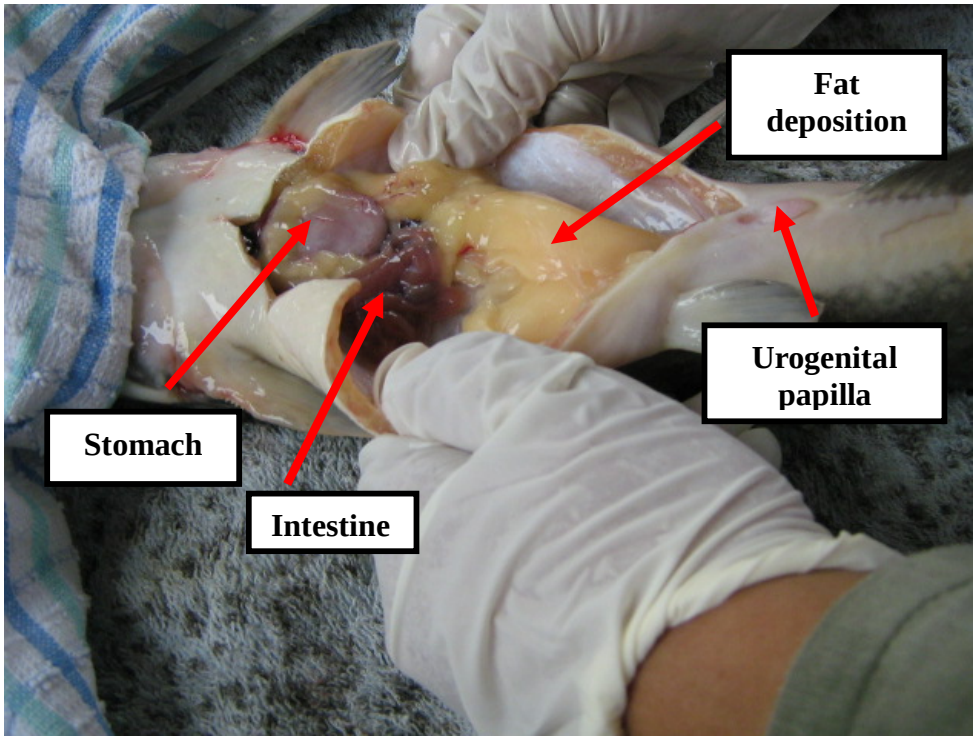

Figure 3.4: Structures of abdominal organs after incision of the male African catfish.

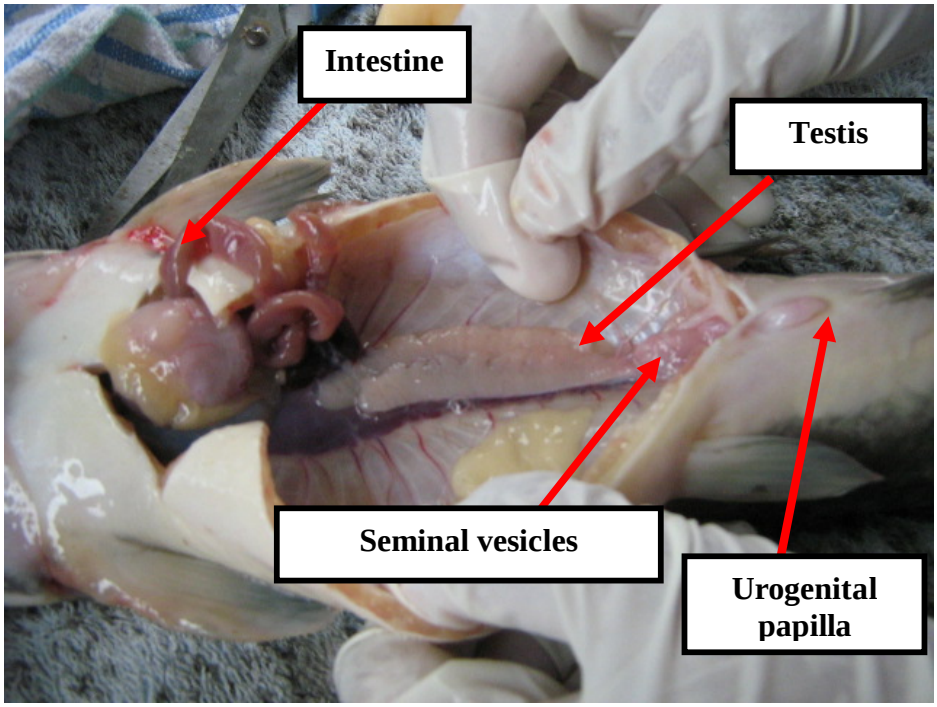

Figure 3.5: The testis (located deep in the abdomen) was taken out from the body cavity after removal of intestine and fats.

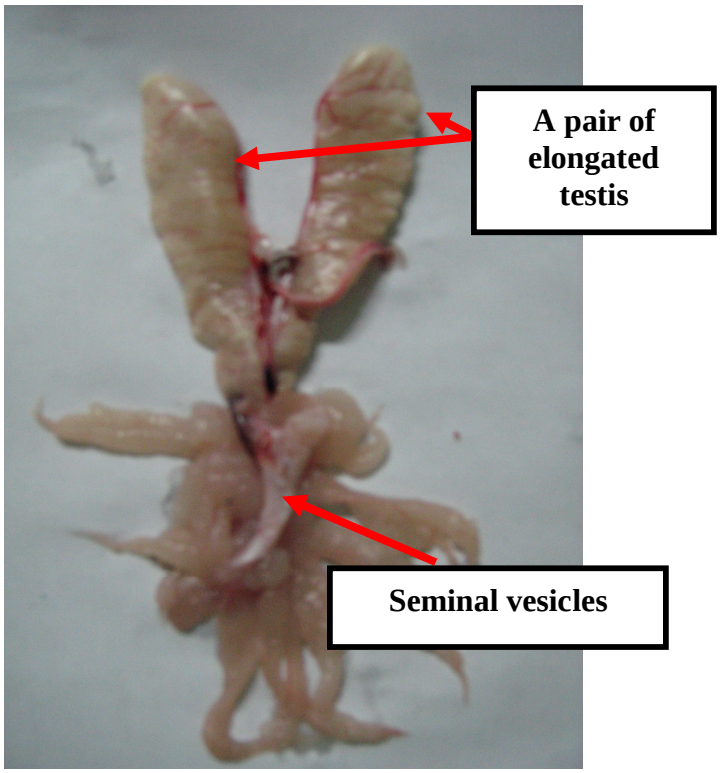

Figure 3.6: Finger-like testis.

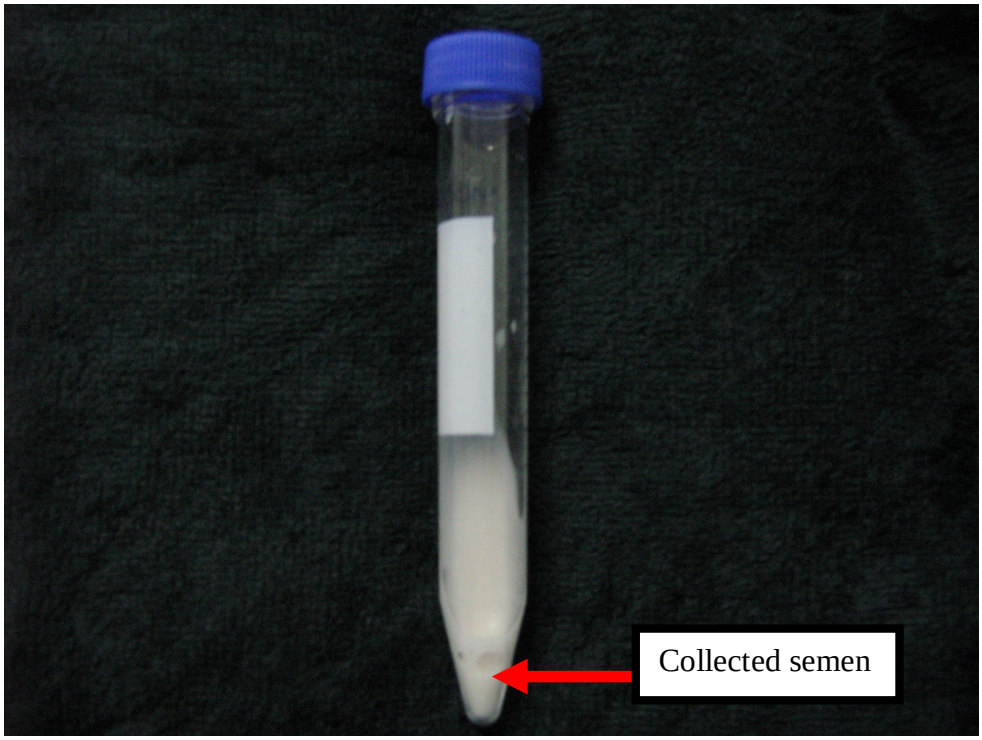

Figure 3.7: Semen was collected into propylene tube.

3.5 SEMEN DILUTION AND LOADING

3.5.1 Sperm Dilution

After collection, the semen was diluted with diluents in a ratio of semen to extender 1:10. Then, the mixture of semen and extender was homogenously mixed.

3.5.2 Sperm Enveloping

The diluted semen was filled into a 0.5 ml or 0.25 ml French straw; some air space was left in between. Each end of the straw was sealed with an electric sealer. Then, these straws were arranged on the special rack for freezing procedure.

3.6 FREEZING AND THAWING

3.6.1 Equilibration

The straw filled with semen-diluent was placed on the rack and being put into the low temperature incubator (Model: L15, Shel lab, Cornelius, Oregon) at 4^{o}C for three equilibration durations (120, 140 or 160 minutes) (Figure 3.8).

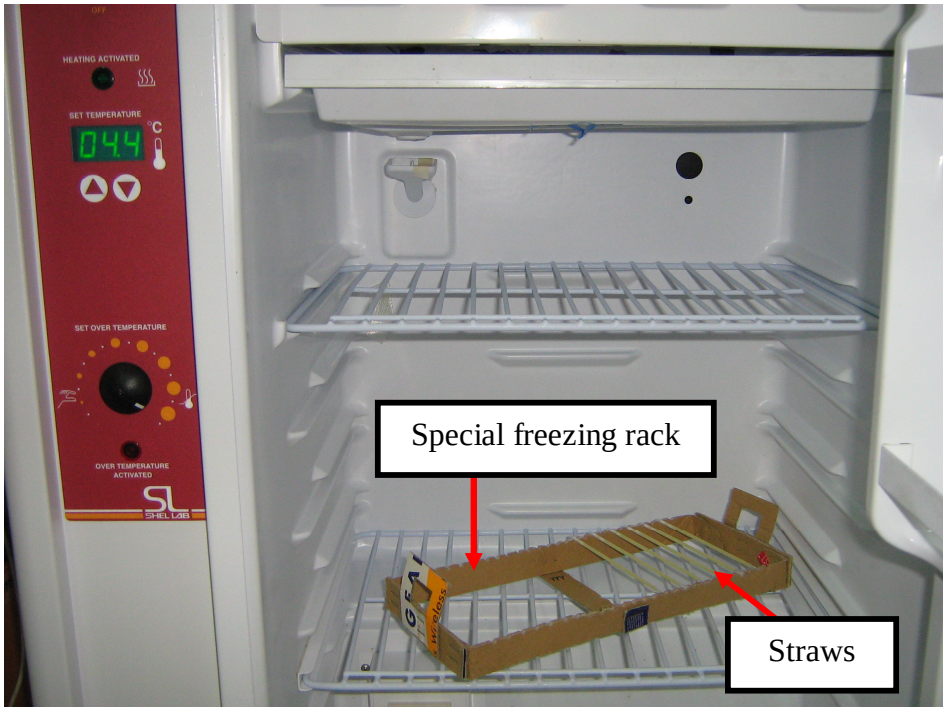

Figure 3.8: Equilibration of straws on a rack in the low temperature incubator at 4^{o}C.

3.6.2 Rapid Freezing

This process comprises of two-stages: a) exposure to liquid nitrogen vapour at different durations (5, 10 or 15 minutes) and temperatures (-80, -90 or -100^{o}C) (Figure 3.9). b) complete submerge of straw into the liquid nitrogen (-196^{o}C) for 10 minutes. The straws were kept in the liquid nitrogen tank for long-term sperm storage (Figure 3.10) until analysis.

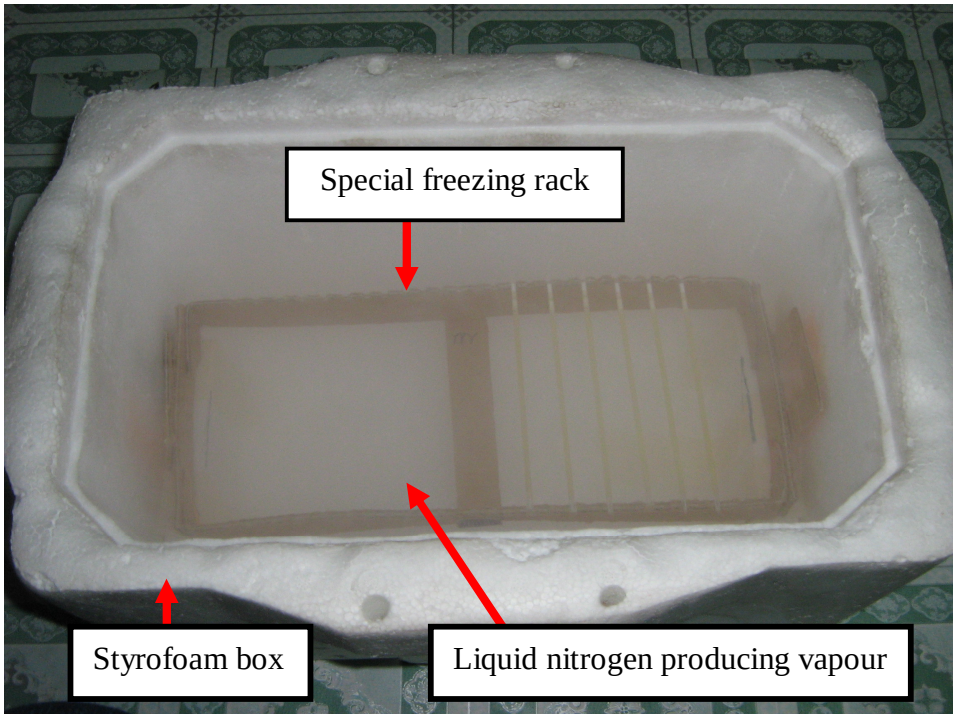

Figure 3.9: Exposure of straws to liquid nitrogen vapour.

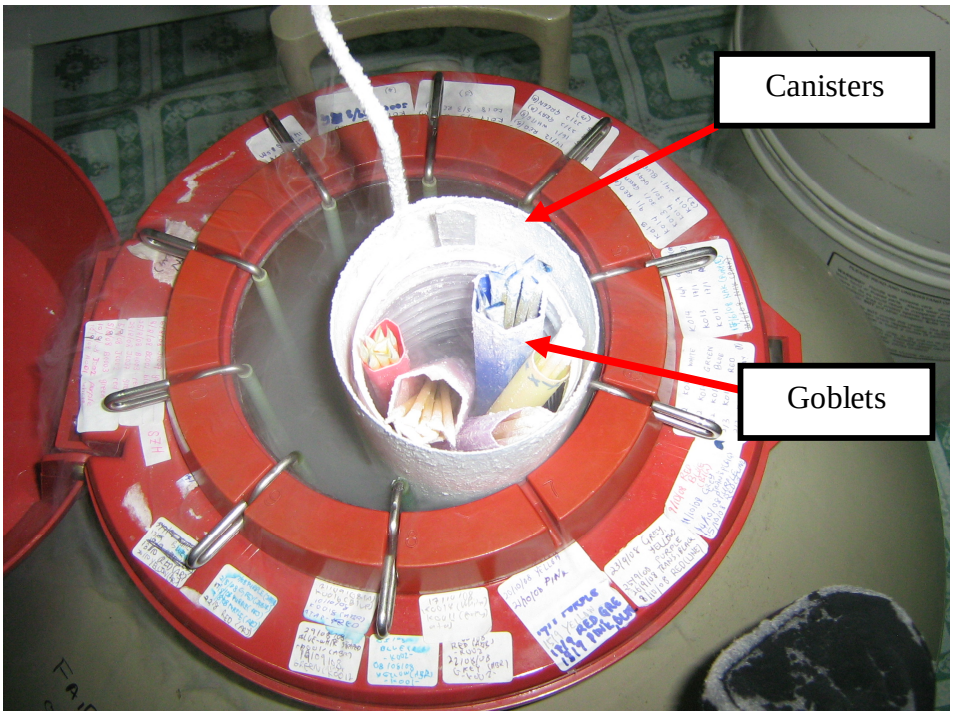

Figure 3.10: Long-term sperm storage in the liquid nitrogen tank.

3.6.3 Thawing

The straws were put into the water bath at 30^{o}C for 30 seconds. After that, the straws were wiped dry with tissue paper and both sealed ends were cut to extract the sperm.

3.7 ANALYSIS OF SPERM

Fresh and post-thawed cryopreserved sperm were analysed using the automated semen analyzer (IVOS; Hamilton-Thorne, USA) (Figure 3.11) to evaluate total motility, progressive motility, velocity distributions and motion characteristics of the sperm.

Figure 3.11: Automated semen analyzer (IVOS; Hamilton-Thorne, USA).

3.7.1 Automated Semen Analyzer (IVOS; Hamilton-Thorne, USA)

Figure 3.12 shows the schematic representation of some of the motility patterns measured by the CASA system. The most useful parameters which were automatically assessed were:

a) Total motility: the number of motile sperm within the analysis field divided by the sum of the motile plus immotile sperm within the analysis field.
b) Progressive motility: total progressive motility of sperm cells.
c) Velocity distributions: total percentage of
    i) Rapid.
    ii) Medium.
    iii) Slow.
    iv) Static.
d) Sperm motion characteristics
    i) VAP {Velocity average path (μm s^{-1})}: Velocity over an average path.
    ii) VSL {straight line velocity (μm s^{-1})}: the straight line distance between the start and end points of the track divided by time taken for the sperm to cover the track.
    iii) VCL {curvilinear velocity (μm s^{-1})}: the sum of the incremental distances moved in each frame along the sampled path divided by time taken for the sperm to cover the track.
    iv) ALH (microns): amplitude of lateral head displacement.
    v) BCF (hertz): beat cross frequency.
    vi) STR (%): straightness (ratio of VSL / VAP).

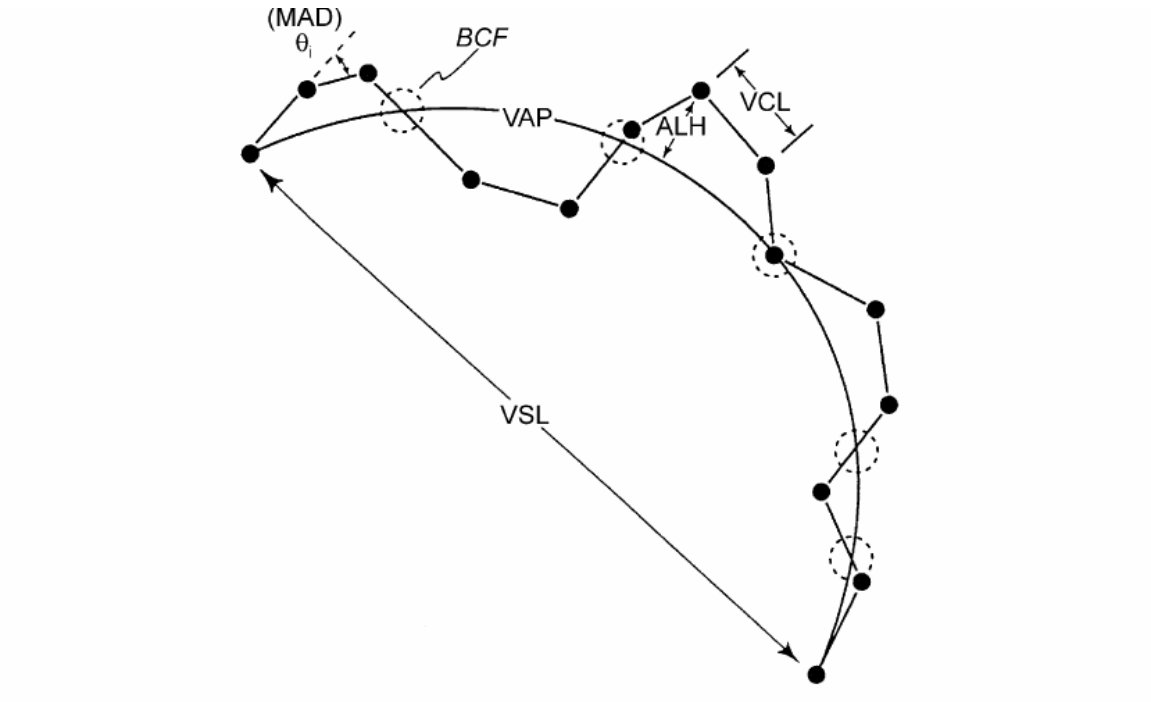

Figure 3.12: Schematic representation of some of the motility patterns measured by the CASA system (Boyer *et al*., 1989). Black circles represent successive images of the head of a motile sperm and are joined by straight path lines. Curved line indicates a smoothed path fitted through sperm track. Analysis includes {MAD: angular displacement, BCF: beating cross frequency, VAP: average path velocity, ALH: amplitude of lateral head displacement, VCL: curvilinear velocity, VSL: straight line velocity}.

vii) LIN {the linearity (%)}: the straight line distance between the start and end points of the track divided by the sum of the incremental distances along the actual path (VSL / VCL x 100).

3.7.2 Technique of Sperm Analysis Using IVOS

To ensure simultaneous activation, 10 µl fresh sperm was dropped onto the markler, followed by addition of 10 µl distilled water which induces the sperm motility. As for frozen-sperm, 10 µl of post-thawed cryopreserved sperm that contained extender as diluent was dropped onto the markler; sperm remains immotile, followed by addition of distilled

water, which induced motility (activation). The dilution with 10 µl water and transfer of diluted activated sperm to the markler were carried out very rapidly (<10 seconds). (Since motility of fish sperm decreases very rapidly after mixing with water, it is essential to get fully stabilised image as quickly as possible ideally approximately 5 seconds after induction of motility).

3.8 EXTENDER

There are two types of extender used in this experiment: a) Tris-Citric Acid Yolk Extender (TCAYE) as described by Asmad *et al.* (2005) and b) Fish-Ringer Extender (FRE) as described by Basavaraja *et al*. (2004).

3.8.1 Tris-Citric Acid Yolk Extender (TCAYE)

3.8.1.1 Tris stabilizer preparation

This preparation comprises of Tris and citric acid. Tris and citric acid were weighed and diluted in milli-Q water. This was followed by the addition of Streptomycin and Penicillin. Then, the pH was adjusted to 6.75 with 10% citric acid.

3.8.1.2 Egg yolk preparation

Fresh chicken egg was used (within 25 hours after laying). The egg shell was cleaned with water and then wiped with cotton soaked in alcohol, and left to dry. Then the shell was broken and the yolk separated from the albumin. The yolk and residues of albumin was rolled on a piece of filter paper to separate the entire remaining albumin. The membrane of the yolk was pierced to enable the liquid interior to be put into a clean beaker. Precaution should be taken to ensure that the yellow liquid is not contaminated with yolk membrane or albumin.

3.8.1.3 Liquid substance preparation

The yolk was mixed thoroughly with Tris-stabilizer with the ratio of (1:4), and the mixture was centrifuged at 2000 rpm for 15 minutes to separate the sediment. The supernatant was separated and mixed with either 2.27, 4.53 or 6.80% glycerol, followed by addition of 1% fructose. After thorough mixing, the liquefied substance was placed in a water bath at 37^{o}C before it could be utilised.

3.8.2 Fish-Ringer Extender (FRE)

Table 3.1: The composition of Fish-Ringer Extender (Basavaraja *et al*., 2004)

Chemical	Amount (g/100 ml)
NaCl	0.75
KCl	0.10
$CaCl_2$	0.016
$MgSO_4$	0.023
NaH_2PO_4	0.041
Glucose	0.10

All the chemicals in Table 3.1 were weighed accurately. To prepare 100 ml FRE extender, first each of the chemicals was added into 90 ml distilled water and was gently stirred. Then, the remaining of 10 ml distilled water was topped up until reached 100 ml. To ensure all the chemicals added were homogenously mixed; the mixture was stirred on the stirrer with magnetic stirrer was placed inside. Then, the pH was adjusted until reached 7.50.

3.9 EXPERIMENTAL DESIGN

The primary objective of this study was to develop a sperm freezing protocol in African catfish (*Clarias gariepinus*) using two different extenders (TCAYE and FRE extenders). This study was divided into 4 experiments which have been illustrated as a flow chart in Figure 3.13. Each experiment has been described in the following sections.

3.9.1 Effects of Individual Fish Body Weight on Fresh Sperm Motility Characteristics in African Catfish (*Clarias gariepinus*) (Experiment 1)

The aim of this experiment was to evaluate sperm motility characteristics before freezing according to individual fish and body weight in order to form the standard baseline information before sperm freezing procedures proper were carried out. Each session of semen collection, 2 male African catfish were sacrificed to collect the milt. First, the individual fish was weighed to get an actual body weight. The actual body weight was used to categorise 3 respective sizes, namely small (<1.0 kg), medium (1.0 -1.5 kg) and large (>1.5 kg). The testis of the sacrificed male African catfish was dissected out from the body cavity and the testis was cleaned with tap water to rinse the blood. Then, the testis was gently perforated with needle to collect the milt. Precaution during perforated of testis has to take into account to avoid the needle pierce into the capillary. The milt collected was diluted with diluents in a ratio of 1:10 to facilitate analysis of sperm motility characteristics. Without dilution, the analysis of sperm using IVOS was difficult because the sperm was too concentrated. Sperm movement characteristics and velocity distributions of pre-freezing semen was evaluated using an automated semen analyzer (IVOS; Hamilton-Thorne, USA).

3.9.2 Optimisation of Molarity of Glycerol in TCAYE Extender, Equilibration Duration, Vapour Temperature and Vapour Exposure Duration on Frozen-thawed Sperm Motility Characteristics of African Catfish (*Clarias gariepinus*) (Experiment 2)

The preliminary trial was conducted with the aim to familiarise the protocol of cryopreservation in red tilapia using TCAYE before being applied to African catfish. Subsequently, after familiarising with the freezing technique, an experiment with the aim to determine the effect of equilibration duration, vapour temperature and vapour exposure duration on post-thawed cryopreserved sperm motility characteristics in African catfish were carried out. Briefly, semen was collected from testis of the sacrificed African catfish, diluted in a ratio of 1:10 semen to diluents using TCAYE in 0.5 ml or 0.25 ml French straws and the straws containing the diluted semen were subjected to freezing process. This research involved a 3 x 3 x 3 x 3 factorial experiment consisting of 3 molarities of glycerol (0.5, 1.0 or 2.0 M), 3 equilibration durations (120, 140 or 160 minutes), 3 vapour temperatures (-80, -90 or -100^{o}C) and 3 vapour exposure durations (5, 10 or 15 minutes). Each of the combination treatments were replicated 3 times with 5 observations per replicate. Sperm motility characteristics after frozen-thawed were evaluated using an automated semen analyzer (IVOS; Hamilton-Thorne, USA).

3.9.3 Optimisation of Equilibration Duration, Vapour Temperature and Vapour Exposure Duration on Frozen-thawed Sperm Motility Characteristics of African Catfish (*Clarias gariepinus*) Using Fish-Ringer Extender (Experiment 3)

The goal of this experiment was to determine the effect of equilibration duration, vapour temperature and vapour exposure duration on post-thawed cryopreserved sperm motility characteristics in African catfish using Fish-Ringer extender. The protocol of semen collection was similar to Experiment 2. Semen was collected from testis of sacrificed

catfish, diluted in a ratio of 1:10 in 0.5 ml or 0.25 ml French straws and the straws containing the diluted semen were subjected to freezing process. This research involved a 3 x 3 x 3 factorial experiment consisting of 3 equilibration durations (120, 140 or 160 minutes), 3 vapour temperatures (-80, -90 or -100^{o}C) and 3 vapour exposure durations (5, 10 or 15 minutes). The molarity of cryoprotectant was fixed at 10% DMSO as described by Basavaraja *et al*. (2004). Each of the combination treatments was replicated 3 times with 5 observations per replicate. Sperm motility characteristics after freezing were evaluated using an automated semen analyzer (IVOS; Hamilton-Thorne, USA).

3.9.4 Comparison of Effects of Different Types of Extender and Cryoprotectant on Frozen-thawed Sperm Motility Characteristics of African Catfish (*Clarias gariepinus*) (Experiment 4)

The aim of this experiment was to compare the effects of different types of extender and cryoprotectant on frozen-thawed sperm of African catfish. TCAYE extender was used with combination of three molarities of glycerol (0.5, 1.0 and 2.0 M). In addition, FRE extender was also studied with a fixed concentration of cryoprotectant at 10% DMSO. Each of the combination treatments was replicated 3 times with 5 observations per replicate. Sperm motility characteristics after freezing were evaluated using an automated semen analyzer (IVOS; Hamilton-Thorne, USA).

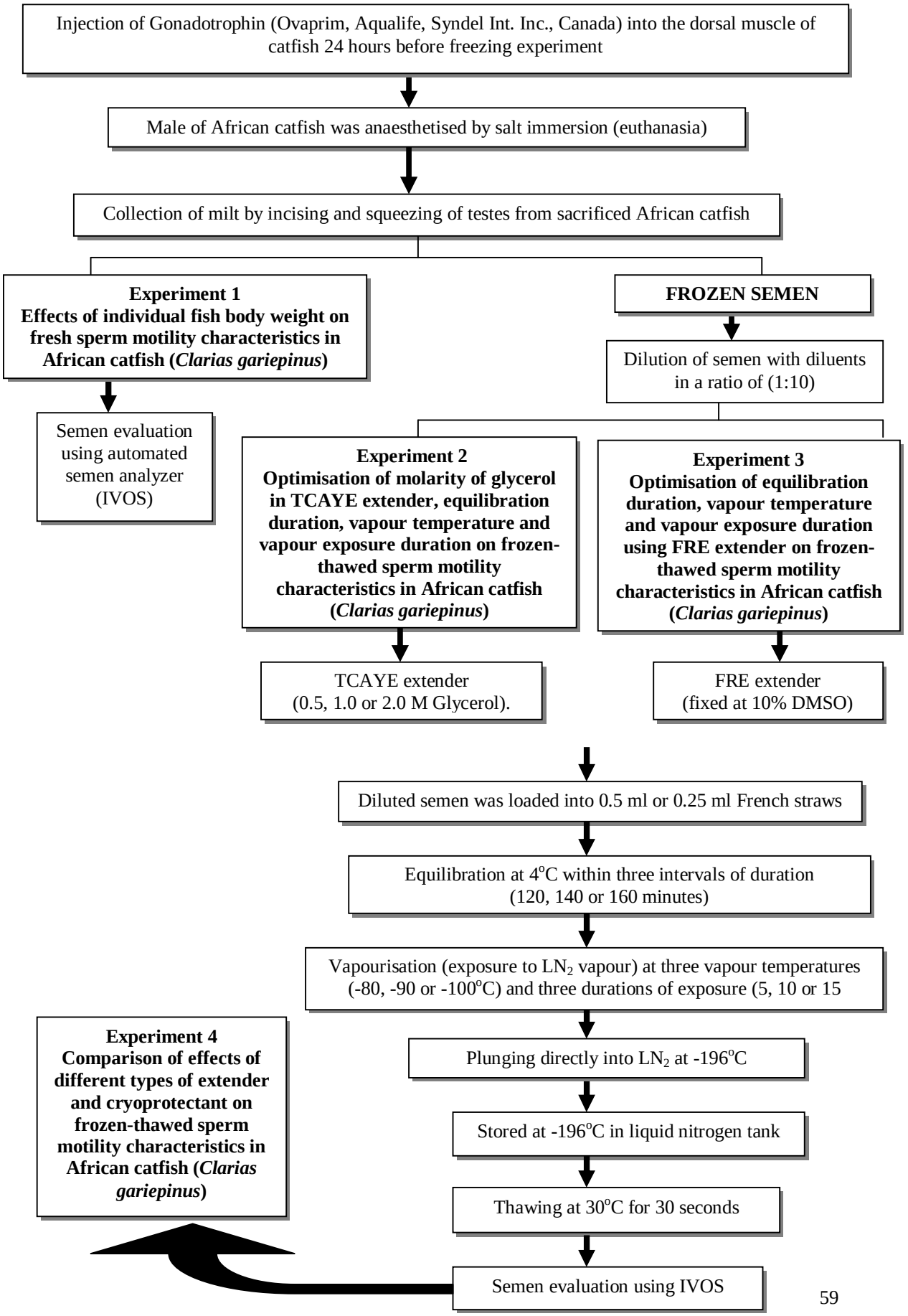

Figure 3.13: Flow chart of experimental design.

3.10 STATISTICAL ANALYSIS

All data were subjected to analysis of variance (ANOVA), followed by comparison of means using Duncan's multiple range test (DMRT). All statistical analysis was performed using SPSS (Statistical Package for Social Sciences) for windows, version 12.0. The data was presented as (mean±SEM). To determine correlations among the parameters measured, Pearson correlation was used which was significant at ($P<0.01$). The factors involved and parameters measured were categorised into dependent and independent variables.

The independent variables involved were:

a) Molarity of glycerol –molarity of glycerol in TCAYE extender.
b) Types of extender – TCAYE and FRE extenders.
c) Equilibration duration – duration for semen and extender to equilibrate.
d) Vapour temperature –vapourisation temperature for freezing process before submerged into liquid nitrogen.
e) Vapour exposure duration – duration for exposure the sperm onto liquid nitrogen vapour.

The dependent variables were:

a) Total motility – total motility of sperm cells.
b) Progressive motility– total progressive motility of sperm cells.
c) Sperm velocity distributions, i.e. total rapid, medium, slow and static sperm cells were estimated.
d) Sperm motion characteristics, i.e. VAP, VCL, VSL, ALH, BCF, STR and LIN.

Chapter 4

RESULTS

Chapter 4

4.0 RESULTS

4.1 EFFECT OF INDIVIDUAL BODY WEIGHT ON FRESH SPERM MOTILITY CHARACTERISTICS IN AFRICAN CATFISH (*Clarias gariepinus*) (EXPERIMENT 1)

The fresh sperm motility characteristics analysed by the IVOS (Hamilton-Thorne, USA) were grouped according to body weight of African catfish, namely called body size: small (<1.0 kg), medium (1.0-1.5 kg) and large (>1.5 kg). Table 4.1 shows the total motility and progressive motility of fresh sperm obtained from 24 randomly selected fish which were grouped into small (5), medium (14) and large (5) body weight groups (BW). Large BW of African catfish gave the highest total motility (82.40±4.59%) followed by medium BW (51.64±9.82%) and small BW (40.40±12.16%), whereby small BW fish were significantly different in total motility compared with the other two groups. The values for progressive motility for small, medium and large BW of fish were 8.20±3.65%, 14.00±4.29% and 17.40±3.36%, respectively (P>0.05).

Table 4.2 shows velocity distributions for fresh sperm of African catfish according to body weight group of fish. The values of sperm with rapid and slow velocities for the three body weight groups did not show any significant differences (P>0.05). However, sperm for large BW group with medium velocity showed the highest significant value (15.40±2.82%) as compared to medium BW group (6.21±1.86%) and small BW group (5.60±2.32%). In static velocity, there were no significant differences among the different BW groups studied (P>0.05). The values for rapid, medium, slow and static velocity distributions for fresh sperm were ranged from 14.00±6.63% to 25.80±4.97%, 5.60±2.32% to 15.40±2.82%, 20.80±6.49% to 41.20±5.18% and 17.60±4.59% to 59.60±12.16%, respectively. Interestingly, large BW group of fish gave significantly higher medium

velocity compared to the other groups. Conversely, small and medium BW groups were significantly higher in static velocity compared to large BW group.

Analysis of sperm motion characteristics for fresh sperm of African catfish are shown in Table 4.3. There were no significant differences for values of ALH (range: 5.36±0.60-6.20±0.49 μm), BCF (range: 10.78±2.82-16.10±2.66 Hz), STR (range: 83.33±1.45-88.36±1.53%) and LIN (range: 62.00±1.97-66.80±7.88%) among the three BW groups ($P>0.05$). However, the respective VAP (83.34±9.31 μm/s), VSL (73.44±11.60 μm/s) and VCL (108.12±5.51 μm/s) values for small BW groups were significantly higher ($P<0.05$) than those of the large BW group (49.70±6.42, 41.90±4.94 and 74.60±9.47 μm/s).

Tables 4.4, 4.5 and 4.6 show correlations of fresh sperm motility characteristics in African catfish for small, medium and large BW groups. For small BW group, positive correlations ($P<0.05$) were shown between medium and slow; VAP and VSL; VAP and LIN; VSL and STR; VSL and LIN and STR and LIN. In contrast, total motility and static; rapid and ALH; VAP and BCF; VSL and BCF and BCF and LIN showed high negative correlations ($P<0.05$). In medium BW group, total motility and progressive motility; total motility and rapid; total motility and medium; progressive motility and rapid; progressive motility and medium; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN as well as ALH and LIN were high positively correlated ($P<0.05$). On the other hand, high negative correlations were shown between total motility and static; progressive motility and static; progressive motility and VAP; progressive motility and VCL; rapid and static; rapid and VAP; rapid and VCL; medium and static; slow and static; VAP and LIN; VSL and LIN; VCL and STR; VCL and LIN and BCF and STR ($P<0.05$). In large BW group, positive correlations ($P<0.05$) were shown between progressive motility and rapid; progressive motility and LIN; rapid and STR;

rapid and LIN; VAP and VSL; VAP and VCL; VAP and ALH; VSL and VCL and VSL and ALH. In contrast, negative correlations ($P<0.05$) were shown between total motility and static; progressive motility and VAP; progressive motility and VSL; progressive motility and VCL; progressive motility and ALH; rapid and VAP; rapid and VSL; rapid and VCL; rapid and ALH; VAP and LIN; VSL and STR; VSL and LIN; VCL and LIN and ALH and LIN.

Table 4.7 shows correlations among fresh sperm motility characteristics of African catfish for overall body weight groups. There were positive correlations ($P<0.05$) 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; rapid and medium; medium and slow; slow and BCF; static and VSL; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown between medium and static; slow and static and BCF and STR.

Table 4.1: Total motility and progressive motility (mean ± SEM) for fresh sperm of African catfish according to body weight group of fish

Body size (BW, kg)	N*	Total motility (%)	Progressive motility (%)
Small (< 1.0)	5	40.40±12.16[a]	8.20±3.65[a]
Medium (1.0-1.5)	14	51.64±9.82[ab]	14.00±4.29[a]
Large (>1.5)	5	82.40±4.59[b]	17.40±3.36[a]

N*= Number of individual fish.
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.2: Velocity distributions (mean ± SEM) for fresh sperm of African catfish according to body weight group of fish

Body size (BW, kg)	N*	Rapid (%)	Medium (%)	Slow (%)	Static (%)
Small (<1.0)	5	14.00±6.63[a]	5.60±2.32[a]	20.80±6.49[a]	59.60±12.16[b]
Medium (1.0-1.5)	14	18.79±6.13[a]	6.21±1.86[a]	26.50±5.25[a]	48.36±9.82[ab]
Large (>1.5)	5	25.80±4.97[a]	15.40±2.82[b]	41.20±5.18[a]	17.60±4.59[a]

N*= Number of individual fish.
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.3: Sperm motion characteristics (mean ± SEM) for fresh sperm of African catfish according to body weight group of fish

Body size (BW, kg)	N*	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
Small (<1.0)	5	83.34 ±9.31[b]	73.44 ±11.60[b]	108.12 ±5.51[b]	6.20 ±0.49[a]	10.78 ±2.82[a]	86.00 ±3.81[a]	66.80 ±7.88[a]
Medium (1.0-1.5)	14	58.82 ±6.22[ab]	52.55 ±5.61[ab]	80.45 ±6.87[ab]	5.36 ±0.60[a]	11.21 ±1.64[a]	88.36 ±1.53[a]	62.00 ±1.97[a]
Large (>1.5)	5	49.70 ±6.42[a]	41.90 ±4.94[a]	74.60 ±9.47[a]	5.53 ±1.47[a]	16.10 ±2.66[a]	83.33 ±1.45[a]	62.67 ±2.32[a]

N*= Number of individual fish.
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.4: Correlations among fresh sperm motility characteristics for small BW group of African catfish

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.768	.632	.857	.845	-.959**	-.324	-.364	-.014	-.375	.362	-.439	-.406
Progressive motility		1	.795	.369	.310	-.669	-.213	-.356	.378	-.471	.240	-.636	-.465
Rapid			1	.202	.245	-.714	-.193	-.325	.313	-.909*	.402	-.569	-.420
Medium				1	.970**	-.818	-.430	-.374	-.406	-.018	.391	-.250	-.346
Slow					1	-.852	-.263	-.203	-.303	-.117	.284	-.087	-.176
Static						1	.328	.357	.069	.561	-.445	.404	.389
VAP							1	.983**	.786	.140	-.948*	.837	.950*
VSL								1	.661	.237	-.945*	.923*	.991**
VCL									1	-.198	-.715	.321	.554
ALH										1	-.425	.403	.302
BCF											1	-.827	-.922*
STR												1	.966**
LIN													1

No. of fish = 5.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.5: Correlations among fresh sperm motility characteristics for medium BW group of African catfish

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.813**	.776**	.783**	.692**	-1.000**	-.335	-.353	-.360	.090	.325	-.167	-.181
Progressive motility		1	.993**	.611*	.149	-.813**	-.289	-.299	-.296	-.001	.098	-.162	-.255
Rapid			1	.519	.103	-.776**	-.269	-.279	-.274	-.006	.060	-.153	-.250
Medium				1	.509	-.783**	-.207	-.227	-.234	.211	.354	-.172	-.078
Slow					1	-.692**	-.262	-.282	-.298	.136	.513	-.090	-.003
Static						1	.335	.353	.360	-.090	-.325	.167	.181
VAP							1	.979**	.963**	.561*	.007	-.370	.577*
VSL								1	.896**	.597*	-.139	-.200	.705**
VCL									1	.435	.116	-.508	.366
ALH										1	.033	-.099	.659*
BCF											1	-.742**	-.392
STR												1	.384
LIN													1

No. of fish = 14.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.6: Correlations among fresh sperm motility characteristics for large BW group of African catfish

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.462	.400	.225	.407	-1.000**	.062	-.056	.025	-.032	.116	.296	.092
Progressive motility		1	.993**	-.004	-.507	-.462	-.984*	-.995**	-.979*	-.998**	.019	.941	.998**
Rapid			1	-.084	-.531	-.400	-.986*	-.999**	-.989*	-.994**	.109	.963*	.989*
Medium				1	-.251	-.225	.170	.138	.233	.067	-.892	-.212	.018
Slow					1	-.407	.847	.822	.797	.869	.452	-.635	-.883
Static						1	-.062	.056	-.025	.032	-.116	-.296	-.092
VAP							1	.993**	.996**	.993**	-.086	-.916	-.978*
VSL								1	.994**	.996**	-.109	-.953*	-.988*
VCL									1	.986*	-.171	-.941	-.968*
ALH										1	-.022	-.928	-.996**
BCF											1	.307	-.039
STR												1	.923
LIN													1

No. of fish = 5.
**Pearson correlations were significant (P<0.01).
*Pearson correlations were significant (P<0.05).

Table 4.7: Correlations among fresh sperm motility characteristics of African catfish (*Clarias gariepinus*) for overall pooled BW groups

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.776**	.739**	.778**	.745**	-.995**	-.407	-.428*	-.375	.027	.419	-.295	-.234
Progressive motility		1	.971**	.509*	.184	-.767**	-.357	-.364	-.346	-.144	.132	-.170	-.155
Rapid			1	.439*	.145	-.748**	-.321	-.336	-.300	-.179	.152	-.195	-.146
Medium				1	.566**	-.774**	-.297	-.318	-.262	.155	.362	-.298	-.152
Slow					1	-.746**	-.294	-.307	-.277	.184	.543**	-.217	-.218
Static						1	.407	.427*	.380	-.011	-.433*	.287	.230
VAP							1	.976**	.940**	.511*	-.266	-.015	.518*
VSL								1	.849**	.507*	-.403	.182	.650**
VCL									1	.454*	-.087	-.288	.247
ALH										1	-.011	-.099	.200
BCF											1	-.736**	-.494*
STR												1	.602**
LIN													1

No. of fish = 24.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

4.2 FAMILIARISING THE PROTOCOL OF SPERM CRYOPRESERVATION USING RED TILAPIA (*Oreochromis niloticus*) AS A MODEL (EXPERIMENT 2)

4.2.1 Fresh Sperm

Table 4.8 shows total motility and progressive motility of fresh sperm for Red tilapia. The mean values for total motility and progressive motility obtained from the three individual fish were 63.67±12.33% and 30.00±7.02%, respectively.

The velocity distributions of fresh sperm for red tilapia are shown in Table 4.9. The mean values of rapid, medium, slow and static velocities obtained were 37.00±8.62%, 6.67±1.20%, 20.00±4.93% and 36.33±12.33%, respectively.

Analysis of sperm motion characteristics of fresh sperm for Red tilapia are shown in Table 4.10. The mean values for VAP, VSL, VCL, ALH, BCF, STR and LIN obtained were 44.27±2.89 µm/s, 37.23±4.51 µm/s, 60.00±4.36 µm/s, 2.77±0.33 µm, 18.13±0.26 Hz, 87.33±1.33% and 63.33±3.18%, respectively.

Table 4.8: Total motility and progressive motility (mean ± SEM) for fresh sperm of Red tilapia (*Oreochromis niloticus*)

Individual Fish	Total Motility (%)	Progressive motility (%)
1	76.00	36.00
2	76.00	38.00
3	39.00	16.00
Mean±SEM	63.67±12.33	30.00±7.02

Total number of fish, N = 3.

Table 4.9: Velocity distributions (mean ± SEM) for fresh sperm of Red tilapia (*Oreochromis niloticus*)

Individual Fish	Rapid (%)	Medium (%)	Slow (%)	Static (%)
1	43.00	5.00	29.00	24.00
2	48.00	6.00	19.00	24.00
3	20.00	9.00	12.00	61.00
Mean±SEM	37.00±8.62	6.67±1.20	20.00±4.93	36.33±12.33

Total number of fish, N = 3.

Table 4.10: Sperm motion characteristics (mean ± SEM) for fresh sperm of Red tilapia (*Oreochromis niloticus*)

Individual Fish	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
1	49.20	44.40	65.90	3.40	18.60	90.00	69.00
2	44.40	38.40	62.60	2.60	18.10	86.00	63.00
3	39.20	28.90	51.50	2.30	17.70	86.00	58.00
Mean	44.27	37.23	60.00	2.77	18.13	87.33	63.33
±SEM	±2.89	±4.51	±4.36	±0.33	±0.26	±1.33	±3.18

Total number of fish, N = 3.

4.2.2 Post-thawed Cryopreserved Sperm

Table 4.11 shows total motility and progressive motility of post-thawed cryopreserved sperm of Red tilapia using 0.5 M glycerol in TCAYE extender for combinations of equilibration duration, vapour temperature and vapour exposure duration. The mean values for total motility and progressive motility obtained from post-thawed cryopreserved sperm were 51.00±7.79% and 22.33±4.06%, respectively.

Table 4.12 shows velocity distributions of post-thawed cryopreserved sperm of Red tilapia using 0.5 M glycerol in TCAYE extender for combinations of equilibration duration, vapour temperature and vapour exposure duration. The mean values for rapid, medium, slow and static velocities obtained from post-thawed cryopreserved sperm were 25.17±4.52%, 3.50±0.92%, 22.17±7.38% and 49.00±7.79%, respectively.

Table 4.13 shows sperm motion characteristics of post-thawed cryopreserved sperm of Red tilapia using 0.5 M glycerol in TCAYE extender for combinations of equilibration duration, vapour temperature and vapour exposure duration. The mean values for VAP, VSL, VCL, ALH, BCF, STR and LIN obtained from post-thawed cryopreserved sperm were 44.50±1.48 μm/s, 40.52±1.66 μm/s, 58.82±1.45 μm/s, 2.88±0.29 μm, 15.63±1.39 Hz, 90.83±0.70% and 69.50±1.15%, respectively.

Table 4.11: Total motility and progressive motility (mean ± SEM) of post-thawed cryopreserved sperm of Red tilapia (*Oreochromis niloticus*) using 0.5 M of glycerol in TCAYE extender for combinations of equilibration duration, vapour temperature and vapour exposure duration

Equilibration duration (min)	Vapour temperature (^{o}C)	Exposure duration (min)	Total motility (%)	Progressive motility (%)
30	-90	7	48.00	30.00
45	-90	5	64.00	29.00
45	-90	10	49.00	5.00
60	-80	10	35.00	29.00
60	-90	5	29.00	16.00
		Mean±SEM	51.00±7.79	22.33±4.06

N*= Each of combination was done once to familiarise the sperm freezing technique.

Table 4.12: Velocity distributions (mean ± SEM) of post-thawed cryopreserved sperm of Red tilapia (*Oreochromis niloticus*) using 0.5 M of glycerol in TCAYE extender for combinations of equilibration duration, vapour temperature and vapour exposure duration

Equilibration duration (min)	Vapour temperature (^{o}C)	Vapour exposure duration (min)	Rapid (%)	Medium (%)	Slow (%)	Static (%)
30	-90	7	34.00	7.00	8.00	52.00
45	-90	5	34.00	4.00	25.00	36.00
45	-90	10	6.00	3.00	40.00	51.00
60	-80	10	29.00	0.00	6.00	65.00
60	-90	5	18.00	4.00	7.00	71.00
		Mean ±SEM	25.17 ±4.52	3.50 ±0.92	22.17 ±7.38	49.00 ±7.79

N* = Each of combination was done once to familiarise the sperm freezing technique.

Table 4.13: Sperm motion characteristics (mean ± SEM) for post-thawed cryopreserved sperm of Red tilapia (*Oreochromis niloticus*) using 0.5 M of glycerol in TCAYE extender for combinations of equilibration duration, vapour temperature and vapour exposure duration

Equilibration duration (min)	Vapour temperature (^{o}C)	Vapour exposure duration (min)	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
30	-90	7	41.40	37.80	56.60	2.60	15.80	90.00	67.00
45	-90	5	44.20	39.00	61.00	2.50	17.40	89.00	67.00
45	-90	10	40.20	37.50	54.80	4.20	16.40	91.00	67.00
60	-80	10	49.90	47.30	64.20	2.80	19.50	94.00	73.00
60	-90	5	43.90	37.80	56.20	2.20	9.40	90.00	71.00
			44.50	40.52±	58.82	2.88	15.63	90.83	69.50
		Mean±SEM	±1.48	1.66	±1.45	±0.29	±1.39	±0.70	±1.15

N* = Each of combination was done once to familiarise the sperm freezing technique.

4.3 EFFECT OF MOLARITY OF GLYCEROL IN TCAYE EXTENDER ON FROZEN-THAWED SPERM MOTILITY OF AFRICAN CATFISH (*Clarias gariepinus*) (EXPERIMENT 2)

Table 4.14 shows total motility and progressive motility of post-thawed cryopreserved sperm of African catfish for 0.5, 1.0 and 2.0 M of glycerol in TCAYE extender. Glycerol with molarity of 0.5 M showed significant highest values of total motility (32.27±2.05%) and progressive motility (3.75±0.41%) as compared to the total motility and progressive motility values of 1.0 M (24.50±1.81% and 2.63±0.29%, respectively). Meanwhile, there were no significant differences (P>0.05) in values of total motility (24.50±1.81% and 26.74±2.14%, respectively) and progressive motility (2.63±0.29% and 2.45±0.37%, respectively) between 1.0 and 2.0 M of glycerol.

Table 4.15 shows velocity distributions of post-thawed cryopreserved sperm of African catfish using TCAYE extender for different molarities of glycerol. There was a significant difference in value of rapid velocity between 0.5 M of glycerol, which gave comparatively the highest value (5.19±0.60%) than 1.0 M (3.46±0.37%) and 2.0 M (3.37±0.51%) of glycerol. For medium and slow velocities, 0.5 M of glycerol gave the significant highest values (1.70±0.14% and 25.39±1.62%, respectively) as compared to 1.0 M of glycerol (1.27±0.13% and 19.76±1.47%, respectively). Conversely, 0.5 M of glycerol gave comparatively the lowest value of static velocity (67.74±2.05%) in comparison with 2.0 M (73.27±2.14%) and 1.0 M of glycerol (75.50±1.81%).

Results for sperm motion characteristics are shown in Table 4.16. The values of VAP and VSL showed significant differences in 2.0 M of glycerol, which were comparatively the lowest (45.84±2.00 µm/s and 40.77±1.85 µm/s, respectively) as compared to 1.0 M (52.80±1.89 µm/s and 47.94±1.81 µm/s, respectively) and 0.5 M of glycerol (56.91±2.27 µm/s and 49.89±2.09 µm/s, respectively). Meanwhile, both 0.5 and

1.0 M did not show any significant differences (P>0.05) in values of VAP (52.80±1.89 and 56.91±2.27 µm/s, respectively) and VSL (47.94±1.81 and 49.89±2.09 µm/s, respectively). There were no significant differences (P>0.05) among the three molarities of glycerol (0.5, 1.0 and 2.0 M) in values of ALH (range: 4.60±0.31-5.34±0.24 µm), BCF (range: 13.61±0.94-14.99±0.89 Hz) and LIN (range: 63.02±1.16-65.18±1.55%). In term of VCL, the three molarities of glycerol showed significant differences, in which 0.5 M of glycerol gave higher value (82.87±3.08 µm/s) as compared to 2.0 M of glycerol (65.43±2.45 µm/s). For value of STR, 0.5 M of glycerol gave the significant lowest value (86.36±0.64%) as compared to 1.0 M (88.78±0.60%) and 2.0 M of glycerol (88.79±0.73%), whereby 1.0 and 2.0 M of glycerol did not show any significant differences in STR values (P>0.05).

Tables 4.17, 4.18 and 4.19 show correlations of sperm motility characteristics of post-thawed cryopreserved sperm of African catfish for 0.5, 1.0 and 2.0 M of glycerol. For 0.5 M of glycerol, 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 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 VAP; slow and VSL; slow and VCL; VAP and VSL; VAP and VCL; VAP and ALH; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; STR and LIN were positively correlated (P<0.05). In contrast, negative correlations (P<0.05) were shown between total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; static and VAP; static and VSL; static and VCL; VCL and STR; VCL and LIN; BCF and STR and BCF and LIN. For 1.0 M of glycerol, positive correlations (P<0.05) were shown among 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; rapid and medium; rapid and slow; medium and slow; 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; ALH and BCF; ALH and LIN and STR and LIN. There were negative correlations ($P<0.05$) in 1.0 M glycerol among total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; slow and BCF; VCL and STR; ALH and STR; BCF and STR and BCF and LIN. In 2.0 M of glycerol, positive correlations ($P<0.05$) were shown among 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; rapid and medium; rapid and slow; medium and slow; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; VCL and BCF; ALH and BCF and STR and LIN. In contrast, total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; VCL and STR; VCL and LIN; ALH and STR; BCF and STR and BCF and LIN were negatively correlated ($P<0.05$).

Table 4.20 shows correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish for overall pooled molarities of glycerol using TCAYE extender. There were positive correlations ($P<0.05$) among 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 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 VAP; slow and VSL; slow and VCL; VAP and VSL; VAP

and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN VCL and ALH; VCL and BCF; ALH and BCF and STR and LIN but negative correlations ($P<0.05$) were shown among total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; static and VAP; static and VSL; static and VCL; slow and static; VCL and STR; VCL and LIN; ALH and STR; BCF and STR and BCF and LIN.

Table 4.14: Total motility and progressive motility (mean ± SEM) of post-thawed cryopreserved sperm of African (*Clarias gariepinus*) catfish using TCAYE extender for different molarities of glycerol

Molarity of glycerol (M)	N*	Total motility (%)	Progressive motility (%)
0.5	135	32.27±2.05[b]	3.75±0.41[b]
1.0	147	24.50±1.81[a]	2.63±0.29[a]
2.0	128	26.74±2.14[ab]	2.45±0.37[a]

N* = Total number of observations (straws).
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.15: Velocity distributions (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using TCAYE extender for different molarities of glycerol

Molarity of glycerol (M)	N*	Rapid (%)	Medium (%)	Slow (%)	Static (%)
0.5	135	5.19±0.60[b]	1.70±0.14[b]	25.39±1.62[b]	67.74±2.05[a]
1.0	147	3.46±0.37[a]	1.27±0.13[a]	19.76±1.47[a]	75.50±1.81[b]
2.0	128	3.37±0.51[a]	1.43±0.16[ab]	21.89±1.70[ab]	73.27±2.14[ab]

N* = Total number of observations (straws).
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.16: Sperm motion characteristics (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using TCAYE extender for different molarities of glycerol

Molarity of glycerol(M)	N*	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
0.5	127	56.91 ±2.27[b]	49.89 ±2.09[b]	82.87 ±3.08[c]	5.34 ±0.24[a]	14.99 ±0.89[a]	86.36 ±0.64[a]	63.02 ±1.16[a]
1.0	135	52.80 ±1.89[b]	47.94 ±1.81[b]	73.80 ±2.22[b]	4.92 ±0.24[a]	13.61 ±0.94[a]	88.78 ±0.60[b]	64.79 ±1.22[a]
2.0	115	45.84 ±2.00[a]	40.77 ±1.85[a]	65.43 ±2.45[a]	4.60 ±0.31[a]	14.55 ±1.21[a]	88.79 ±0.73[b]	65.18 ±1.55[a]

N* = Total number of observations (straws).
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.17: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish using glycerol (0.5 M) in TCAYE

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.667** (135)	.638** (135)	.685** (135)	.879** (135)	-.927** (135)	.267** (127)	.266** (126)	.253** (127)	.145 (127)	.012 (127)	-.027 (127)	.025 (127)
Progressive motility		1	.959** (135)	.702** (135)	.378** (135)	-.627** (135)	.318** (127)	.327** (126)	.266** (127)	.149 (127)	-.076 (127)	.066 (127)	.104 (127)
Rapid			1	.738** (135)	.429** (135)	-.679** (135)	.288** (127)	.286** (126)	.257** (127)	.114 (127)	-.057 (127)	.045 (127)	.067 (127)
Medium				1	.591** (135)	-.746** (135)	.099 (127)	.118 (126)	.072 (127)	.067 (127)	-.069 (127)	.072 (127)	.037 (127)
Slow					1	-.953** (135)	.217* (127)	.217* (126)	.208* (127)	.131 (127)	.033 (127)	-.045 (127)	.014 (127)
Static						1	-.265** (127)	-.265** (126)	-.245** (127)	-.143 (127)	-.002 (127)	.017 (127)	-.036 (127)
VAP							1	.965** (126)	.870** (127)	.499** (127)	-.067 (127)	-.119 (127)	.117 (127)
VSL								1	.753** (126)	.547** (126)	-.136 (126)	.057 (126)	.257** (126)
VCL									1	.375** (127)	.103 (127)	-.385** (127)	-.278** (127)
ALH										1	.158 (127)	-.141 (127)	.141 (127)
BCF											1	-.524** (127)	-.337** (127)
STR												1	.680** (127)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.18: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish using glycerol (1.0 M) in TCAYE

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.744** (147)	.701** (147)	.576** (147)	.752** (147)	-.794** (147)	.035 (135)	.032 (135)	.011 (135)	.044 (135)	-.129 (135)	.019 (135)	.001 (135)
Progressive motility		1	.940** (147)	.622** (147)	.539** (147)	-.675** (147)	-.016 (135)	-.014 (135)	-.016 (135)	.050 (135)	-.038 (135)	.056 (135)	-.058 (135)
Rapid			1	.664** (147)	.633** (147)	-.767** (147)	.011 (135)	.012 (135)	.003 (135)	.055 (135)	-.054 (135)	.058 (135)	-.039 (135)
Medium				1	.579** (147)	-.677** (147)	.094 (135)	.091 (135)	.047 (135)	.118 (135)	-.123 (135)	-.009 (135)	-.006 (135)
Slow					1	-.979** (147)	.081 (135)	.082 (135)	.049 (135)	.033 (135)	-.179* (135)	.038 (135)	.025 (135)
Static						1	-.070 (135)	-.071 (135)	-.040 (135)	-.041 (135)	.155 (135)	-.043 (135)	-.010 (135)
VAP							1	.982** (135)	.872** (135)	.507** (135)	-.078 (135)	.070 (135)	.483** (135)
VSL								1	.792** (135)	.480** (135)	-.145 (135)	.223** (135)	.579** (135)
VCL									1	.475** (135)	.099 (135)	-.185* (135)	.083 (135)
ALH										1	.192* (135)	-.190* (135)	.204* (135)
BCF											1	-.417** (135)	-.267** (135)
STR												1	.572** (135)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.19: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish using glycerol (2.0 M) in TCAYE

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.719** (128)	.703** (128)	.674** (128)	.862** (128)	-.903** (128)	.078 (115)	.065 (115)	.117 (115)	.077 (115)	.145 (115)	-.048 (115)	-.147 (115)
Progressive motility		1	.976** (128)	.772** (128)	.522** (128)	-.706** (128)	-.082 (115)	-.097 (115)	-.015 (115)	-.064 (115)	.142 (115)	-.060 (115)	-.160 (115)
Rapid			1	.776** (128)	.550** (128)	-.735** (128)	-.062 (115)	-.081 (115)	.012 (115)	-.072 (115)	.139 (115)	-.082 (115)	-.177 (115)
Medium				1	.583** (128)	-.720** (128)	-.071 (115)	-.084 (115)	.008 (115)	-.041 (115)	.085 (115)	-.079 (115)	-.182 (115)
Slow					1	-.969** (128)	.088 (115)	.085 (115)	.109 (115)	.091 (115)	.112 (115)	-.020 (115)	-.116 (115)
Static						1	-.049 (115)	-.042 (115)	-.090 (115)	-.054 (115)	-.129 (115)	.041 (115)	.148 (115)
VAP							1	.982** (115)	.893** (115)	.613** (115)	.058 (115)	-.176 (115)	.215* (115)
VSL								1	.827** (115)	.582** (115)	-.029 (115)	-.016 (115)	.312** (115)
VCL									1	.666** (115)	.257** (115)	-.423** (115)	-.191* (115)
ALH										1	.393** (115)	-.324** (115)	-.112 (115)
BCF											1	-.512** (115)	-.446** (115)
STR												1	.587** (115)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

Table 4.20: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm in African catfish (*Clarias gariepinus*) for overall pooled molarities of glycerol using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.707** (410)	.675** (410)	.650** (410)	.835** (410)	-.879** (410)	.145** (377)	.133** (376)	.156** (377)	.097 (377)	.024 (377)	-.041 (377)	-.053 (377)
Progressive motility		1	.960** (410)	.698** (410)	.476** (410)	-.668** (410)	.122* (377)	.116* (376)	.133** (377)	.054 (377)	.018 (377)	.002 (377)	-.046 (377)
Rapid			1	.721** (410)	.524** (410)	-.718** (410)	.129* (377)	.116* (376)	.147** (377)	.042 (377)	.019 (377)	-.014 (377)	-.056 (377)
Medium				1	.589** (410)	-.717** (410)	.053 (377)	.051 (376)	.058 (377)	.050 (377)	-.024 (377)	-.022 (377)	-.065 (377)
Slow					1	-.967** (410)	.141** (377)	.134** (376)	.143** (377)	.092 (377)	.003 (377)	-.030 (377)	-.038 (377)
Static						1	-.147** (377)	-.138** (376)	-.154** (377)	-.087 (377)	-.007 (377)	.028 (377)	.048 (377)
VAP							1	.975** (376)	.879** (377)	.538** (377)	-.027 (377)	-.095 (377)	.251** (377)
VSL								1	.786** (376)	.535** (376)	-.101 (376)	.070 (376)	.365** (376)
VCL									1	.496** (377)	.148** (377)	-.350** (377)	-.144** (377)
ALH										1	.263** (377)	-.235** (377)	.055 (377)
BCF											1	-.483** (377)	-.358** (377)
STR												1	.610** (377)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

4.4 EFFECT OF EQUILIBRATION DURATION ON FROZEN-THAWED SPERM MOTILITY OF AFRICAN CATFISH (*Clarias gariepinus*) USING TCAYE EXTENDER (EXPERIMENT 2)

Table 4.21 shows total motility and progressive motility of post-thawed cryopreserved sperm of African catfish using TCAYE extender for different equilibration durations. At 140 minutes equilibration duration, the value of total motility (31.69±2.19%) was significantly higher as compared to 120 minutes which gave the significant lower value (25.26±1.76%). Furthermore, the values of total motility were not significantly different ($P>0.05$) at 140 minutes (31.69±2.19%) and 160 minutes (28.17±2.11%). Meanwhile, the value of progressive motility did not show a significant difference ($P>0.05$) for the three respective equilibration durations (120, 140 and 160 minutes) ranging from 2.60±0.32 to 3.50±0.41%.

Table 4.22 shows analysis of velocity distributions of post-thawed cryopreserved sperm of African catfish using TCAYE extender for different equilibration durations. There were no significant differences for values of rapid and medium velocities at the three equilibration durations ($P>0.05$) which were ranged from 3.67±0.46 to 4.62±0.53% and 1.35±0.12 to 1.76±0.17%, respectively. Equilibration duration at 140 minute showed that slow velocity had higher significant value (25.37±1.77%) as compared to 120 minutes which gave the lower value (19.92±1.35%). Furthermore, the value of static velocity showed higher value at 120 minutes (74.76±1.76%) as compared to 140 minutes (68.31±2.19%).

Table 4.23 represents analysis of sperm motion characteristics of post-thawed cryopreserved sperm of African catfish using TCAYE extender for different equilibration durations. There were no significant differences ($P>0.05$) in values of VAP (range: 49.64±1.64 to 53.76±2.44 μm/s), VSL (range: 44.21±1.50 to 48.37±2.37 μm/s), VCL

(range: 71.37±2.19 to 77.74±3.09 µm/s), STR (range: 87.47±0.66 to 88.23±0.61%) and LIN (range: 62.64±1.32 to 65.13±1.24%) for the three equilibration intervals (120, 140 and 160 minutes). The values of ALH were not significantly different at 140 minutes (5.40±0.28 µm) and 160 minutes (5.35±0.29 µm) equilibration intervals, but both intervals gave significantly higher values as compared to 120 minutes (4.47±0.22%).

Tables 4.24, 4.25 and 4.26 show correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish at 120, 140 and 160 minutes equilibration durations. At 120 minutes equilibration, positive correlations ($P<0.05$) were shown between total motility and progressive motility; 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 slow; progressive motility and ALH; rapid and medium; rapid and slow; medium and slow; static and STR; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; VCL and BCF; ALH and BCF and STR and LIN, but negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; progressive motility and static; rapid and static; medium and static; medium and STR; slow and static; slow and STR; VCL and STR; VCL and LIN; ALH and STR; BCF and STR and ALH and LIN. At 140 minutes, positive correlations ($P<0.05$) were shown among 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; rapid and medium; rapid and slow; medium and slow; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; ALH and BCF; ALH and LIN and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; progressive motility and static; rapid and static; medium and static;

slow and static; VCL and STR; BCF and STR and BCF and LIN. Equilibration duration of 160 minutes gave positive correlations ($P<0.05$) among 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 BCF; rapid and medium; rapid and slow; medium and slow; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; ALH and BCF and STR and LIN. In contrast, total motility and static; total motility and STR; progressive motility and static; rapid and static; rapid and ALH; medium and static; slow and static; VAP and BCF; VSL and BCF; VCL and STR; ALH and STR; BCF and STR and BCF and LIN were negatively correlated ($P<0.05$).

Table 4.27 demonstrates correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish for overall pooled equilibration durations using TCAYE extender. Positive correlations ($P<0.05$) were shown 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 slow; rapid and medium; rapid and slow; rapid and VCL; medium and slow; slow and VAP; slow and VSL; slow and VCL; 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 LIN; VCL and ALH; VCL and BCF; ALH and BCF and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; progressive motility and static; progressive motility and STR; rapid and static; rapid and STR; medium and static; medium and STR; slow and static; slow and STR; static and

VAP; static and VSL; static and VCL; VCL and STR; VCL and LIN; ALH and STR; BCF and STR and BCF and LIN.

Table 4.21: Total motility and progressive motility (mean ± SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using TCAYE extender for different equilibration durations

Equilibration duration (min)	N*	Total motility (%)	Progressive motility (%)
120	184	25.26±1.76[a]	2.87±0.34[a]
140	125	31.69±2.19[b]	3.50±0.41[a]
160	107	28.17±2.11[ab]	2.60±0.32[a]

N* = Total number of observations (straws).
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.22: Velocity distributions (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using TCAYE extender for different equilibration durations

Equilibration duration (min)	N*	Rapid (%)	Medium (%)	Slow (%)	Static (%)
120	184	3.94±0.49[a]	1.35±0.12[a]	19.92±1.35[a]	74.76±1.76[b]
140	125	4.62±0.53[a]	1.76±0.17[a]	25.37±1.77[b]	68.31±2.19[a]
160	107	3.67±0.46[a]	1.40±0.15[a]	23.07±1.74[ab]	71.83±2.11[ab]

N* = Total number of observations (straws).
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.23: Sperm motion characteristics (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using TCAYE extender for different equilibration durations

Equilibration duration (min)	N*	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
120	173	49.64 ±1.64[a]	44.21 ±1.50[a]	71.37 ±2.19[a]	4.47 ±0.22[a]	14.47 ±0.91[ab]	88.23 ±0.61[a]	65.13 ±1.24[a]
140	113	53.76 ±2.44[a]	48.37 ±2.37[a]	75.66 ±2.86[a]	5.40 ±0.28[b]	12.75 ±0.86[a]	87.77 ±0.68[a]	64.12 ±1.26[a]
160	100	53.73 ±2.32[a]	47.33 ±2.07[a]	77.74 ±3.09[a]	5.35 ±0.29[b]	16.27 ±1.21[b]	87.47 ±0.66[a]	62.64 ±1.32[a]

N* = Total number of observations (straws).
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.24: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for 120 min equilibration duration using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.752** (184)	.735** (184)	.731** (184)	.892** (184)	-.936** (184)	.151* (173)	.124 (173)	.138 (173)	.138 (173)	.063 (173)	-.168* (173)	-.041 (173)
Progressive motility		1	.981** (184)	.750** (184)	.532** (184)	-.730** (184)	.131 (173)	.120 (173)	.123 (173)	.152* (173)	.100 (173)	-.105 (173)	-.051 (173)
Rapid			1	.769** (184)	.554** (184)	-.753** (184)	.147 (173)	.133 (173)	.141 (173)	.138 (173)	.070 (173)	-.113 (173)	-.040 (173)
Medium				1	.678** (184)	-.795** (184)	.126 (173)	.103 (173)	.109 (173)	.130 (173)	.091 (173)	-.172* (173)	-.003 (173)
Slow					1	-.964** (184)	.148 (173)	.121 (173)	.123 (173)	.132 (173)	.022 (173)	-.155* (173)	.000 (173)
Static						1	-.164* (173)	-.139 (173)	-.141 (173)	-.148 (173)	-.040 (173)	.159* (173)	.009 (173)
VAP							1	.973** (173)	.850** (173)	.544** (173)	.076 (173)	-.125 (173)	.208** (173)
VSL								1	.741** (173)	.529** (173)	-.029 (173)	.072 (173)	.351** (173)
VCL									1	.557** (173)	.276** (173)	-.469** (173)	-.240** (173)
ALH										1	.327** (173)	-.241** (173)	-.069 (173)
BCF											1	-.505** (173)	-.403** (173)
STR												1	.730** (173)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.25: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for 140 min equilibration duration using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.675** (125)	.689** (125)	.656** (125)	.954** (125)	-.991** (125)	.068 (113)	.077 (113)	.061 (113)	-.038 (113)	-.030 (113)	-.036 (113)	-.076 (113)
Progressive motility		1	.974** (125)	.720** (125)	.465** (125)	-.672** (125)	-.003 (113)	-.006 (113)	.040 (113)	-.103 (113)	-.025 (113)	-.083 (113)	-.119 (113)
Rapid			1	.743** (125)	.483** (125)	-.693** (125)	.028 (113)	.025 (113)	.067 (113)	-.100 (113)	-.021 (113)	-.081 (113)	-.102 (113)
Medium				1	.509** (125)	-.674** (125)	-.012 (113)	-.005 (113)	-.017 (113)	-.113 (113)	.021 (113)	-.003 (113)	-.053 (113)
Slow					1	-.963** (125)	.080 (113)	.096 (113)	.052 (113)	.002 (113)	-.027 (113)	.000 (113)	-.037 (113)
Static						1	-.073 (113)	-.085 (113)	-.061 (113)	.030 (113)	.025 (113)	.019 (113)	.059 (113)
VAP							1	.988** (113)	.927** (113)	.665** (113)	.003 (113)	-.066 (113)	.308** (113)
VSL								1	.888** (113)	.644** (113)	-.057 (113)	.061 (113)	.367** (113)
VCL									1	.642** (113)	.113 (113)	-.220* (113)	.020 (113)
ALH										1	.200* (113)	-.152 (113)	.228* (113)
BCF											1	-.404** (113)	-.187* (113)
STR												1	.507** (113)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.26: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for 160 min equilibration duration using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.673** (107)	.525** (107)	.502** (107)	.550** (107)	-.598** (107)	.115 (100)	.088 (100)	.170 (100)	.057 (100)	.146 (100)	-.262** (100)	-.017 (100)
Progressive motility		1	.877** (107)	.573** (107)	.374** (107)	-.533** (107)	.000 (100)	-.020 (100)	.118 (100)	-.137 (100)	.210* (100)	-.138 (100)	-.034 (100)
Rapid			1	.628** (107)	.539** (107)	-.700** (107)	-.027 (100)	-.057 (100)	.117 (100)	-.199* (100)	.136 (100)	-.100 (100)	-.109 (100)
Medium				1	.565** (107)	-.662** (107)	.066 (100)	.052 (100)	.149 (100)	-.024 (100)	.182 (100)	-.136 (100)	-.089 (100)
Slow					1	-.978** (107)	.102 (100)	.072 (100)	.165 (100)	.156 (100)	.179 (100)	-.180 (100)	-.097 (100)
Static						1	-.082 (100)	-.051 (100)	-.169 (100)	-.082 (100)	-.185 (100)	.176 (100)	.106 (100)
VAP							1	.962** (100)	.861** (100)	.309** (100)	-.213* (100)	-.043 (100)	.299** (100)
VSL								1	.723** (100)	.339** (100)	-.260** (100)	.120 (100)	.439** (100)
VCL									1	.202* (100)	-.003 (100)	-.276** (100)	-.143 (100)
ALH										1	.308** (100)	-.346** (100)	.069 (100)
BCF											1	-.537** (100)	-.430** (100)
STR												1	.480** (100)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.27: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for overall pooled equilibration durations using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.709** (416)	.677** (416)	.656** (416)	.836** (416)	-.881** (416)	.122* (386)	.108* (386)	.129* (386)	.080 (386)	.053 (386)	-.152** (386)	-.048 (386)
Progressive motility		1	.961** (416)	.703** (416)	.477** (416)	-.672** (416)	.059 (386)	.051 (386)	.094 (386)	.016 (386)	.078 (386)	-.103* (386)	-.065 (386)
Rapid			1	.726** (416)	.524** (416)	-.720** (416)	.073 (386)	.060 (386)	.112* (386)	.000 (386)	.054 (386)	-.101* (386)	-.068 (386)
Medium				1	.594** (416)	-.723** (416)	.065 (386)	.056 (386)	.077 (386)	.021 (386)	.078 (386)	-.108* (386)	-.036 (386)
Slow					1	-.967** (416)	.123* (386)	.110* (386)	.120* (386)	.115* (386)	.046 (386)	-.118* (386)	-.037 (386)
Static						1	-.122* (386)	-.108* (386)	-.130* (386)	-.092 (386)	-.053 (386)	.124* (386)	.047 (386)
VAP							1	.975** (386)	.877** (386)	.529** (386)	-.024 (386)	-.089 (386)	.249** (386)
VSL								1	.785** (386)	.525** (386)	-.098 (386)	.075 (386)	.362** (386)
VCL									1	.493** (386)	.155** (386)	-.350** (386)	-.149** (386)
ALH										1	.281** (386)	-.241** (386)	.036 (386)
BCF											1	-.484** (386)	-.362** (386)
STR												1	.621** (386)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

4.6 EFFECT OF VAPOUR TEMPERATURE ON FROZEN-THAWED SPERM MOTILITY OF AFRICAN CATFISH (*Clarias gariepinus*) USING TCAYE EXTENDER (EXPERIMENT 2)

Table 4.28 shows total motility and progressive motility of post-thawed cryopreserved sperm of African catfish using TCAYE extender for different vapour temperatures. There were no significant differences (P>0.05) in values of total motility and progressive motility for -80, -90 and -100^{o}C which were ranged from 25.95±2.34 to 29.41±1.69% and 2.73±0.39 to 3.25±0.32%, respectively.

Table 4.29 shows sperm velocity distributions of post-thawed cryopreserved sperm of African catfish using TCAYE extender for different vapour temperatures. Post-thawed sperm that were cryopreserved under the three respective vapour temperatures (-80, -90 and -100^{o}C) did not show any significant differences (P>0.05) in values of rapid (range: 3.67±0.55-4.39±0.43%), medium (range: 1.32±0.14-1.60±0.13%), slow (range: 20.43±1.94-23.43±1.30%) and static velocities (range: 70.59±1.69-74.06±2.34%).

Table 4.30 shows sperm motion characteristics of post-thawed cryopreserved sperm of African catfish using TCAYE extender for different vapour temperatures. The values of VAP, VSL, ALH and BCF were not significantly different (P>0.05) for -80, -90 and -100^{o}C which were ranged from 50.13±2.20 to 55.66±2.57 µm/s, 45.62±2.12 to 48.67±2.22 µm/s, 4.81±0.22 to 5.40±0.30 µm and 14.03±0.78 to 15.62±1.13 Hz, respectively. Furthermore, under -80 and -90^{o}C, the respective values of VCL (81.50±4.20 and 75.13±2.08 µm/s), STR (86.23±0.89 and 87.21±0.56%) and LIN (62.16±1.68 and 62.80±1.07%) were not significantly different (P>0.05). For VCL and LIN values, -80^{o}C (81.50±4.20 µm/s and 62.16±1.68%, respectively) and -90^{o}C (75.13±2.08 µm/s and 62.80±1.07%, respectively) were higher values as compared to -100^{o}C (69.60±2.44 µm/s and 67.14±1.30%, respectively). In contrast, the value of STR was significantly the highest

under -100^{o}C (89.77±0.60%) as compared to -80^{o}C (86.23±0.89%) and -90^{o}C (87.21±0.56%).

Tables 4.31, 4.32 and 4.33 show correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish using TCAYE extender for -80, -90 and -100^{o}C vapour temperatures. For -80^{o}C, positive correlations were ($P<0.05$) indicated by total motility and progressive motility; rapid and medium; rapid and slow; medium and slow; slow and BCF; VAP and VSL; VAP and VCL; VAP and ALH; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; ALH and BCF and STR and LIN. Conversely, negative correlations were shown by progressive motility and VCL; rapid and static; rapid and STR; medium and static; slow and static; VCL and STR; VCL and LIN; BCF and STR and BCF and LIN. Vapour temperature of -90^{o}C shows positive correlations among total motility and progressive motility; total motility and slow; rapid and medium; rapid and slow; rapid and ALH; medium and slow; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; VCL and BCF; ALH and BCF and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown by total motility and static; rapid and static; medium and static; slow and static; static and ALH; VCL and STR; ALH and STR; BCF and STR and BCF and LIN. For -100^{o}C, positive correlations ($P<0.05$) were shown by total motility and progressive motility; total motility and rapid; total motility and medium; progressive motility and rapid; progressive motility and medium; progressive motility and VCL; rapid and medium; rapid and slow; rapid and VCL; medium and slow; medium and VCL; static and STR; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; ALH and BCF and STR and LIN. In contrast, total motility and static; total motility and STR; total motility and LIN; progressive motility and static; progressive motility and STR; rapid and static; rapid and STR; medium and static;

medium and STR; slow and static; slow and STR; static and VCL; static and STR; VSL and BCF; VCL and STR; ALH and STR; BCF and STR and BCF and LIN were negatively correlated ($P<0.05$).

Table 4.34 demonstrates correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish for overall pooled vapour temperatures using TCAYE extender. Positive correlations ($P<0.05$) were shown by total motility and progressive motility; total motility and rapid; total motility and slow; progressive motility and rapid; progressive motility and medium; progressive motility and slow; rapid and medium; rapid and slow; rapid and VCL; medium and slow; medium 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 LIN; VCL and ALH; VCL and BCF; ALH and BCF and STR and LIN. In contrast, total motility and static; total motility and STR; total motility and LIN; progressive motility and static; rapid and static; rapid and STR; rapid and LIN; medium and static; medium and STR; slow and static; static and ALH; VSL and BCF; VCL and STR; VCL and LIN; ALH and STR; BCF and STR and BCF and LIN show negative correlation ($P<0.05$).

Table 4.28: Total motility and progressive motility (mean ± SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using TCAYE extender for different vapour temperatures

Vapour temperature (^oC)	N*	Total motility (%)	Progressive motility (%)
-80	77	25.95±2.34[a]	2.82±0.31[a]
-90	193	29.41±1.69[a]	3.25±0.32[a]
-100	146	27.04±1.16[a]	2.73±0.39[a]

N* = Total number of observations (straws).
[a]Means with same superscript within a column were not significantly different (P>0.05).

Table 4.29: Velocity distributions (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using TCAYE extender for different vapour temperatures

Vapour temperature (^oC)	N*	Rapid (%)	Medium (%)	Slow (%)	Static (%)
-80	77	4.04±0.50[a]	1.53±0.14[a]	20.43±1.94[a]	74.06±2.34[a]
-90	193	4.39±0.43[a]	1.60±0.13[a]	23.43±1.30[a]	70.59±1.69[a]
-100	146	3.67±0.55[a]	1.32±0.14[a]	21.98±1.69[a]	72.97±2.09[a]

N* = Total number of observations (straws).
[a]Means with same superscript within a column were not significantly different (P>0.05).

Table 4.30: Sperm motion characteristics (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using TCAYE extender for different vapour temperatures

Vapour temperature (^oC)	N*	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
-80	75	55.66 ±2.57[a]	48.67 ±2.22[a]	81.50 ±4.20[b]	5.40 ±0.30[a]	15.62 ±1.13[a]	86.23 ±0.89[a]	62.16 ±1.68[a]
-90	177	51.96 ±1.68[a]	45.94 ±1.56[a]	75.13 ±2.08a[b]	4.81 ±0.22[a]	14.03 ±0.78[a]	87.21 ±0.56[a]	62.80 ±1.07[a]
-100	131	50.13 ±2.20[a]	45.62 ±2.12[a]	69.60 ±2.44[a]	4.99 ±0.27[a]	14.46 ±1.15[a]	89.77 ±0.60[b]	67.14 ±1.30[b]

N* = Total number of observations (straws).
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.31: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at -80^{o}C vapour temperature using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.705** (77)	.047 (77)	.011 (77)	-.087 (77)	.066 (77)	-.128 (75)	-.115 (75)	-.154 (75)	-.049 (75)	-.041 (75)	.030 (75)	-.001 (75)
Progressive motility		1	.047 (77)	.006 (77)	-.035 (77)	.022 (77)	-.212 (75)	-.186 (75)	-.231* (75)	-.117 (75)	-.053 (75)	.038 (75)	.038 (75)
Rapid			1	.552** (77)	.578** (77)	-.721** (77)	.147 (75)	.138 (75)	.110 (75)	.134 (75)	.093 (75)	-.267* (75)	-.127 (75)
Medium				1	.567** (77)	-.639** (77)	.061 (75)	.106 (75)	-.059 (75)	.126 (75)	.057 (75)	-.016 (75)	.047 (75)
Slow					1	-.981** (77)	.083 (75)	.084 (75)	.060 (75)	.125 (75)	.242* (75)	-.159 (75)	-.083 (75)
Static						1	-.100 (75)	-.101 (75)	-.068 (75)	-.135 (75)	-.225 (75)	.191 (75)	.092 (75)
VAP							1	.944** (75)	.848** (75)	.399** (75)	-.084 (75)	.013 (75)	.091 (75)
VSL								1	.669** (75)	.501** (75)	-.123 (75)	.226 (75)	.310** (75)
VCL									1	.279* (75)	.078 (75)	-.303** (75)	-.330** (75)
ALH										1	.350** (75)	.052 (75)	.108 (75)
BCF											1	-.308** (75)	-.295* (75)
STR												1	.807** (75)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.32: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at -90^{o}C vapour temperature using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.762** (193)	.037 (193)	.008 (193)	.196** (193)	-.159* (193)	-.035 (177)	-.058 (177)	.027 (177)	-.033 (177)	.087 (177)	-.104 (177)	-.073 (177)
Progressive motility		1	.046 (193)	.001 (193)	.168* (193)	-.138 (193)	-.047 (177)	-.057 (177)	-.001 (177)	-.095 (177)	.117 (177)	-.033 (177)	-.097 (177)
Rapid			1	.779** (193)	.604** (193)	-.781** (193)	.024 (177)	.018 (177)	.032 (177)	.166* (177)	.097 (177)	-.096 (177)	-.066 (177)
Medium				1	.602** (193)	-.738** (193)	-.044 (177)	-.029 (177)	-.040 (177)	.139 (177)	.029 (177)	-.051 (177)	-.094 (177)
Slow					1	-.967** (193)	.009 (177)	.027 (177)	-.007 (177)	.142 (177)	.005 (177)	.026 (177)	-.034 (177)
Static						1	-.005 (177)	-.018 (177)	.005 (177)	-.158* (177)	-.029 (177)	.003 (177)	.047 (177)
VAP							1	.976** (177)	.891** (177)	.526** (177)	.087 (177)	-.104 (177)	.294** (177)
VSL								1	.812** (177)	.510** (177)	-.024 (177)	.074 (177)	.395** (177)
VCL									1	.558** (177)	.285** (177)	-.351** (177)	-.092 (177)
ALH										1	.337** (177)	-.256** (177)	-.018 (177)
BCF											1	-.610** (177)	-.401** (177)
STR												1	.575** (177)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.33: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at -100^{o}C vapour temperature using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.655** (146)	.294** (146)	.216** (146)	.125 (146)	-.191* (146)	-.020 (131)	-.071 (131)	.066 (131)	.150 (131)	.086 (131)	-.316** (131)	-.191* (131)
Progressive motility		1	.704** (146)	.432** (146)	.135 (146)	-.323** (146)	.130 (131)	.083 (131)	.210* (131)	.105 (131)	-.034 (131)	-.220* (131)	-.144 (131)
Rapid			1	.707** (146)	.428** (146)	-.657** (146)	.109 (131)	.053 (131)	.221* (131)	.016 (131)	.085 (131)	-.291** (131)	-.148 (131)
Medium				1	.604** (146)	-.738** (146)	.130 (131)	.087 (131)	.202* (131)	.116 (131)	.047 (131)	-.232** (131)	-.074 (131)
Slow					1	-.961** (146)	.081 (131)	.055 (131)	.145 (131)	.162 (131)	-.005 (131)	-.206* (131)	-.102 (131)
Static						1	-.103 (131)	-.064 (131)	-.190* (131)	-.142 (131)	-.020 (131)	.258** (131)	.128 (131)
VAP							1	.990** (131)	.907** (131)	.573** (131)	-.135 (131)	-.098 (131)	.324** (131)
VSL								1	.862** (131)	.538** (131)	-.184* (131)	.022 (131)	.389** (131)
VCL									1	.556** (131)	.044 (131)	-.342** (131)	-.035 (131)
ALH										1	.174* (131)	-.398** (131)	.075 (131)
BCF											1	-.475** (131)	-.379** (131)
STR												1	.524** (131)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.34: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for overall pooled vapour temperatures using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.709** (416)	.148** (416)	.090 (416)	.129** (416)	-.144** (416)	-.045 (383)	-.074 (382)	.001 (383)	.033 (383)	.065 (383)	-.151** (383)	-.106* (383)
Progressive motility		1	.330** (416)	.172** (416)	.134** (416)	-.199** (416)	.006 (383)	-.012 (382)	.040 (383)	-.019 (383)	.029 (383)	-.092 (383)	-.100 (383)
Rapid			1	.726** (416)	.524** (416)	-.720** (416)	.079 (383)	.049 (382)	.118* (383)	.095 (383)	.090 (383)	-.196** (383)	-.111* (383)
Medium				1	.594** (416)	-.723** (416)	.043 (383)	.037 (382)	.048 (383)	.127* (383)	.040 (383)	-.117* (383)	-.074 (383)
Slow					1	-.967** (416)	.048 (383)	.046 (382)	.058 (383)	.143** (383)	.034 (383)	-.088 (383)	-.068 (383)
Static						1	-.058 (383)	-.048 (382)	-.076 (383)	-.144** (383)	-.051 (383)	.124* (383)	.086 (383)
VAP							1	.975** (382)	.877** (383)	.525** (383)	-.033 (383)	-.091 (383)	.254** (383)
VSL								1	.784** (382)	.521** (382)	-.107* (382)	.074 (382)	.368** (382)
VCL									1	.489** (383)	.148** (383)	-.351** (383)	-.146** (383)
ALH										1	.271** (383)	-.246** (383)	.037 (383)
BCF											1	-.490** (383)	-.368** (383)
STR												1	.615** (383)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

4.6 EFFECT OF VAPOUR EXPOSURE DURATION ON FROZEN-THAWED SPERM MOTILITY OF AFRICAN CATFISH (*Clarias gariepinus*) USING TCAYE EXTENDER (EXPERIMENT 2)

Table 4.35 shows total motility and progressive motility of post-thawed cryopreserved sperm of African catfish using TCAYE extender for different vapour exposure durations. The values of total motility and progressive motility did not show any significant differences (P>0.05) among the three vapour exposure durations (5, 10 and 15 minutes), which were ranged from 27.63±2.02 to 28.45±2.14% and 2.79±0.29 to 3.18±0.35%, respectively.

Table 4.36 shows velocity distributions of post-thawed cryopreserved sperm of African catfish using TCAYE extender for different vapour exposure durations. There were no significant differences (P>0.05) in values of rapid, medium, slow and static at 5, 10 and 15 minutes exposure which were ranged from 3.72±0.38 to 4.47±0.64%, 1.34±0.12 to 1.62±0.16%, 21.78±1.5 to 22.70±1.53% and 71.80±2.20 to 72.26±1.88%, respectively.

Table 4.37 shows sperm motion characteristics of post-thawed cryopreserved sperm of African catfish using TCAYE extender for different vapour exposure durations. The values of VAP, VSL, VCL, ALH, BCF and STR did not show any significant differences (P>0.05) among 5, 10 and 15 minutes, which were ranged from 50.46±1.94 to 53.80±2.55 µm/s, 44.51±1.79 to 48.37±2.38 µm/s, 73.28±2.42 to 75.10±3.31 µm/s, 4.84±0.26 to 5.15±0.23 µm, 13.06±0.95 to 15.74±1.07 Hz and 86.87±0.65 to 88.65±0.70%. However, the value of LIN gave higher significant value at 15 minutes vapour exposure (65.97±1.50%) as compared to 10 minutes vapour exposure duration, which attained lower value (61.93±1.23%).

Tables 4.38, 4.39 and 4.40 show correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish for 5, 10 and 15 minutes vapour

exposure durations. At 5 minutes vapour exposure duration, positive correlations ($P<0.05$) were shown among total motility and progressive motility; total motility and rapid; total motility and medium; total motility and slow; rapid and medium; rapid and slow; medium and slow; medium and BCF; slow and BCF; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; VCL and BCF and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown among total motility and static; rapid and static; medium and static; slow and static; static and BCF; VCL and STR; VCL and LIN; ALH and STR; BCF and STR and BCF and LIN. At 10 minutes vapour exposure duration, 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; rapid and medium; rapid and slow; rapid and VAP; rapid and VSL; rapid and VCL; medium and slow; slow and VAP; slow and VSL; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; ALH and BCF and STR and LIN were positively correlated ($P<0.05$). Conversely, negative correlations ($P<0.05$) were shown among total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; static and VAP; static and VSL; static and VCL; VCL and STR; BCF and STR and STR and LIN. Vapour exposure duration of 15 minutes showed positive correlations ($P<0.05$) among total motility and progressive motility; progressive motility and rapid; progressive motility and BCF; rapid and medium; rapid and slow; medium and slow; slow and VSL; slow and LIN; VAP and VSL; VAP and VCL; VAP and ALH; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; ALH and BCF and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown among rapid and static; medium and static; slow and static; VCL and STR; VCL and LIN; ALH and STR; BCF and STR and BCF and LIN.

Table 4.41 shows correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish for overall pooled vapour exposure durations using TCAYE extender. Positive correlations ($P<0.05$) were shown among 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; rapid and medium; rapid and slow; rapid and VAP; rapid and VSL; rapid and VCL; medium and slow; medium and BCF; slow and static; slow and VAP; slow and VSL; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; VCL and BCF; ALH and BCF and STR and LIN. Conversely, total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; static and VAP; static and VSL; VSL and BCF; VCL and STR; VCL and LIN; ALH and STR; BCF and STR and BCF and LIN showed negative correlations ($P<0.05$).

Table 4.35: Total motility and progressive motility (mean ± SEM) of post-thawed cryopreserved sperm of African (*Clarias gariepinus*) catfish using TCAYE extender for different vapour exposure durations

Vapour exposure duration (min)	N*	Total motility (%)	Progressive motility (%)
5	148	27.74±1.88[a]	2.79±0.29[a]
10	131	27.63±2.02[a]	3.02±0.46[a]
15	137	28.45±2.14[a]	3.18±0.35[a]

N* = Total number of observations (straws).
[a]Means with same superscript within a column were not significantly different (P>0.05).

Table 4.36: Velocity distributions (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using TCAYE extender for different vapour exposure durations

Vapour exposure duration (min)	N*	Rapid (%)	Medium (%)	Slow (%)	Static (%)
5	148	3.72±0.38[a]	1.34±0.12[a]	22.70±1.53[a]	72.26±1.88[a]
10	136	4.47±0.64[a]	1.62±0.16[a]	21.78±1.51[a]	72.12±1.97[a]
15	132	4.06±0.48[a]	1.52±0.15[a]	22.59±1.75[a]	71.80±2.20[a]

N* = Total number of observations (straws).
[a]Means with same superscript within a column were not significantly different (P>0.05).

Table 4.37: Sperm motion characteristics (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using TCAYE extender 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	137	51.97 ±1.71[a]	46.26 ±1.59[a]	75.04 ±2.20[a]	5.15 ±0.23[a]	14.62 ±0.98[a]	88.15 ±0.62[a]	64.57 ±1.15[ab]
10	124	50.46 ±1.94[a]	44.51 ±1.79[a]	73.28 ±2.42[a]	4.84 ±0.26[a]	15.74 ±1.07[a]	86.87 ±0.65[a]	61.93 ±1.23[a]
15	122	53.80 ±2.55[a]	48.37 ±2.38[a]	75.10 ±3.31[a]	4.95 ±0.29[a]	13.06 ±0.95[a]	88.65 ±0.70[a]	65.97 ±1.50[b]

N* = Total number of observations (straws).
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.38: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at 5 min vapour exposure duration using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.717** (148)	.191* (148)	.204* (148)	.184* (148)	-.195* (148)	.115 (137)	.104 (137)	.142 (137)	.064 (137)	.024 (137)	-.056 (137)	.011 (137)
Progressive motility		1	.090 (148)	.110 (148)	.138 (148)	-.127 (148)	.095 (137)	.079 (137)	.141 (137)	.125 (137)	-.002 (137)	-.109 (137)	-.086 (137)
Rapid			1	.655** (148)	.615** (148)	-.747** (148)	.050 (137)	.043 (137)	.042 (137)	-.034 (137)	.011 (137)	-.023 (137)	.043 (137)
Medium				1	.696** (148)	-.762** (148)	-.023 (137)	-.041 (137)	-.018 (137)	-.045 (137)	.205* (137)	-.069 (137)	-.034 (137)
Slow					1	-.981** (148)	-.013 (137)	-.032 (137)	-.008 (137)	-.010 (137)	.223** (137)	-.085 (137)	-.049 (137)
Static						1	.006 (137)	.024 (137)	.002 (137)	.024 (137)	-.193* (137)	.076 (137)	.034 (137)
VAP							1	.975** (137)	.846** (137)	.652** (137)	-.034 (137)	-.143 (137)	.267** (137)
VSL								1	.757** (137)	.623** (137)	-.120 (137)	.045 (137)	.379** (137)
VCL									1	.585** (137)	.196* (137)	-.453** (137)	-.182* (137)
ALH										1	.105 (137)	-.244** (137)	.112 (137)
BCF											1	-.472** (137)	-.335** (137)
STR												1	.626** (137)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

Table 4.39: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at 10 min vapour exposure duration using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.679** (131)	.353** (131)	.243** (131)	.197* (131)	-.289** (131)	.050 (124)	.036 (123)	.096 (124)	-.053 (124)	-.051 (124)	-.135 (124)	-.074 (124)
Progressive motility		1	.563** (131)	.321** (131)	.138 (131)	-.317** (131)	.076 (124)	.067 (123)	.110 (124)	.009 (124)	-.020 (124)	-.055 (124)	-.065 (124)
Rapid			1	.737** (131)	.394** (131)	-.685** (131)	.226* (124)	.198* (123)	.237** (124)	.138 (124)	.011 (124)	-.119 (124)	.023 (124)
Medium				1	.579** (131)	-.759** (131)	.087 (124)	.083 (123)	.079 (124)	.129 (124)	.031 (124)	-.100 (124)	-.009 (124)
Slow					1	-.938** (131)	.209* (124)	.228* (123)	.164 (124)	.158 (124)	-.068 (124)	.025 (124)	.098 (124)
Static						1	-.240** (124)	-.245** (123)	-.208* (124)	-.173 (124)	.046 (124)	.028 (124)	-.083 (124)
VAP							1	.975** (123)	.875** (124)	.445** (124)	-.040 (124)	-.091 (124)	.388** (124)
VSL								1	.792** (123)	.463** (123)	-.135 (123)	.091 (123)	.499** (123)
VCL									1	.422** (124)	.138 (124)	-.294** (124)	-.007 (124)
ALH										1	.287** (124)	-.118 (124)	.142 (124)
BCF											1	-.475** (124)	-.368** (124)
STR												1	.495** (124)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

Table 4.40: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at 15 min vapour exposure duration using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.767** (137)	.123 (132)	.055 (132)	.091 (132)	-.102 (132)	-.132 (122)	-.104 (122)	-.176 (122)	-.048 (122)	.091 (122)	.174 (122)	.135 (122)
Progressive motility		1	.185* (137)	.076 (132)	.145 (132)	-.160 (132)	-.134 (122)	-.130 (122)	-.153 (122)	.091 (122)	.222* (122)	.026 (122)	.047 (122)
Rapid			1	.786** (132)	.655** (132)	-.795** (132)	.090 (122)	.080 (122)	.096 (122)	.124 (122)	.148 (122)	-.125 (122)	-.032 (122)
Medium				1	.532** (132)	-.664** (132)	.128 (122)	.115 (122)	.121 (122)	.040 (122)	.072 (122)	-.080 (122)	.006 (122)
Slow					1	-.978** (132)	.156 (122)	.182* (122)	.074 (122)	.102 (122)	-.013 (122)	.078 (122)	.205* (122)
Static						1	-.153 (122)	-.171 (122)	-.088 (122)	-.112 (122)	-.027 (122)	-.030 (122)	-.159 (122)
VAP							1	.975** (122)	.896** (122)	.504** (122)	-.012 (122)	-.068 (122)	.150 (122)
VSL								1	.796** (122)	.502** (122)	-.062 (122)	.071 (122)	.268** (122)
VCL									1	.478** (122)	.133 (122)	-.332** (122)	-.217* (122)
ALH										1	.435** (122)	-.370** (122)	-.105 (122)
BCF											1	-.517** (122)	-.393** (122)
STR												1	.693** (122)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

Table 4.41: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for overall pooled vapour exposure durations using TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.709** (416)	.228** (411)	.164** (411)	.153** (411)	-.189** (411)	-.005 (383)	-.001 (382)	-.003 (383)	-.015 (383)	.019 (383)	.002 (383)	.035 (383)
Progressive motility		1	.349** (411)	.189** (411)	.137** (411)	-.207** (411)	.002 (383)	-.003 (382)	.016 (383)	.065 (383)	.052 (383)	-.043 (383)	-.029 (383)
Rapid			1	.726** (411)	.524** (411)	-.720** (411)	.127* (383)	.110* (382)	.130* (383)	.085 (383)	.050 (383)	-.096 (383)	.005 (383)
Medium				1	.594** (411)	-.723** (411)	.071 (383)	.059 (382)	.066 (383)	.044 (383)	.100* (383)	-.086 (383)	-.015 (383)
Slow					1	-.967** (411)	.120* (383)	.130* (382)	.073 (383)	.083 (383)	.051 (383)	.010 (383)	.097 (383)
Static						1	-.131* (383)	-.135** (382)	-.094 (383)	-.088 (383)	-.059 (383)	.021 (383)	-.078 (383)
VAP							1	.975** (382)	.877** (383)	.525** (383)	-.033 (383)	-.091 (383)	.254** (383)
VSL								1	.784** (382)	.521** (382)	-.107* (382)	.074 (382)	.368** (382)
VCL									1	.489** (383)	.148** (383)	-.351** (383)	-.146** (383)
ALH										1	.271** (383)	-.246** (383)	.037 (383)
BCF											1	-.490** (383)	-.368** (383)
STR												1	.615** (383)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

4.7 EFFECT OF EQUILIBRATION DURATION ON FROZEN-THAWED SPERM MOTILITY OF AFRICAN CATFISH (*Clarias gariepinus*) USING FRE EXTENDER (EXPERIMENT 3)

Table 4.42 demonstrates total motility and progressive motility of post-thawed cryopreserved sperm of African catfish using FRE extender for different equilibration durations. There were no significant differences (P>0.05) in values of total motility at 120 minutes (76.65±2.27%) and 160 minutes equilibrations (76.01±2.04%), but these durations gave comparatively higher values of total motility than 140 minutes (66.90±2.60%). For progressive motility, 140 minutes (15.85±1.06%) and 160 minutes (16.97±0.89%) did not show any significant differences (P>0.05), but were significantly lower values as compared to 120 minutes (21.60±1.09%).

Results for velocity distributions of post-thawed cryopreserved sperm of African catfish using FRE extender for different equilibration durations are shown in Table 4.43. At 120 minutes equilibration duration of sperm with extender showed to have significantly better rapid and medium velocity distributions of post-thawed cryopreserved sperm. However, at 140 and 160 minutes equilibrations, there were no significant differences (P>0.05) in values of rapid velocity which gave 20.56±1.35% and 22.05±1.15%, respectively. The values of medium and static velocities did not show any significant differences (P>0.05) at 120 minutes (10.02±0.51% and 23.01±2.26%, respectively) and 160 minutes equilibrations (9.64±0.42% and 23.92±2.07%, respectively). At 140 minutes equilibration, the value of medium velocity was significantly the lowest (7.98±0.48%) as compared to 160 minutes (9.64±0.42%) and 120 minutes (10.02±0.51%). Conversely, the value of static velocity was significantly the highest at 140 minutes (33.14±2.55%) as compared to 120 minutes (23.01±2.26%) and 160 minutes (23.92±2.07%). For slow velocity, equilibration duration of 160 minute was significantly the highest value

(44.44±1.11%) as compared to 120 minutes (38.25±1.17%) and 140 minutes (38.32±1.50%), which both values of slow velocities at 120 and 140 minutes were not significantly different (P>0.05).

Table 4.44 demonstrates sperm motion characteristics of post-thawed cryopreserved sperm of African catfish using FRE extender for different equilibration durations. There were no significant differences (P>0.05) for values of VAP, VSL, VCL, ALH, STR and LIN at 120, 140 and 160 minutes, which were ranged from 54.68±1.58 to 58.58±1.28 μm/s, 47.91±1.43 to 51.17±1.09 μm/s, 75.88±2.01 to 80.59±1.56 μm/s, 4.97±0.20 to 5.42±0.20 μm, 87.28±0.39 to 87.54±0.45% and 63.44±0.64 to 64.36±0.66%, respectively. At 160 minute equilibration duration, the value of BCF was significantly the highest (13.73±0.51 Hz) as compared to 120 minutes (12.59±0.42 Hz) and 140 minutes (12.11±0.47 Hz).

Tables 4.45, 4.46 and 4.47 show correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish at 120, 140 and 160 minutes equilibration durations. Equilibration duration of 120 minutes showed positive correlations (P<0.05) among total motility and progressive motility; total motility and rapid; total motility and medium; total motility and slow; total motility and VAP; total motility and VCL; progressive motility and rapid; progressive motility and medium; progressive motility and slow; rapid and medium; rapid and VCL; medium and slow; medium and VAP; medium 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 LIN; VCL and ALH; ALH and BCF and STR and LIN. Conversely, negative correlations (P<0.05) were shown among total motility and static; total motility and STR; progressive motility and static; progressive motility and STR; rapid and static; rapid and STR; medium and static; slow and static; static and VAP; static and VSL; static and VCL; VAP and STR; VSL and STR; VCL and

STR; ALH and STR; BCF and STR and BCF and LIN. Equilibration duration of 140 minutes showed positive correlations ($P<0.05$) among total motility and progressive motility; total motility and rapid; total motility and medium; total motility and slow; progressive motility and rapid; progressive motility and VAP; progressive motility and VSL; progressive motility and VCL; rapid and medium; rapid and slow; rapid and VCL; medium and slow; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. In contrast, total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; VAP and STR; VCL and STR; ALH and STR; BCF and STR and BCF and LIN were negatively correlated ($P<0.05$). Equilibration duration of 160 minutes showed a positive correlations ($P<0.05$) among 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; rapid and medium; rapid and slow; rapid and VCL; medium and slow; static and STR; static and LIN; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. Conversely, negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; total motility and LIN; progressive motility and static; rapid and static; medium and static; medium and VSL; medium and LIN; slow and LIN; VCL and STR and ALH and STR.

Table 4.48 demonstrates correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish for overall pooled equilibration durations using FRE extender. Positive correlations ($P<0.05$) were shown among total motility and progressive motility; total motility and rapid; total motility and medium; total motility and slow; total motility and VCL; 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 slow; rapid and VAP; rapid and VSL; rapid and VCL; medium and slow; static and STR; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; progressive motility and static; progressive motility and STR; rapid and static; rapid and STR; medium and static; medium and STR; slow and static; slow and STR; static and VAP; static and VCL; VAP and STR; VCL and STR; ALH and STR; BCF and STR and BCF and LIN.

Table 4.42: Total motility and progressive motility (mean ± SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using FRE extender for different equilibration durations

Equilibration duration (min)	N*	Total motility (%)	Progressive motility (%)
120	115	76.65±2.27[b]	21.60±1.09[b]
140	92	66.90±2.60[a]	15.85±1.06[a]
160	100	76.01±2.04[b]	16.97±0.89[a]

N* = Total number of observations (straws).
[abc]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.43: Velocity distributions (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using FRE extender for different equilibration durations

Equilibration duration (min)	N*	Rapid (%)	Medium (%)	Slow (%)	Static (%)
120	114	28.72±1.42[b]	10.02±0.51[b]	38.25±1.17[a]	23.01±2.26[a]
140	94	20.56±1.35[a]	7.98±0.48[a]	38.32±1.50[a]	33.14±2.55[b]
160	97	22.05±1.15[a]	9.64±0.42[b]	44.44±1.11[b]	23.92±2.07[a]

N* = Total number of observations (straws).
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.44: Sperm motion characteristics (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using FRE extender for different equilibration durations

Equilibration duration (min)	N*	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
120	112	58.58 ±1.28[a]	51.17 ±1.09[a]	80.59 ±1.56[a]	5.00 ±0.16[a]	12.59 ±0.42[ab]	87.48 ±0.36[a]	64.36 ±0.66[a]
140	91	54.68 ±1.58[a]	47.91 ±1.43[a]	76.30 ±1.93[a]	4.97 ±0.20[a]	12.11 ±0.47[a]	87.54 ±0.45[a]	63.65 ±0.89[a]
160	97	55.63 ±1.40[a]	48.66 ±1.24[a]	75.88 ±2.01[a]	5.42 ±0.20[a]	13.73 ±0.51[b]	87.28 ±0.39[a]	63.44 ±0.64[a]

N* = Total number of observations (straws).
[ab]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.45: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for 120 min equilibration duration using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.784** (115)	.761** (114)	.628** (114)	.696** (114)	-.978** (114)	.205* (112)	.178 (112)	.187* (112)	.151 (112)	.048 (112)	-.252** (112)	-.022 (112)
Progressive motility		1	.892** (114)	.373** (114)	.215* (114)	-.753** (114)	.164 (112)	.144 (112)	.170 (112)	.091 (112)	.125 (112)	-.212* (112)	-.073 (112)
Rapid			1	.368** (114)	.169 (114)	-.797** (114)	.169 (112)	.152 (112)	.186* (112)	.099 (112)	.132 (112)	-.207* (112)	-.102 (112)
Medium				1	.327** (114)	-.623** (114)	.192* (112)	.173 (112)	.147 (112)	.204* (112)	-.083 (112)	-.179 (112)	.026 (112)
Slow					1	-.697** (114)	.132 (112)	.106 (112)	.101 (112)	.073 (112)	-.014 (112)	-.180 (112)	.069 (112)
Static						1	-.219* (112)	-.192* (112)	-.203* (112)	-.146 (112)	-.055 (112)	.258** (112)	.019 (112)
VAP							1	.978** (112)	.947** (112)	.439** (112)	.039 (112)	-.406** (112)	.247** (112)
VSL								1	.891** (112)	.410** (112)	-.002 (112)	-.238* (112)	.335** (112)
VCL									1	.410** (112)	.158 (112)	-.512** (112)	-.039 (112)
ALH										1	.236* (112)	-.428** (112)	.007 (112)
BCF											1	-.416** (112)	-.365** (112)
STR												1	.359** (112)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.46: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for 140 min equilibration duration using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.763** (92)	.257* (92)	.283** (92)	.233* (92)	-.328** (92)	.170 (91)	.165 (91)	.176 (91)	.143 (91)	.003 (91)	-.017 (91)	-.018 (91)
Progressive motility		1	.443** (92)	.165 (92)	-.089 (92)	-.212* (92)	.255* (91)	.238* (91)	.249* (91)	.157 (91)	.000 (91)	-.043 (91)	.078 (91)
Rapid			1	.526** (94)	.226* (94)	-.763** (94)	.190 (91)	.150 (91)	.224* (91)	.047 (91)	.104 (91)	-.151 (91)	.019 (91)
Medium				1	.375** (94)	-.693** (94)	.051 (91)	.027 (91)	.096 (91)	-.007 (91)	-.016 (91)	-.098 (91)	-.058 (91)
Slow					1	-.783** (94)	.034 (91)	.043 (91)	.028 (91)	.059 (91)	-.068 (91)	.010 (91)	.066 (91)
Static						1	-.124 (91)	-.104 (91)	-.148 (91)	-.055 (91)	-.011 (91)	.092 (91)	-.038 (91)
VAP							1	.979** (91)	.926** (91)	.457** (91)	-.079 (91)	-.236* (91)	.206* (91)
VSL								1	.871** (91)	.415** (91)	-.128 (91)	-.098 (91)	.273** (91)
VCL									1	.530** (91)	.100 (91)	-.439** (91)	-.097 (91)
ALH										1	.139 (91)	-.523** (91)	-.189 (91)
BCF											1	-.447** (91)	-.591** (91)
STR												1	.662** (91)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.47: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for 160 min equilibration duration using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.776** (100)	.810** (97)	.712** (97)	.725** (97)	-.980** (97)	-.019 (97)	-.086 (97)	.129 (97)	.033 (97)	-.072 (97)	-.216* (97)	-.228* (97)
Progressive motility		1	.971** (97)	.565** (97)	.214* (97)	-.765** (97)	.133 (97)	.094 (97)	.192 (97)	.045 (97)	-.149 (97)	-.116 (97)	-.033 (97)
Rapid			1	.621**	.251* (97)	-.813** (97)	.139 (97)	.088 (97)	.225* (97)	.080 (97)	-.107 (97)	-.195 (97)	-.108 (97)
Medium				1	.376** (97)	-.741** (97)	-.181 (97)	-.222* (97)	-.031 (97)	-.088 (97)	-.092 (97)	-.097 (97)	-.225* (97)
Slow					1	-.750** (97)	-.089 (97)	-.148 (97)	.056 (97)	.037 (97)	.035 (97)	-.189 (97)	-.256* (97)
Static						1	.005 (97)	.073 (97)	-.152 (97)	-.048 (97)	.053 (97)	.229* (97)	.241* (97)
VAP							1	.977** (97)	.874** (97)	.599** (97)	-.172 (97)	-.164 (97)	.243* (97)
VSL								1	.817** (97)	.587** (97)	-.185 (97)	.011 (97)	.365** (97)
VCL									1	.580** (97)	-.169 (97)	-.325** (97)	.032 (97)
ALH										1	-.107 (97)	-.235* (97)	.013 (97)
BCF											1	-.100 (97)	-.184 (97)
STR												1	.681** (97)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.48: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for overall pooled equilibration durations using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.769** (307)	.614** (303)	.553** (303)	.522** (303)	-.762** (303)	.143* (300)	.112 (300)	.171** (300)	.119* (300)	.013 (299)	-.160** (300)	-.066 (300)
Progressive motility		1	.793** (303)	.375** (303)	.082 (303)	-.588** (303)	.204** (300)	.181** (300)	.218** (300)	.085 (300)	.002 (299)	-.125* (300)	.002 (300)
Rapid			1	.485** (305)	.171** (305)	-.781** (305)	.190** (300)	.156** (300)	.227** (300)	.065 (300)	.047 (299)	-.177** (300)	-.045 (300)
Medium				1	.349** (305)	-.683** (305)	.062 (300)	.033 (300)	.087 (300)	.060 (300)	-.048 (299)	-.130* (300)	-.057 (300)
Slow					1	-.724** (305)	.026 (300)	.004 (300)	.043 (300)	.080 (300)	.013 (299)	-.114* (300)	-.018 (300)
Static						1	-.137* (300)	-.100 (300)	-.174** (300)	-.094 (300)	-.026 (299)	.191** (300)	.047 (300)
VAP							1	.978** (300)	.913** (300)	.488** (300)	-.070 (299)	-.269** (300)	.234** (300)
VSL								1	.857** (300)	.460** (300)	-.105 (299)	-.110 (300)	.322** (300)
VCL									1	.496** (300)	.011 (299)	-.418** (300)	-.031 (300)
ALH										1	.096 (299)	-.397** (300)	-.066 (300)
BCF											1	-.318** (299)	-.382** (299)
STR												1	.561** (300)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P< 0.05).

4.8 EFFECT OF VAPOUR TEMPERATURE ON FROZEN-THAWED SPERM MOTILITY OF AFRICAN CATFISH (*Clarias gariepinus*) USING FRE EXTENDER (EXPERIMENT 3)

Table 4.49 demonstrates total motility and progressive motility of post-thawed cryopreserved sperm of African catfish using FRE extender for different vapour temperatures. The values of total motility and progressive motility for vapour temperatures of -90^{o}C (74.07±2.02% and 16.99±0.85%, respectively) and -100^{o}C (74.95±1.88% and 20.55±0.93%, respectively) did not show any significant differences (P>0.05), but they were significantly better than -80^{o}C vapour temperature, which gave comparatively lower values (64.59±5.08% and 13.19±1.54%, respectively).

Table 4.50 demonstrates velocity distributions of post-thawed cryopreserved sperm of African catfish using FRE extender for different vapour temperatures. There were no significant differences (P>0.05) in values of medium and slow velocities for the three respective vapour temperatures (-80, -90 and -100^{o}C) which were ranged from 8.28±1.11% to 9.49±0.43% and 38.97±2.80% to 41.65±1.25%, respectively. In rapid velocity, vapour temperature of -80^{o}C gave the lowest value (17.38±2.09%) as compared to -90^{o}C (22.79±1.10%) and -100^{o}C (26.60±1.22%), which in both temperatures were not significantly different (P>0.05). In static velocity, there were no significant differences (P>0.05) between -90 and -100^{o}C, but both showed significant differences with -80^{o}C which gave comparatively the highest value (35.41±5.08%).

Table 4.51 demonstrates sperm motion characteristics of post-thawed cryopreserved sperm of African catfish using FRE extender for different vapour temperatures. The respective values of VCL, ALH, BCF and LIN did not show any significant differences (P>0.05) for -80, -90 and -100^{o}C, which were ranged from 73.03±3.64 to 79.59±1.64 μm/s, 4.98±0.34 to 5.31±0.16 μm, 12.33±0.82 to 12.94±0.42 Hz

and 63.24±0.52 to 64.38±0.59%. The vapour temperature of -100^{o}C gave the highest significant values of VAP and VSL (58.40±1.19 µm/s and 51.28±1.05 µm/s, respectively) as compared to -80^{o}C (51.21±3.26 µm/s and 44.95±2.96 µm/s, respectively) and -90^{o}C (55.34±1.11 µm/s and 48.12±0.96 µm/s, respectively). In contrast, the value of STR gave the highest value at -80^{o}C (89.13±0.84%) as compared to -90^{o}C (86.85±0.32%) and -100^{o}C (87.56±0.33%).

Tables 4.52, 4.53 and 4.54 show correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish for -80, -90 and -100^{o}C vapour temperatures. Vapour temperature of -80^{o}C gave positive correlations ($P<0.05$) among 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 slow; progressive motility and VAP; progressive motility and VSL; rapid and medium; rapid and slow; rapid and VAP; rapid and VSL; medium and slow; medium and VAP; medium and VSL; medium and VCL; slow and VAP; slow and VSL; VAP and VSL; VAP and VCL; VAP and ALH; VSL and VCL; VSL and ALH; VCL and ALH and ALH and BCF. Conversely, negative correlations ($P<0.05$) were shown among total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; static and VAP; static and VSL; static and VCL; VAP and STR; VSL and STR; VCL and STR; ALH and STR and BCF and STR. At -90^{o}C vapour temperature, there were positive correlations ($P<0.05$) among total motility and progressive motility; total motility and rapid; total motility and medium; total motility and slow; total motility and VCL; total motility and BCF; 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; static and STR; VAP and VSL; VAP and VCL; VAP and ALH; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. In contrast, negative correlations ($P<0.05$) were indicated among total motility and static; total motility and STR; progressive motility and static; rapid and static; medium and static; medium and STR; medium and LIN; slow and static; static and VCL; static and BCF; VAP and STR; VCL and STR; ALH and STR; BCF and STR and BCF and LIN. Vapour temperature of -100^{o}C gave positive correlations ($P<0.05$) among 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 VCL; 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 LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. Conversely, negative correlations ($P<0.05$) were shown among total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; static and VAP; static and VCL; VAP and STR; VCL and STR; ALH and STR; ALH and LIN; BCF and STR and BCF and LIN.

Table 4.55 demonstrates correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish for overall pooled vapour temperatures using FRE extender. Positive correlations ($P<0.05$) were shown among total motility and progressive motility; total motility and rapid; total motility and medium; total motility and slow; total motility and VAP; total motility and VCL; progressive motility and rapid; 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; static and STR; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL;

VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; progressive motility and static; rapid and static; rapid and STR; medium and static; medium and STR; slow and static; static and VAP; static and VSL; static and VCL; VAP and STR; VCL and STR; ALH and STR; BCF and STR and BCF and LIN.

Table 4.49: Total motility and progressive motility (mean ± SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using FRE extender for different vapour temperatures

Vapour temperature (^{o}C)	N*	Total motility (%)	Progressive motility (%)
-80	32	64.59±5.08^{a}	13.19±1.54^{a}
-90	122	74.07±2.02^{b}	16.99±0.85^{b}
-100	153	74.95±1.88^{b}	20.55±0.93^{b}

N* = Total number of observations (straws).
^{ab}Means with different superscripts within a column were significantly different (P<0.05).

Table 4.50: Velocity distributions (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using FRE extender for different vapour temperatures

Vapour temperature (^{o}C)	N*	Rapid (%)	Medium (%)	Slow (%)	Static (%)
-80	32	17.38±2.09^{a}	8.28±1.11^{a}	38.97±2.80^{a}	35.41±5.08^{b}
-90	124	22.79±1.10^{b}	9.49±0.43^{a}	41.65±1.25^{a}	26.08±1.99^{a}
-100	149	26.60±1.22^{b}	9.30±0.38^{a}	39.34±0.94^{a}	24.77±1.90^{a}

N* = Total number of observations (straws).
^{ab}Means with different superscripts within a column were significantly different (P<0.05).

Table 4.51: Sperm motion characteristics (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using FRE extender for different vapour temperatures

Vapour temperature (^{o}C)	N*	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
-80	30	51.21 ±3.26^{a}	44.95 ±2.96^{a}	73.03 ±3.64^{a}	4.98 ±0.34^{a}	12.33 ±0.82^{a}	89.13 ±0.84^{b}	63.63 ±2.17^{a}
-90	121	55.34 ±1.11^{ab}	48.12 ±0.96^{ab}	76.70 ±1.37^{a}	5.31 ±0.16^{a}	12.77 ±0.39^{a}	86.85 ±0.32^{a}	63.24 0.52^{a}
-100	149	58.40 ±1.19^{b}	51.28 ±1.05^{b}	79.59 ±1.64^{a}	5.01 ±0.15^{a}	12.94 ±0.42^{a}	87.56 ±0.33^{a}	64.38 ±0.59^{a}

N* = Total number of observations (straws).
^{ab}Means with different superscripts within a column were significantly different (P<0.05).

Table 4.52: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at -80^{o}C vapour temperature using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.850** (32)	.833** (32)	.697** (32)	.917** (32)	-1.000** (32)	.500** (30)	.467** (30)	.408* (30)	.342 (30)	-.216 (30)	-.244 (30)	.202 (30)
Progressive motility		1	.987** (32)	.435* (32)	.635** (32)	-.850** (32)	.405* (30)	.395* (30)	.297 (30)	.229 (30)	-.251 (30)	-.012 (30)	.233 (30)
Rapid			1	.429* (32)	.596** (32)	-.833** (32)	.376* (30)	.364* (30)	.300 (30)	.204 (30)	-.265 (30)	-.011 (30)	.156 (30)
Medium				1	.552** (32)	-.697** (32)	.449* (30)	.393* (30)	.431* (30)	.326 (30)	-.089 (30)	-.331 (30)	.090 (30)
Slow					1	-.917** (32)	.448* (30)	.417* (30)	.342 (30)	.349 (30)	-.152 (30)	-.312 (30)	.227 (30)
Static						1	-.500** (30)	-.467** (30)	-.408* (30)	-.342 (30)	.216 (30)	.244 (30)	-.202 (30)
VAP							1	.984** (30)	.923** (30)	.592** (30)	.094 (30)	-.521** (30)	.324 (30)
VSL								1	.901** (30)	.601** (30)	.114 (30)	-.433* (30)	.310 (30)
VCL									1	.574** (30)	.157 (30)	-.621** (30)	-.031 (30)
ALH										1	.499** (30)	-.529** (30)	.111 (30)
BCF											1	-.515** (30)	-.221 (30)
STR												1	.240 (30)
LIN													1

() Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

Table 4.53: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at -90^{o}C vapour temperature using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.708** (122)	.640** (122)	.539** (122)	.691** (122)	-.906** (122)	.143 (119)	.076 (119)	.218* (119)	.008 (119)	.216* (119)	-.293** (119)	-.147 (119)
Progressive motility		1	.865** (122)	.350** (122)	.119 (122)	-.626** (122)	.240** (119)	.196* (119)	.266** (119)	-.104 (119)	.074 (119)	-.139 (119)	.081 (119)
Rapid			1	.462** (124)	.135 (124)	-.736** (124)	.258** (121)	.199* (121)	.314** (121)	-.047 (121)	.107 (121)	-.174 (121)	.038 (121)
Medium				1	.183* (124)	-.587** (124)	-.045 (121)	-.090 (121)	.018 (121)	-.002 (121)	.230* (121)	-.194* (121)	-.265** (121)
Slow					1	-.743** (124)	.071 (121)	.043 (121)	.132 (121)	.109 (121)	.174 (121)	-.160 (121)	-.128 (121)
Static						1	-.172 (121)	-.113 (121)	-.254** (121)	-.040 (121)	-.215* (121)	.233** (121)	.116 (121)
VAP							1	.973** (121)	.959** (121)	.541** (121)	-.093 (121)	-.326** (121)	.168 (121)
VSL								1	.897** (121)	.518** (121)	-.155 (121)	-.141 (121)	.279** (121)
VCL									1	.510** (121)	.053 (121)	-.465** (121)	-.057 (121)
ALH										1	-.076 (121)	-.378** (121)	.038 (121)
BCF											1	-.338** (121)	-.538** (121)
STR												1	.586** (121)
LIN													1

() Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

Table 4.54: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at -100^{o}C vapour temperature using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.812** (153)	.509** (148)	.361** (148)	.222** (148)	-.511** (148)	.033 (148)	-.012 (148)	.037 (148)	-.097 (148)	-.098 (147)	-.067 (148)	-.040 (148)
Progressive motility		1	.581** (148)	.314** (148)	.057 (148)	-.466** (148)	.193* (148)	.151 (148)	.190* (148)	-.046 (148)	-.052 (147)	-.051 (148)	.023 (148)
Rapid			1	.535** (149)	.133 (149)	-.813** (149)	.347** (148)	.298** (148)	.370** (148)	-.104 (148)	-.073 (147)	-.106 (148)	.078 (148)
Medium				1	.440** (149)	-.756** (149)	-.081 (148)	-.107 (148)	-.022 (148)	-.150 (148)	-.010 (147)	.012 (148)	-.100 (148)
Slow					1	-.666** (149)	-.064 (148)	-.092 (148)	.004 (148)	-.014 (148)	.051 (147)	.016 (148)	-.151 (148)
Static						1	-.176* (148)	-.126 (148)	-.237** (148)	.103 (148)	.019 (147)	.060 (148)	.046 (148)
VAP							1	.979** (149)	.891** (149)	.458** (149)	-.105 (148)	-.163* (149)	.227** (149)
VSL								1	.829** (149)	.423** (149)	-.140 (148)	.008 (149)	.344** (149)
VCL									1	.494** (149)	-.044 (149)	-.353** (149)	-.031 (149)
ALH										1	.141 (149)	-.375** (149)	-.181* (149)
BCF											1	-.271** (148)	-.369** (148)
STR												1	.689** (149)
LIN													1

() Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

Table 4.55: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for overall pooled vapour temperatures using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.769** (307)	.590** (302)	.485** (302)	.517** (302)	-.734** (302)	.158** (297)	.110 (297)	.158** (297)	-.001 (297)	.004 (296)	-.184** (297)	-.020 (297)
Progressive motility		1	.718** (302)	.333** (302)	.129* (302)	-.562** (302)	.257** (297)	.221** (297)	.245** (297)	-.048 (297)	-.018 (296)	-.086 (297)	.078 (297)
Rapid			1	.485** (305)	.171** (305)	-.781** (305)	.342** (299)	.297** (299)	.361** (299)	-.059 (299)	-.021 (298)	-.129* (299)	.083 (299)
Medium				1	.349** (305)	-.683** (305)	.024 (299)	-.011 (299)	.061 (299)	-.025 (299)	.068 (298)	-.125* (299)	-.111 (299)
Slow					1	-.724** (305)	.063 (299)	.036 (299)	.094 (299)	.088 (299)	.075 (298)	-.111 (299)	-.070 (299)
Static						1	-.239** (299)	-.191** (299)	-.276** (299)	-.008 (299)	-.046 (298)	.162** (299)	.014 (299)
VAP							1	.978** (300)	.913** (300)	.488** (300)	-.070 (299)	-.269** (300)	.234** (300)
VSL								1	.857** (300)	.460** (300)	-.105 (299)	-.110 (300)	.322** (300)
VCL									1	.496** (300)	.011 (299)	-.418** (300)	-.031 (300)
ALH										1	.096 (299)	-.397** (300)	-.066 (300)
BCF											1	-.318** (299)	-.382** (299)
STR												1	.561** (300)
LIN													1

() Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

4.9 EFFECT OF VAPOUR EXPOSURE DURATION ON FROZEN-THAWED SPERM MOTILITY OF AFRICAN CATFISH (*Clarias gariepinus*) USING FRE EXTENDER (EXPERIMENT 3)

Table 4.56 demonstrates the total motility and progressive motility of post-thawed cryopreserved sperm of African catfish using FRE extender for different vapour exposure durations. There were no significant differences (P>0.05) in values of total motility and progressive motility for 5, 10 and 15 minutes which were ranged from 72.67±2.27% to 73.99±2.34% and 18.11±1.07% to 18.61±1.04%, respectively.

Table 4.57 demonstrates the velocity distributions of post-thawed cryopreserved sperm of African catfish using FRE extender for different vapour exposure durations. The values of rapid, medium, slow and static velocities did not show any significant differences (P>0.05) at 5, 10 and 15 minutes vapour exposure durations, which were ranged from 23.62±1.35% to 24.34±1.36%, 8.75±0.48% to 9.92±0.52%, 39.70±1.28% to 40.66±1.18% and 26.06±2.41% to 26.94±2.26%, respectively.

Table 4.58 demonstrates the sperm motion characteristics of post-thawed cryopreserved sperm of African catfish using FRE extender for different vapour exposure durations. There were no significant differences (P>0.05) for values of VAP, VSL, VCL, ALH, BCF, STR and LIN at 5, 10 and 15 minutes, which were ranged from 55.64±1.43 to 57.95±1.44 µm/s, 48.48±1.27 to 50.70±1.29 µm/s, 76.43±1.95 to 80.34±1.71 µm/s, 4.92±0.19 to 5.29±0.18 µm, 12.52±0.48 to 13.04±0.45 Hz, 87.08±0.43 to 87.68±0.37% and 63.31±0.66 to 64.77±0.74%, respectively.

Tables 4.59, 4.60 and 4.61 show correlations among post-thawed cryopreserved sperm of African catfish at 5, 10 and 15 minutes vapour exposure durations using FRE extender. At 5 minutes vapour exposure duration, positive correlations (P<0.05) were shown among 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 VCL; rapid and medium; rapid and slow; rapid and VAP; rapid and VCL; medium and slow; static and STR; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. Conversely, negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; progressive motility and static; rapid and static; rapid and STR; medium and static; medium and VAP; medium and VSL; medium and VCL; slow and static; slow and STR; slow and LIN; VCL and STR; ALH and STR; BCF and STR and BCF and LIN. Vapour exposure duration of 10 minutes gave positive correlations ($P<0.05$) among 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; rapid and medium; rapid and slow; medium and slow; medium and VAP; medium and VCL; static and STR; VAP and VSL; VAP and VCL; VAP and ALH; VSL and VCL; VSL and ALH; VCL and ALH; ALH and BCF and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; progressive motility and STR; rapid and static; medium and static; medium and STR; slow and static; slow and STR; static and VAP; static and VCL; VAP and STR; VCL and STR; ALH and STR; BCF and STR and STR and LIN. At 15 minutes vapour exposure duration, positive correlations ($P<0.05$) were shown among 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; total motility and BCF; progressive motility and rapid; progressive motility and medium; progressive motility and VAP; progressive motility and VSL; progressive motility and

VCL; progressive motility and BCF; rapid and medium; rapid and VAP; rapid and VSL; rapid and VCL; medium and slow; medium and BCF; slow and BCF; static and STR; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. Conversely, negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; progressive motility and static; progressive motility and STR; rapid and static; rapid and STR; medium and static; medium and STR; medium and LIN; slow and static; slow and VSL; static and VCL; static and BCF; VAP and STR; VSL and STR; VCL and STR; ALH and STR; BCF and STR and BCF and LIN.

Table 4.62 demonstrates correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish for overall pooled vapour exposure durations using FRE extender. Positive correlations ($P<0.05$) were shown among 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; total motility and BCF; 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 slow; rapid and VAP; rapid and VSL; rapid and VCL; medium and slow; medium and BCF; static and STR; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; total motility and LIN; progressive motility and static; progressive motility and STR; rapid and static; rapid and STR; medium and static; medium and STR; medium and LIN; slow and static; slow and STR; slow and LIN; static and VAP; static and VCL; static and BCF; VAP and STR; VCL and STR; ALH and STR; BCF and STR and BCF and LIN.

Table 4.56: Total motility and progressive motility (mean ± SEM) of post-thawed cryopreserved sperm of African (*Clarias gariepinus*) catfish using FRE extender for different vapour exposure durations

Vapour exposure duration (min)	N*	Total motility (%)	Progressive motility (%)
5	102	73.94±2.41[a]	18.39±1.06[a]
10	105	72.67±2.27[a]	18.11±1.07[a]
15	307	73.99±2.34[a]	18.61±1.04[a]

N* = Total number of observations (straws).
[a]Means with same superscript within a column were not significantly different (P>0.05).

Table 4.57: Velocity distributions (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using FRE extender for different exposure vapour durations

Vapour exposure duration (min)	N*	Rapid (%)	Medium (%)	Slow (%)	Static (%)
5	102	24.34±1.36[a]	9.92±0.52[a]	39.70±1.28[a]	26.06±2.41[a]
10	104	23.62±1.35[a]	8.75±0.48[a]	40.66±1.18[a]	26.94±2.26[a]
15	99	24.31±1.41[a]	9.14±0.43[a]	40.35±1.41[a]	26.24±2.36[a]

N* = Total number of observations (straws).
[a]Means with same superscript within a column were not significantly different (P>0.05).

Table 4.58: Sperm motion characteristics (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using FRE extender 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	100	57.95 ±1.44[a]	50.70 ±1.29[a]	80.34 ±1.71[a]	5.29 ±0.18[a]	13.04 ±0.45[a]	87.08 ±0.43[a]	63.44 ±0.76[a]
10	101	55.64 ±1.43[a]	48.48 ±1.27[a]	76.53 ±1.80[a]	4.92 ±0.19[a]	12.52 ±0.48[a]	87.68 ±0.37[a]	64.77 ±0.74[a]
15	99	55.74 ±1.37[a]	48.93 ±1.17[a]	76.43 ±1.95[a]	5.18 ±0.18[a]	12.88 ±0.48[a]	87.54 ±0.38[a]	63.31 ±0.66[a]

N* = Total number of observations (straws).
[a]Means with same superscript within a column were not significantly different (P>0.05).

Table 4.59: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at 5 min vapour exposure duration using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.787** (102)	.777** (102)	.682** (102)	.743** (102)	-.981** (102)	.057 (100)	-.029 (100)	.094 (100)	-.043 (100)	-.040 (99)	-.297** (100)	-.139 (100)
Progressive motility		1	.928** (102)	.481** (102)	.255** (102)	-.761** (102)	.247* (100)	.192 (100)	.233* (100)	-.173 (100)	-.114 (99)	-.103 (100)	.133 (100)
Rapid			1	.526** (102)	.242* (102)	-.805** (102)	.226* (100)	.146 (100)	.240* (100)	-.134 (100)	-.047 (99)	-.207* (100)	.067 (100)
Medium				1	.347** (102)	-.696** (102)	-.284** (100)	-.293** (100)	-.277** (100)	-.170 (100)	-.043 (99)	-.021 (100)	-.188 (100)
Slow					1	-.744** (102)	-.019 (100)	-.089 (100)	.024 (100)	.136 (100)	-.005 (99)	-.315** (100)	-.238* (100)
Static						1	-.055 (100)	.030 (100)	-.089 (100)	.039 (100)	.031 (99)	.293** (100)	.134 (100)
VAP							1	.972** (100)	.923** (100)	.499** (100)	-.088 (99)	-.103 (100)	.331** (100)
VSL								1	.835** (100)	.454** (100)	-.123 (100)	.094 (100)	.461** (100)
VCL									1	.566** (100)	-.036 (99)	-.357** (100)	.006 (100)
ALH										1	.116 (99)	-.357** (100)	-.083 (100)
BCF											1	-.235* (99)	-.236* (99)
STR												1	.657** (100)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.60: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at 10 min vapour exposure duration using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.787** (105)	.453** (104)	.306** (104)	.227* (104)	-.454** (104)	.246* (101)	.220* (101)	.254* (101)	.064 (101)	.039 (101)	-.197* (101)	-.101 (101)
Progressive motility		1	.648** (104)	.226* (104)	.009 (104)	-.440** (104)	.103 (101)	.095 (101)	.105 (101)	.000 (101)	.102 (101)	-.106 (101)	-.060 (101)
Rapid			1	.465** (104)	.201* (104)	-.800** (104)	.154 (101)	.117 (101)	.171 (101)	.125 (101)	.053 (101)	-.133 (101)	.018 (101)
Medium				1	.374** (104)	-.685** (104)	.237* (101)	.179 (101)	.251* (101)	.122 (101)	-.015 (101)	-.250* (101)	-.015 (101)
Slow					1	-.721** (104)	.159 (101)	.104 (101)	.167 (101)	.080 (101)	-.124 (101)	-.199* (101)	.010 (101)
Static						1	-.229* (101)	-.166 (101)	-.245* (101)	-.143 (101)	.037 (101)	.235* (101)	-.015 (101)
VAP							1	.981** (101)	.965** (101)	.550** (101)	-.072 (101)	-.314** (101)	.101 (101)
VSL								1	.927** (101)	.543** (101)	-.103 (101)	-.189 (101)	.155 (101)
VCL									1	.562** (101)	.059 (101)	-.448** (101)	-.113 (101)
ALH										1	.211* (101)	-.425** (101)	-.170 (101)
BCF											1	-.415** (101)	-.521** (101)
STR												1	.692** (101)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.61: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) at 15 min vapour exposure duration using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.731** (100)	.591** (99)	.604** (99)	.420** (99)	-.718** (99)	.328** (99)	.259** (99)	.462** (99)	.058 (99)	.507** (99)	-.448** (99)	-.185 (99)
Progressive motility		1	.809** (99)	.415** (99)	.024 (99)	-.577** (99)	.521** (99)	.475** (99)	.558** (99)	-.049 (99)	.212* (99)	-.328** (99)	.056 (99)
Rapid			1	.466** (99)	.082 (99)	-.738** (99)	.377** (99)	.314** (99)	.411** (99)	-.035 (99)	.184 (99)	-.399** (99)	-.002 (99)
Medium				1	.350** (99)	-.677**	-.100 (99)	-.157 (99)	.063 (99)	.028 (99)	.515** (99)	-.264** (99)	-.289** (99)
Slow					1	-.715**	-.178 (99)	-.209* (99)	-.070 (99)	.051 (99)	.370** (99)	-.188 (99)	-.197 (99)
Static						1	-.099 (99)	-.032 (99)	-.216* (99)	-.014 (99)	-.427** (99)	.402** (99)	.175 (99)
VAP							1	.983** (99)	.858** (99)	.401** (99)	-.059 (99)	-.412** (99)	.292** (99)
VSL								1	.817** (99)	.365** (99)	-.100 (99)	-.265** (99)	.375** (99)
VCL									1	.367** (99)	-.008 (99)	-.448** (99)	.032 (99)
ALH										1	-.055 (99)	-.409** (99)	.098 (99)
BCF											1	-.306** (99)	-.377** (99)
STR												1	.285** (99)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.62: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) for overall pooled vapour exposure durations using FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.769** (307)	.609** (305)	.529** (305)	.469** (305)	-.725** (305)	.206** (300)	.144* (300)	.274** (300)	.028 (300)	.170** (299)	-.312** (300)	-.141* (300)
Progressive motility		1	.791** (305)	.369** (305)	.095 (305)	-.593** (305)	.280** (300)	.242** (300)	.297** (300)	-.071 (300)	.071 (299)	-.172** (300)	.040 (300)
Rapid			1	.485** (305)	.171** (305)	-.781** (305)	.249** (300)	.188** (300)	.276** (300)	-.010 (300)	.067 (299)	-.244** (300)	.027 (300)
Medium				1	.349** (305)	-.683** (305)	-.043 (300)	-.082 (300)	.020 (300)	-.002 (300)	.138* (299)	-.169** (300)	-.159** (300)
Slow					1	-.724** (305)	-.019 (300)	-.069 (300)	.029 (300)	.085 (300)	.091 (299)	-.234** (300)	-.141* (300)
Static						1	-.127* (300)	-.055 (300)	-.183** (300)	-.040 (300)	-.120* (299)	.308** (300)	.097 (300)
VAP							1	.978** (300)	.913** (300)	.488** (300)	-.070 (299)	-.269** (300)	.234** (300)
VSL								1	.857** (300)	.460** (300)	-.105 (299)	-.110 (300)	.322** (300)
VCL									1	.496** (300)	.011 (299)	-.418** (300)	-.031 (300)
ALH										1	.096 (299)	-.397** (300)	-.066 (300)
BCF											1	-.318** (299)	-.382** (299)
STR												1	.561** (300)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

4.10 EFFECTS OF COMBINATION FACTORS OF EQUILIBRATION DURATION, VAPOUR TEMPERATURE AND VAPOUR EXPOSURE DURATION ON FROZEN-THAWED SPERM MOTILITY CHARACTERISTICS OF AFRICAN CATFISH (*Clarias gariepinus*) USING FRE EXTENDER

Table 4.63 showed total motility and progressive motility of post-thawed cryopreserved sperm of African catfish using 10% DMSO in FRE extender for combination factors of equilibration duration, vapour temperature and vapour exposure duration. It is apparent from table 4.63 that the highest values of total motility and progressive motility obtained were combination factors of 120 minutes equilibration duration, -100^{o}C vapour temperature and 15 minutes vapour exposure duration (87.44±2.07% and 28.22±2.16%, respectively). Combinations of 140 minutes, -100^{o}C and 10 minutes showed the lowest values of total motility (59.27±8.00%), but the lowest value of progressive motility was gained by combination of 160 minutes, -90^{o}C and 10 minutes (12.20±2.24%).

Table 4.64 showed velocity distributions of post-thawed cryopreserved sperm of African catfish using 10% DMSO in FRE extender for combination factors of equilibration duration, vapour temperature and vapour exposure duration. As table 4.64 shows, combination of 120 minutes, -100^{o}C and 15 minutes gave the highest value of rapid velocity (38.56±3.10%), while combination of 120 minutes, -80^{o}C and 10 minutes gave the lowest value (16.33±3.21%). For medium, slow and static velocities, the highest value were attained by combination of 120 minutes, -100^{o}C and 5 minutes (11.75±1.29%), 160 minutes, -80^{o}C and 10 minutes (44.92±3.34%) and 140 minutes, -100^{o}C and 10 minutes (40.73±8.00%), respectively. Both the lowest values of medium and slow velocities were shown by combination of 140 minutes, -80^{o}C and 10 minutes (5.67±0.92% and 29.83±4.58%, respectively). For static velocity, the highest value of static velocity was

gained by combination of 140 minutes, -100^{o}C and 10 minutes (40.73±8.00%), whereas the lowest value was combination of 120 minutes, -100^{o}C and 15 minutes (12.56±2.07%).

Table 4.65 shows the sperm motion characteristics of post-thawed cryopreserved sperm of African catfish using 10% DMSO in FRE extender for combination of equilibration duration, vapour temperature and vapour exposure duration.The highest values of VAP, VSL and VCL were attained by combination of 120 minutes, -80^{o}C and 15 minutes which resulted 68.76±6.42%, 60.49±6.01% and 94.71±6.27%, respectively. Whereas, the lowest values of VAP, VSL, VCL and ALH were shown by combination of 140 minutes, -100^{o}C and 15 minutes with respective values, 47.95±5.49%, 43.06±4.88%, 67.52±7.36% and 3.76±0.70%. In contrast, the combination of 160 minutes, -80^{o}C and 15 minutes gave the highest value of ALH. For value of BCF, combination of 120 minutes, -80^{o}C and 10 minutes was the highest (15.26±4.17%), while 120 minutes, -80^{o}C and 5 minutes obtained the lowest (10.18±1.50%). The velocity of STR and LIN demonstrated the highest value in combination of 140 minutes, -100^{o}C and 15 minutes (90.20±0.97%) and 160 minutes, -100^{o}C and 5 minutes (66.98±1.91%), respectively. On the other hand, the combination of 140 minutes, -90^{o}C and 5 minutes, and 160 minutes, -90^{o}C and 15 minutes gave the lowest value of STR (85.47±0.57%) and LIN (61.11±1.67%), respectively.

Table 4.63: Total motility and progressive motility (mean ± SEM) of post-thawed cryopreserved sperm of African (*Clarias gariepinus*) catfish using 10% DMSO in FRE extender for combination of equilibration duration, vapour temperature and vapour exposure duration

Equilibration duration (min)	Vapour temperature (^{o}C)	Vapour exposure duration (min)	N*	Total motility (%)	Progressive motility (%)
120	-80	5	15	74.00±4.69^{abc}	20.00±2.59^{abc}
		10	15	62.73±6.95^{ab}	12.80±2.49^{a}
		15	13	59.92±9.11^{a}	15.77±3.63^{a}
	-90	5	13	71.38±9.76^{abc}	15.62±2.54^{a}
		10	15	80.80±4.54^{abc}	19.93±2.73^{abc}
		15	15	72.60±7.45^{abc}	19.93±2.71^{abc}
	-100	5	20	84.00±2.86^{bc}	25.95±2.36^{bc}
		10	20	80.95±3.71^{abc}	25.95±2.74^{bc}
		15	18	87.44±2.07^{c}	28.22±2.16^{c}
140	-80	5	7	62.86±7.74^{ab}	14.00±3.51^{a}
		10	6	59.67±13.56^{a}	16.00±6.54^{a}
		15	7	78.29±7.51^{abc}	19.57±3.72^{abc}
	-90	5	15	73.73±5.17^{abc}	18.73±2.83^{ab}
		10	15	68.00±5.89^{abc}	16.13±2.35^{a}
		15	18	76.33±4.59^{abc}	20.00±2.18^{abc}
	-100	5	11	61.27±8.10^{a}	13.27±3.21^{a}
		10	15	59.27±8.00^{a}	13.53±2.87^{a}
		15	10	60.80±9.43^{a}	13.80±3.21^{a}
160	-80	5	14	76.07±5.57^{abc}	15.64±2.67^{a}
		10	12	78.75±6.04^{abc}	20.08±2.74^{abc}
		15	12	73.75±4.76^{abc}	16.08±2.82^{a}
	-90	5	13	74.15±6.40^{abc}	16.08±1.95^{a}
		10	15	60.20±7.12^{a}	12.20±2.24^{a}
		15	19	73.21±4.50^{abc}	14.05±2.23^{a}
	-100	5	20	71.45±6.69^{abc}	18.60±2.66^{ab}
		10	19	80.00±3.65^{abc}	20.16±2.04^{abc}
		15	20	73.75±4.86^{abc}	17.80±2.00^{ab}

N* = Total number of observations (straws).
^{abc}Means with different superscripts within a column were significantly different (P<0.05).

Table 4.64: Velocity distributions (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using 10% DMSO in FRE extender for combination of equilibration duration, vapour temperature and vapour exposure duration

Equilibration duration (min)	Vapour temperature (^{o}C)	Vapour exposure duration (min)	N*	Rapid (%)	Medium (%)	Slow (%)	Static (%)
120	-80	5	15	25.13±3.18^{abc}	6.87±0.90^{abcd}	41.87±3.64^{abc}	26.00±4.69^{abcd}
		10	15	16.33±3.21^{a}	6.47±1.84^{abc}	39.93±3.90^{abc}	37.27±6.95^{bcd}
		15	13	21.54±5.08^{ab}	5.92±1.24^{ab}	32.54±3.79^{ab}	40.08±9.11^{d}
	-90	5	13	21.31±3.71^{ab}	11.23±1.74^{de}	38.85±5.49^{abc}	28.62±9.76^{abcd}
		10	15	26.73±3.28^{abc}	10.73±1.34^{cde}	43.40±2.67^{bc}	19.20±4.54^{abcd}
		15	15	25.60±3.53^{abc}	10.47±1.53^{bcde}	36.60±3.77^{abc}	27.40±7.45^{abcd}
	-100	5	20	33.65±2.78^{bcd}	11.75±1.29^{e}	38.70±1.81^{abc}	16.00±2.86^{ab}
		10	19	34.21±3.53^{cd}	9.21±0.82^{abcde}	39.68±1.80^{abc}	16.79±3.10^{abc}
		15	18	38.56±3.10^{d}	10.22±0.53^{abcde}	38.56±1.75^{abc}	12.56±2.07^{a}
140	-80	5	7	19.00±5.18^{a}	9.00±2.49^{abcde}	34.71±2.74^{abc}	37.14±7.74^{bcd}
		10	6	24.00±9.94^{abc}	5.67±0.92^{a}	29.83±4.58^{a}	40.33±13.56^{d}
		15	7	26.71±5.57^{abc}	9.86±1.79^{abcde}	42.00±4.55^{abc}	21.71±7.51^{abcd}
	-90	5	15	25.80±3.92^{abc}	8.80±0.94^{abcde}	39.07±3.35^{abc}	26.27±5.17^{abcd}
		10	15	20.27±2.71^{a}	6.27±0.91^{abc}	41.60±3.89^{abc}	32.00±5.89^{abcd}
		15	20	25.55±2.60^{abc}	10.20±0.89^{abcde}	39.30±3.17^{abc}	24.80±4.23^{abcd}
	-100	5	11	17.91±4.14^{a}	7.36±1.65^{abcde}	36.09±4.10^{abc}	38.73±8.10^{cd}
		10	15	17.13±3.54^{a}	7.20±1.43^{abcde}	34.80±4.19^{abc}	40.73±8.00^{d}
		15	10	16.40±3.84^{a}	6.70±1.49^{abcd}	37.90±5.80^{abc}	39.20±9.43^{d}
160	-80	5	14	21.29±3.81^{ab}	10.50±1.10^{bcde}	44.36±4.63^{bc}	24.00±5.56^{abcd}
		10	12	25.08±2.93^{abc}	8.67±1.39^{abcde}	44.92±3.34^{bc}	21.25±4.76^{abcd}
		15	12	21.00±3.91^{ab}	8.58±1.20^{abcde}	44.33±4.03^{bc}	26.25±6.04^{abcd}
	-90	5	13	22.38±2.80^{abc}	10.46±1.44^{bcde}	41.08±3.48^{abc}	25.85±6.40^{abcd}
		10	15	16.80±3.08^{a}	8.87±2.13^{abcde}	34.80±4.25^{abc}	39.80±7.12^{d}
		15	19	19.95±3.27^{a}	6.74±0.67^{abcd}	46.53±3.14^{c}	26.79±4.50^{abcd}
	-100	5	20	23.20±3.33^{abc}	9.10±1.02^{abcde}	39.20±3.26^{abc}	28.55±6.69^{abcd}
		10	19	26.21±2.48^{abc}	10.21±0.85^{abcde}	43.79±1.37^{bc}	19.68±3.47^{abcd}
		15	17	21.59±2.74^{ab}	9.35±1.07^{abcde}	42.71±2.56^{bc}	26.59±5.47^{abcd}

N* = Total number of observations (straws).

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

Table 4.65: Sperm motion characteristics (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using 10% DMSO in FRE extender for combination of equilibration duration, vapour temperature and vapour exposure duration

Equilibration duration (min)	Vapour Temperature (^{o}C)	Vapour exposure duration (min)	N*	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
120	-80	5	15	66.92±5.13^{def}	54.30±3.76^{abc}	91.67±7.10^{de}	5.03±0.40^{abc}	10.18±1.50^{a}	86.33±2.70^{ab}	63.93±2.24^{ab}
		10	13	58.99±5.95^{abcdef}	51.65±4.93^{abc}	81.42±7.40^{abcde}	4.92±0.41^{abc}	15.26±4.17^{a}	87.69±1.88^{ab}	64.38±2.73^{ab}
		15	12	68.76±6.42^{f}	60.49±6.01^{c}	94.71±6.27^{e}	5.33±0.60^{abc}	10.64±1.59^{a}	88.00±1.64^{ab}	62.67±3.44^{ab}
	-90	5	13	52.37±3.10^{abcd}	47.11±2.97^{ab}	72.58±3.78^{abc}	4.76±0.66^{abc}	12.52±1.27^{a}	88.92±1.54^{ab}	63.46±2.25^{ab}
		10	15	58.58±3.06^{abcdef}	50.54±2.89^{abc}	79.93±3.91^{abcde}	4.98±0.38^{abc}	11.94±0.71^{a}	87.00±0.78^{ab}	64.80±1.35^{ab}
		15	15	55.65±3.81^{abcdef}	48.21±3.22^{abc}	75.75±4.76^{abcd}	5.72±0.65^{bc}	12.06±1.64^{a}	86.73±0.66^{ab}	64.93±2.13^{ab}
	-100	5	20	60.57±2.59^{abcdef}	52.94±2.19^{abc}	82.77±3.32^{abcde}	4.84±0.27^{abc}	12.50±0.81^{a}	87.35±0.63^{ab}	64.95±0.90^{ab}
		10	19	61.02±2.10^{abcdef}	53.22±1.71^{abc}	83.33±3.12^{abcde}	5.08±0.40^{abc}	13.27±1.23^{a}	87.53±0.77^{ab}	65.42±1.67^{ab}
		15	18	64.50±2.79^{bcdef}	55.72±2.25^{abc}	89.50±3.45^{bcde}	5.08±0.20^{abc}	13.68±0.70^{a}	85.78±0.70^{a}	62.72±0.78^{ab}
140	-80	5	7	50.14±4.97^{ab}	44.11±4.63^{a}	74.70±4.64^{abcd}	5.11±0.61^{abc}	13.31±1.58^{a}	86.86±1.10^{ab}	58.57±4.15^{a}
		10	6	57.98±10.62^{abcdef}	47.20±9.49^{ab}	80.03±15.64^{abcde}	6.47±1.60^{c}	11.83±2.70^{a}	86.67±3.30^{ab}	66.83±6.18^{b}
		15	7	67.19±6.98^{ef}	58.99±5.94^{bc}	92.17±6.35^{de}	5.54±0.29^{abc}	11.20±1.46^{a}	87.00±1.15^{ab}	63.00±2.50^{ab}
	-90	5	15	61.57±2.45^{abcdef}	52.93±2.23^{abc}	85.21±3.08^{abcde}	5.61±0.23^{bc}	12.53±0.63^{a}	85.47±0.57^{a}	62.40±0.88^{ab}
		10	14	51.77±4.04^{ab}	45.36±3.29^{a}	71.36±4.64^{ab}	4.61±0.43^{ab}	12.86±1.01^{a}	88.50±0.89^{ab}	64.57±1.32^{ab}
		15	18	57.97±2.16^{abcdef}	50.68±1.79^{abc}	79.58±2.84^{abcde}	5.13±0.28^{abc}	13.04±0.98^{a}	87.11±0.79^{ab}	64.11±0.96^{ab}
	-100	5	11	58.30±4.22^{abcdef}	50.45±4.41^{abc}	84.67±4.83^{abcde}	5.91±0.69^{bc}	11.13±1.58^{a}	84.64±2.10^{a}	61.36±4.12^{ab}
		10	15	53.55±4.86^{abcde}	47.96±4.52^{abc}	72.70±5.34^{abc}	4.75±0.66^{abc}	11.91±1.46^{a}	88.20±0.93^{ab}	63.73±2.49^{ab}
		15	10	47.95±5.49^{a}	43.06±4.88^{a}	67.52±7.36^{a}	3.76±0.70^{a}	10.31±1.62^{a}	90.20±0.97^{b}	65.90±1.67^{ab}

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160	-80	5	14	53.34±4.49^{abcde}	47.30±4.00^{ab}	75.36±4.67^{abcd}	5.39±0.51^{abc}	12.61±1.13^{a}	88.64±1.37^{ab}	61.86±2.70^{ab}
		10	12	66.71±4.67^{cdef}	58.89±4.03^{bc}	90.73±4.81^{cde}	6.22±0.47^{bc}	12.48±1.33^{a}	87.92±1.12^{ab}	64.17±1.01^{ab}
		15	11	59.29±2.68^{abcdef}	51.94±2.64^{abc}	82.18±3.69^{abcde}	6.03±0.26^{bc}	12.66±1.64^{a}	86.27±0.75^{ab}	61.55±1.12^{ab}
	-90	5	13	52.45±2.85^{abcd}	45.11±2.34^{a}	74.88±3.78^{abcd}	5.40±0.54^{abc}	12.90±1.39^{a}	86.00±0.60^{ab}	61.92±1.63^{ab}
		10	15	52.21±4.48^{abc}	44.77±3.88^{a}	72.57±5.32^{abc}	4.96±0.62^{abc}	14.24±2.25^{a}	86.67±1.26^{ab}	62.93±2.54^{ab}
		15	19	54.67±3.40^{abcdef}	47.17±2.91^{ab}	77.35±4.21^{abcde}	5.29±0.44^{abc}	13.77±1.24^{a}	85.74±0.79^{a}	61.11±1.67^{ab}
	-100	5	18	62.52±5.17^{abcdef}	55.44±4.54^{abc}	82.39±6.21^{abcde}	5.28±0.58^{abc}	14.34±1.60^{a}	88.67±0.98^{ab}	66.89±1.91^{b}
		10	19	57.36±2.54^{abcdef}	49.84±2.16^{abc}	79.38±3.48^{abcde}	4.78±0.41^{abc}	13.25±1.42^{a}	87.47±0.79^{ab}	65.11±1.34^{ab}
		15	19	54.25±2.31^{abcde}	48.32±2.02^{abc}	69.51±5.98^{a}	5.33±0.40^{abc}	14.11±0.79^{a}	88.37±1.05^{ab}	62.68±1.35^{ab}

N* = Total number of observations (straws).
^{abc}Means with different superscripts within a column were significantly different (P<0.05).

4.11 EFFECTS OF DIFFERENT EXTENDERS AND CRYOPROTECTANTS ON FROZEN-THAWED SPERM MOTILITY CHARACTERISTICS OF AFRICAN CATFISH (*Clarias gariepinus*) (EXPERIMENT 4)

Table 4.66 demonstrates total motility and progressive motility of post-thawed cryopreserved sperm of African catfish using different types of extender and cryoprotectant. There were no significant differences (P>0.05) for values of total motility between 1.0 M (24.50±1.81%) and 2.0 M of glycerol in TCAYE (26.74±2.14%), but they were comparatively lower than 0.5 M of glycerol that showed higher significant value (32.27±2.05%). On the other hand, combination of DMSO (10%) in FRE extender showed the highest significant values of total motility and progressive motility (73.52±1.35% and 18.37±0.61%, respectively) as compared to the three molarities of glycerol (0.5, 1.0 and 2.0 M) in TCAYE extender. There were no significant differences (P>0.05) in values of progressive motility for the three respective molarities (0.5, 1.0 and 2.0 M glycerol), which gave 3.75±0.41%, 2.63±0.29% and 2.45±0.37%.

Table 4.67 demonstrates the velocity distributions of post-thawed cryopreserved sperm of African catfish using different types of extender and cryoprotectant. There were no significant differences (P>0.05) in values of rapid and medium velocities for 0.5, 1.0 and 2.0 M glycerol in TCAYE extender, which were ranged from 3.37±0.51 to 5.19±0.60% and 1.27±0.13 to 1.70±0.14%, respectively. Furthermore, 0.5, 1.0 and 2.0 M glycerol showed significant differences with DMSO (10%) in FRE, which gave comparatively the highest value of rapid and medium velocities (24.09±0.79% and 9.27±0.28%, respectively). As for the values of slow and static velocities, there were no significant differences (P>0.05) among 1.0 M (19.76±1.47% and 75.50±1.81%, respectively) and 2.0 M glycerol (21.89±1.70% and 73.27±2.14%, respectively), but these were significant with 0.5 M of glycerol in TCAYE extender (25.39±1.62% and

67.74±2.05%, respectively) and DMSO (10%) in FRE extender (40.24±0.74% and 26.42±1.35%, respectively).

Table 4.68 demonstrates sperm motion characteristics of post-thawed cryopreserved sperm of African catfish using different types of extender and cryoprotectant. There were no significant differences (P>0.05) in values of BCF and LIN for 0.5 M (14.99±0.89% and 63.02±1.16%, respectively), 1.0 M (13.61±0.94% and 64.79±1.22%, respectively) and 2.0 M (14.55±1.21% and 65.18±1.55%, respectively) glycerol in TCAYE extender and DMSO (10%) in FRE extender (12.81±0.27% and 63.85±0.42%, respectively). There were significant differences for values of VAP and VSL between 2.0 M glycerol (45.84±2.00% and 40.77±1.85%, respectively) and 0.5 M (56.91±2.27% and 49.89±2.09%, respectively) as well as 1.0 M glycerol (52.80±1.89% and 47.94±1.81%, respectively) in TCAYE extender and DMSO (10%) in FRE extender (56.44±0.82% and 49.37±0.72%, respectively). However, the values of VAP and VSL for 0.5 and 1.0 M glycerol in TCAYE extender as well as 10% DMSO in FRE extender did not show significant differences (P>0.05).

Tables 4.69, 4.70, 4.71 and 4.72 show correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish using 0.5, 1.0 and 2.0 M glycerol in TCAYE extender as well as 10% DMSO in FRE extender. For 0.5 M glycerol, positive correlations (P<0.05) were shown among 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 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 VAP; slow and VSL; slow and VCL; VAP and VSL; VAP and VCL; VAP

and ALH; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH and STR and LIN. In contrast, negative correlations ($P<0.05$) were shown among total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; static and VAP; static and VSL; static and VCL; VCL and STR; VCL and LIN; BCF and STR and BCF and LIN. In 1.0 M glycerol, there were positive correlations ($P<0.05$) among 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; rapid and medium; rapid and slow; medium and slow; 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; ALH and BCF; ALH and LIN and STR and LIN. In contrast, there were negative correlations ($P<0.05$) among total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; VCL and STR; ALH and STR; BCF and STR and STR and LIN. In 2.0 M glycerol, positive correlations ($P<0.05$) were shown among 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; rapid and medium; rapid and slow; medium and slow; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; VCL and BCF; ALH and BCF and STR and LIN. Conversely, negative correlations ($P<0.05$) were shown among total motility and static; progressive motility and static; rapid and static; medium and static; slow and static; VCL and STR; VCL and LIN; ALH and STR; BCF and STR and BCF and LIN. In 10% DMSO in FRE extender, there were positive correlations ($P<0.05$) among total motility and progressive motility; total motility and rapid; total motility and medium; total motility and slow; total motility and VAP; total motility and VCL; progressive motility and rapid; progressive motility and

medium; progressive motility and slow; rapid and medium; rapid and slow; medium and slow; medium 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 LIN; VCL and ALH and STR and LIN. Conversely, negative correlations ($P<0.05$) were shown among total motility and static; total motility and STR; progressive motility and static; progressive motility and STR; rapid and static; rapid and STR; medium and static; medium and STR; slow and static; slow and STR; static and VAP; VAP and STR; VCL and STR; ALH and STR; BCF and STR and BCF and LIN.

Table 4.66: Total motility and Progressive motility (mean ± SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using different types of extender and cryoprotectant

Types of extender	N*	Total motility (%)	Progressive motility (%)
Glycerol (0.5 M) in TCAYE	135	32.27±2.05[b]	3.75±0.41[a]
Glycerol (1.0 M) in TCAYE	147	24.50±1.81[a]	2.63±0.29[a]
Glycerol (2.0 M) in TCAYE	128	26.74±2.14[a]	2.45±0.37[a]
DMSO (10%) in FRE	307	73.52±1.35[c]	18.37±0.61[b]

N* =Total number of observations (straws).
[abc]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.67: Velocity distributions (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using different types of extender and cryoprotectant

Types of Extender	*N	Rapid (%)	Medium (%)	Slow (%)	Static (%)
Glycerol (0.5 M) in TCAYE	135	5.19±0.60[a]	1.70±0.14[a]	25.39±1.62[b]	67.74±2.05[b]
Glycerol (1.0 M) in TCAYE	147	3.46±0.37[a]	1.27±0.13[a]	19.76±1.47[a]	75.50±1.81[c]
Glycerol (2.0 M) in TCAYE	128	3.37±0.51[a]	1.43±0.16[a]	21.89±1.70[ab]	73.27±2.14[c]
DMSO (10%) in FRE	307	24.09±0.79[b]	9.27±0.28[b]	40.24±0.74[c]	26.42±1.35[a]

*N = Total number of observations (straws).
[abc]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.68: Sperm motion characteristics (mean±SEM) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using different types of extender and cryoprotectant

Types of Extender	*N	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
Glycerol (0.5 M) in TCAYE	127	56.91 ±2.27[b]	49.89 ±2.09[b]	82.87 ±3.08[c]	5.34 ±0.24[b]	14.99 ±0.89[a]	86.36 ±0.64[a]	63.02 ±1.16[a]
Glycerol (1.0 M) in TCAYE	135	52.80 ±1.89[b]	47.94 ±1.81[b]	73.80 ±2.22[b]	4.92 ±0.24[ab]	13.61 ±0.94[a]	88.78 ±0.60[b]	64.79 ±1.22[a]
Glycerol (2.0 M) in TCAYE	115	45.84 ±2.00[a]	40.77 ±1.85[a]	65.43 ±2.45[a]	4.60 ±0.31[a]	14.55 ±1.21[a]	88.79 ±0.73[b]	65.18 ±1.55[a]
DMSO (10%) in FRE	300	56.44 ±0.82[b]	49.37 ±0.72[b]	77.77 ±1.05[bc]	5.13 ±0.11[ab]	12.81 ±0.27[a]	87.43 ±0.23[ab]	63.85 ±0.42[a]

*N= Total number of observations (straws).
[abc]Means with different superscripts within a column were significantly different (P<0.05).

Table 4.69: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using 0.5 M glycerol in TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.667** (135)	.638** (135)	.685** (135)	.879** (135)	-.927** (135)	.267** (127)	.266** (126)	.253** (127)	.145 (127)	.012 (127)	-.027 (127)	.025 (127)
Progressive motility		1	.959** (135)	.702** (135)	.378** (135)	-.627** (135)	.318** (127)	.327** (126)	.266** (127)	.149 (127)	-.076 (127)	.066 (127)	.104 (127)
Rapid			1	.738** (135)	.429** (135)	-.679** (135)	.288** (127)	.286** (126)	.257** (127)	.114 (127)	-.057 (127)	.045 (127)	.067 (127)
Medium				1	.591** (135)	-.746** (135)	.099 (127)	.118 (126)	.072 (127)	.067 (127)	-.069 (127)	.072 (127)	.037 (127)
Slow					1	-.953** (135)	.217* (127)	.217* (126)	.208* (127)	.131 (127)	.033 (127)	-.045 (127)	.014 (127)
Static						1	-.265** (127)	-.265** (126)	-.245** (127)	-.143 (127)	-.002 (127)	.017 (127)	-.036 (127)
VAP							1	.965** (126)	.870** (127)	.499** (127)	-.067 (127)	-.119 (127)	.117 (127)
VSL								1	.753** (126)	.547** (126)	-.136 (126)	.057 (126)	.257** (126)
VCL									1	.375** (127)	.103 (127)	-.385** (127)	-.278** (127)
ALH										1	.158 (127)	-.141 (127)	.141 (127)
BCF											1	-.524** (127)	-.337** (127)
STR												1	.680** (127)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.70: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using 1.0 M glycerol in TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.744** (147)	.701** (147)	.576** (147)	.752** (147)	-.794** (147)	.035 (135)	.032 (135)	.011 (135)	.044 (135)	-.129 (135)	.019 (135)	.001 (135)
Progressive motility		1	.940** (147)	.622** (147)	.539** (147)	-.675** (147)	-.016 (135)	-.014 (135)	-.016 (135)	.050 (135)	-.038 (135)	.056 (135)	-.058 (135)
Rapid			1	.664** (147)	.633** (147)	-.767** (147)	.011 (135)	.012 (135)	.003 (135)	.055 (135)	-.054 (135)	.058 (135)	-.039 (135)
Medium				1	.579** (147)	-.677** (147)	.094 (135)	.091 (135)	.047 (135)	.118 (135)	-.123 (135)	-.009 (135)	-.006 (135)
Slow					1	-.979** (147)	.081 (135)	.082 (135)	.049 (135)	.033 (135)	-.179* (135)	.038 (135)	.025 (135)
Static						1	-.070 (135)	-.071 (135)	-.040 (135)	-.041 (135)	.155 (135)	-.043 (135)	-.010 (135)
VAP							1	.982** (135)	.872** (135)	.507** (135)	-.078 (135)	.070 (135)	.483** (135)
VSL								1	.792** (135)	.480** (135)	-.145 (135)	.223** (135)	.579** (135)
VCL									1	.475** (135)	.099 (135)	-.185* (135)	.083 (135)
ALH										1	.192* (135)	-.190* (135)	.204* (135)
BCF											1	-.417** (135)	-.267** (135)
STR												1	.572** (135)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P<0.01).
* Pearson correlations were significant (P<0.05).

Table 4.71: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using 2.0 M glycerol in TCAYE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.719** (128)	.703** (128)	.674** (128)	.862** (128)	-.903** (128)	.078 (115)	.065 (115)	.117 (115)	.077 (115)	.145 (115)	-.048 (115)	-.147 (115)
Progressive motility		1	.976** (128)	.772** (128)	.522** (128)	-.706** (128)	-.082 (115)	-.097 (115)	-.015 (115)	-.064 (115)	.142 (115)	-.060 (115)	-.160 (115)
Rapid			1	.776** (128)	.550** (128)	-.735** (128)	-.062 (115)	-.081 (115)	.012 (115)	-.072 (115)	.139 (115)	-.082 (115)	-.177 (115)
Medium				1	.583** (128)	-.720** (128)	-.071 (115)	-.084 (115)	.008 (115)	-.041 (115)	.085 (115)	-.079 (115)	-.182 (115)
Slow					1	-.969** (128)	.088 (115)	.085 (115)	.109 (115)	.091 (115)	.112 (115)	-.020 (115)	-.116 (115)
Static						1	-.049 (115)	-.042 (115)	-.090 (115)	-.054 (115)	-.129 (115)	.041 (115)	.148 (115)
VAP							1	.982** (115)	.893** (115)	.613** (115)	.058 (115)	-.176 (115)	.215* (115)
VSL								1	.827** (115)	.582** (115)	-.029 (115)	-.016 (115)	.312** (115)
VCL									1	.666** (115)	.257** (115)	-.423** (115)	-.191* (115)
ALH										1	.393** (115)	-.324** (115)	-.112 (115)
BCF											1	-.512** (115)	-.446** (115)
STR												1	.587** (115)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

Table 4.72: Correlations among sperm motility characteristics of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using 10% DMSO in FRE extender

	Total motility	Progressive motility	Rapid	Medium	Slow	Static	VAP	VSL	VCL	ALH	BCF	STR	LIN
Total motility	1	.769** (307)	.504** (305)	.410** (305)	.421** (305)	-.611** (305)	.131* (300)	.112 (300)	.133* (300)	.085 (300)	.063 (299)	-.135* (300)	-.062 (300)
Progressive motility		1	.604** (305)	.287** (305)	.136* (305)	-.488** (305)	.099 (300)	.084 (300)	.113 (300)	.011 (300)	.097 (299)	-.143* (300)	-.101 (300)
Rapid			1	.485** (305)	.171** (305)	-.781** (305)	.105 (300)	.080 (300)	.098 (300)	.043 (300)	.053 (299)	-.175** (300)	-.100 (300)
Medium				1	.349** (305)	-.683** (305)	.104 (300)	.092 (300)	.068 (300)	.153** (300)	-.049 (299)	-.135* (300)	-.008 (300)
Slow					1	-.724** (305)	.067 (300)	.052 (300)	.024 (300)	.050 (300)	.071 (299)	-.118* (300)	.011 (300)
Static						1	-.121* (300)	-.096 (300)	-.085 (300)	-.084 (300)	-.061 (299)	.196** (300)	.055 (300)
VAP							1	.978** (300)	.913** (300)	.488** (300)	-.070 (299)	-.269** (300)	.234** (300)
VSL								1	.857** (300)	.460** (300)	-.105 (299)	-.110 (300)	.322** (300)
VCL									1	.496** (300)	.011 (299)	-.418** (300)	-.031 (300)
ALH										1	.096 (299)	-.397** (300)	-.066 (300)
BCF											1	-.318** (299)	-.382** (299)
STR												1	.561** (300)
LIN													1

( ) Represents no. of samples.
** Pearson correlations were significant (P< 0.01).
* Pearson correlations were significant (P<0.05).

Chapter 5

DISCUSSION

Chapter 5

5.0 DISCUSSION

5.1 EFFECT OF INDIVIDUAL BODY WEIGHT ON FRESH SPERM MOTILITY CHARACTERISTICS IN AFRICAN CATFISH (*Clarias gariepinus*) (EXPERIMENT 1)

The current study found that body weight of African catfish affects the fresh sperm motility characteristics before freezing. The most interesting finding was that large BW of African catfish with (>1.5kg) gave the highest total motility (82.40±4.59%) as compared to the other two groups. Another important finding was that small BW with (<1.5kg) gave the lowest sperm total motility (40.40±12.16%). It is difficult to explain this result, but it might be related to a numbers of factors such as the variations of fresh sperm among individual broodstock fish and the effects of body weight of African catfish on the percent total sperm motility produced. The presumptive reasons behind effects of body weight might be associated with the age of male African catfish broodstocks which can produce a high sperm total motility, but this is not an absolute reason since a limited sample size of fish were used for each groups. These findings further support the idea of Vuthiphandchai and Zohar (1999) who suggested age of broodstocks have a significant influence on the sperm quality and may affect the success of storing sperm. In captive-reared striped bass (*Morone saxatilis*), 3-year-old fish had higher sperm quality than the 1-year or 12-month-old fish, based on higher sperm production and increased sperm longevity during short-term storage. However, the fertilising capacity of virgin and repeat spawners was comparable in Atlantic cod, *G. morhua* (Trippel and Neilson, 1992). Another possible explanation for this is that the nutrition shows an important factor that affects the body weight of African catfish. Nutrition is susceptible to affect not only fecundity and gametogenesis, but also gamete quality, and existing work has been extensively reviewed

(Kjorsvik *et al*., 1990; Brooks *et al*., 1997; Izquierdo *et al*., 2001). Improvement in broodstock nutrition and feeding greatly improves gamete quality and seed production (Izquierdo *et al*., 2001). According to Alavi *et al*. (2009), found that significant relationships were observed between weight and length of broodfish (*Barbus barbus* L.) with total number of sperm and sperm concentration, but not with sperm volume. The evidence of this was associated with endocrinological mechanism regulating hydration of sperm during spermiation (Nagahama, 1994; Billard *et al*., 1995; Alavi and Cosson, 2006).

The pattern of positive correlations among sperm motility for respective small, medium and large BW group was different. In small BW group, the pattern of sperm motility characteristics correlations were medium and slow; VAP and VSL; VAP and LIN; VSL and STR; VSL and LIN and STR and LIN. This pattern of correlations differ in medium and large BW group, which showed correlations of total motility and progressive motility; total motility and rapid; total motility and medium; progressive motility and rapid; progressive motility and medium; VAP and VSL; VAP and VCL; VAP and ALH; VAP and LIN; VSL and VCL; VSL and ALH; VSL and LIN as well as ALH and LIN. In large BW group, the patterns of correlations was progressive motility and rapid; progressive motility and LIN; rapid and STR; rapid and LIN; VAP and VSL; VAP and VCL; VAP and ALH; VSL and VCL and VSL and ALH. In general, therefore, percent total motility and sperm velocity have been correlated to reproductive success in fish; reductions in these parameters may decrease fecundity (Blaxhall *et al*., 1973; Bustos-Obregon *et al*., 1975; Brunette *et al*., 2001; Cabrita *et al*., 2003). The variation of pattern in correlation among sperm motility characteristics across the body weight group was assumed to be related with the average gamete quality of each group.

5.2 EFFECTS OF MOLARITY OF GLYCEROL IN TCAYE EXTENDER, EQUILIBRATION DURATION, VAPOUR TEMPERATURE AND VAPOUR EXPOSURE DURATION ON FROZEN-THAWED SPERM MOTILITY OF AFRICAN CATFISH (*Clarias gariepinus*) (EXPERIMENT 2)

Molarities of glycerol in TCAYE extender and equilibration duration are identified as the superior factors that gave significant differences in cryopreservation of African catfish sperm. However, the sperm total motility results for vapour temperature and vapour exposure duration using TCAYE extender were not statistically significant. Refrigeration temperature (4^{o}C) has been used for equilibration since it was postulated to be optimal for cryoprotectant addition and equilibration, and it is routinely been used in avian sperm cryopreservation protocols (Donoghue *et al*., 2000).

Three molarities of glycerol were studied in order to have an optimum result for molarity of frozen-thawed sperm. The results of this study indicated that glycerol with molarity of 0.5 M showed significant highest values of sperm total motility (32.27±2.05%) as compared to the total motility values of 1.0 M (24.50±1.81%). The present findings seem to be consistent with Asmad *et al*. (2008), who found that there were significant differences between three molarities of glycerol (0.5, 1.0 or 1.5 M) whereby, 0.5 M glycerol showed the highest sperm total motility (54.92±0.93%) for frozen-thawed red tilapia sperm (*Oreochromis niloticus*) compared with 1.0 M (46.90±0.76%) and 1.5 M (35.65±0.71%). These results may be explained by the fact that lower molarity of glycerol is more suitable for cryopreservation of African catfish and red tilapia sperm. This finding is also in agreement with Huang *et al*. (2004a), who showed higher concentrations of glycerol (14 to 17%) yielded the highest sperm motility (77%) immediately after thawing, but lower concentrations (8 to 11%) retained sperm motility longer when stored at 4^{o}C, as was found in previous study with *Xiphophorus helleri*. Other finding showed that glycerol

at a concentration of 10% was found to be the best cryoprotectant for European catfish ejaculated sperm, which obtained 36% motility for frozen-thawed sperm (Linhart *et al*., 1993). Glycerol (10%) also proved effective for freezing Asian catfish, *Heteropneustes fossilis* and *Clarias batrachus* sperm, yielding 69 to 84% of control hatching rates (Padhi *et al*., 1995). However, glycerol was toxic to salmonid sperm, whereas DMSO could be used for cryopreservation (Stoss *et al*., 1981).

The correlations of sperm motility characteristics observed for the three respective molarities of glycerol (0.5, 1.0 and 2.0 M) showed a similar pattern, but 0.5 M glycerol showed a slightly different pattern for positive correlations among 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 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 VAP; slow and VSL; slow and VCL; VAP and VSL; VAP and VCL; VAP and ALH; VSL and VCL; VSL and ALH; VSL and LIN; VCL and ALH; STR and LIN. Both 1.0 and 2.0 M glycerol showed similarities of pattern of correlations among sperm motility characteristics.

On the question of optimum equilibration duration, this study found that at 140 minutes equilibration, the value of total motility (31.69±2.19%) was significantly higher as compared to 120 minutes which gave the significant lower value (25.26±1.76%). Contrary to expectations, this study did not find a significant difference between the values of sperm total motility at 140 minutes (31.69±2.19%) and 160 minutes (28.17±2.11%) equilibration. A possible explanation for this might be that longer equilibration permitted the sperm-diluent mixture to allow the maximum adaptation phase in a slow shock process without

giving toxicity effect of cryoprotectant on sperm. Another possible explanation is that longer equilibration at optimal osmotic, ionic and pH conditions could enable the reconstitution of the ATP content in sperm (Linhart *et al*., 2005). It can thus be suggested that equilibration duration is an important process for slow reduction of temperature within an ideal duration in order to avoid cold shock during sperm freezing. In agreement with Huang *et al*. (2004b), optimal equilibration duration before freezing is necessary to allow permeating cryoprotectant to penetrate the sperm while minimising toxicity. The most commonly equilibration duration used for fish sperm cryopreservation is 10 to 20 minutes (Billard, 2001). In red tilapia, 60 minutes equilibration gave the highest sperm motility (50.85±1.01%) of frozen-thawed sperm as compared to shorter durations (30 and 45 minutes) (Asmad *et al*., 2008). According to Steyn, (1993), best results for the cryopreservation of sperm of African sharp-tooth catfish, *Clarias gariepinus*, were obtained using a two-step cooling regime, including a cooling rate of 5^{o}C/min. Further study by Christensen *et al*. (2005), they found that a cooling rate of 45^{o}C/min yielded higher post-thawed motility than 3^{o}C/min.

The correlations among sperm motility characteristics for the three equilibration durations (120, 140 and 160 minutes) showed similarities in the pattern except there were differences in correlations of total motility and VAP; progressive motility and ALH; static and STR and VCL and BCF at 120 minutes; ALH and LIN at 140 minutes and progressive motility and BCF at 160 minutes.

Surprisingly, vapour temperature and vapour exposure duration did not give any significant effects on frozen-thawed sperm of African catfish. The rates of total motility for frozen-thawed sperm of African catfish for the three vapour temperatures (-80, -90 and -100^{o}C) obtained were ranged from 25.95±2.34% to 29.41±1.69%. Although, these results differ from findings reported by Asmad *et al*. (2008), the research indicated that vapour

temperature gave a significant results for sperm total motility of frozen-thawed in red tilapia, in which -80^{o}C and -90^{o}C produced a higher values of total motility (51.03±1.12%) and (50.22±1.06%), respectively, but the findings were consistent with the present study of vapour exposure duration in which no significant difference was observed at the three vapour exposure durations (5, 10 and 15 minutes).

Among -80, -90 and -100^{o}C vapour temperature, there were similarities in the pattern of correlations between the sperm motility characteristics, except one correlation at -80^{o}C was not found at -90 and -100^{o}C, between slow and BCF. Other variations of pattern were observed for vapour temperature of -90^{o}C as with those of -80 and -100^{o}C, in which it showed correlations of total motility and slow; rapid and ALH and VCL and BCF. At -100^{o}C vapour temperature, it differs from -80 and -90^{o}C for correlations of total motility and rapid; total motility and medium; progressive motility and rapid; progressive motility and medium; progressive motility and VCL; rapid and VCL; medium and VCL and static and STR.

For 5 minutes vapour exposure relative to 10 and 15 minutes, the patterns of correlation were different among medium and BCF; slow and BCF and VAP and LIN. A correlations among progressive motility and rapid; progressive motility and medium; rapid and VAP; rapid and VSL; rapid and VCL; slow and VAP; slow and VSL; VAP and LIN and ALH and BCF were only noticeably be shown at 10 minutes vapour exposure, whereas 15 minutes was found to give correlations among progressive motility and rapid; progressive motility and BCF; slow and VSL; slow and LIN and ALH and BCF.

5.3 EFFECTS OF EQUILIBRATION DURATION, VAPOUR TEMPERATURE AND VAPOUR EXPOSURE DURATION ON FROZEN-THAWED SPERM MOTILITY OF AFRICAN CATFISH (*Clarias gariepinus*) USING FRE EXTENDER (EXPERIMENT 3)

The results for the present study indicated that the main factors affect the survivability of frozen-thawed sperm of African catfish using FRE extender were equilibration duration and vapour temperature whereby both factors gave significant results of total motility. Contrary to expectations, this study did not find a significant difference in value of sperm total motility after frozen-thawed for vapour exposure duration.

The current study found that the total motility at 120 minutes (76.65±2.27%) and 160 minutes equilibrations (76.01±2.04%) were comparatively higher values than 140 minutes (66.90±2.60%). It is probable, therefore, those 120 minutes is the most suitable duration to equilibrate the sperm because it produced relatively the highest total motility frozen-thawed sperm. Babiak *et al*. (2001) suggested that the effect of equilibration was significantly dependent on the type of cryoprotectant used; they observed that equilibration was not harmful in the case of DMA, whereas it significantly lowered the fertilising ability of cryopreserved sperm in rainbow trout when DMSO or ethylene glycol was used as cryoprotectants. Interaction with extender constituents could be the reason for conflicting information about the use of the equilibration in cryopreservation of fish sperm (Stoss, 1983; Piironen, 1993).

The pattern of sperm motility characteristics correlations among 120, 140 and 160 minutes equilibration durations showed a different pattern, in which 120 minutes gave a contrast correlations between total motility and VAP; total motility and VCL; medium and VAP; medium and ALH and ALH and BCF. However, at 140 minutes marked differences were found among correlations of progressive motility and VAP; progressive motility and

VSL and progressive motility and VCL. The differences in pattern of correlations were shown also at 160 minutes among progressive motility and slow and static and LIN.

The effects of vapour temperature were also significant, in which the values of total motility and progressive motility for vapour temperatures of both -90^{o}C and -100^{o}C (74.07±2.02% and 16.99±0.85%, respectively) and (74.95±1.88% and 20.55±0.93%, respectively) did not show any significant differences, but they were significantly different with -80^{o}C, which gave comparatively lower values (64.59±5.08% and 13.19±1.54%, respectively). This finding suggested that -90 and -100^{o}C are two optimum temperatures which appeared to be suitable for frozen-thawed sperm of African catfish. This study produced results which corroborate with the Asmad *et al*. (2008) findings that suggested -80^{o}C (51.03±1.12%) and -90^{o}C (50.22±1.06%) showed higher value of total motility of frozen-thawed sperm of red tilapia using TCAYE extender. Differences in freezing requirements may have depended on membrane composition, sperm water content or metabolism (Lahnsteiner *et al*., 2000). Best results for cryopreservation of sperm in channel catfish, *Ictalurus punctatus*, were obtained at 45^{o}C/min of cooling rate which gave higher frozen-thawed motility compared to 3^{o}C/min (Christensen and Tiersch, 2005).

Correlations among sperm motility characteristics of -80, -90 and -100^{o}C showed a slightly different in which -80^{o}C was found to have correlations among total motility and VAP; total motility and VSL; progressive motility and slow; rapid and slow; medium and VAP; medium and VSL; medium and VCL; slow and VAP; slow and VSL and ALH and BCF. However, at -90^{o}C, it showed correlations among total motility and BCF; medium and BCF and static and STR. At -100^{o}C, only one correlation differ from -80^{o}C and -90^{o}C that was shown correlation among VAP and LIN.

However, the observed differences of total motility at 5, 10 and 15 minutes vapour exposure durations in this study were not statistically significant. The rates of the frozen-

thawed sperm total motility obtained at 5 to 15 minutes were 72.67±2.27% to 73.99±2.34%. At 5 minutes, only one correlation was different from 10 and 15 minutes which was correlation among progressive motility and slow. The correlations among medium and VAP; medium and VCL and ALH and BCF were different at 10 minutes vapour exposure duration. Marked differences of pattern correlations were found at 15 minutes vapour exposure, between correlations of total motility and BCF; progressive motility and VSL; progressive motility and BCF; rapid and VSL; medium and BCF and slow and BCF.

5.4 EFFECTS OF DIFFERENT EXTENDERS AND CRYOPROTECTANTS ON FROZEN-THAWED SPERM MOTILITY CHARACTERISTICS OF AFRICAN CATFISH (*Clarias gariepinus*) (EXPERIMENT 4)

It is interesting to note that DMSO (10%) in FRE extender was relatively better and gave more consistent frozen-thawed sperm motility results in comparison with TCAYE extender. The current study found that the combination of DMSO (10%) in FRE extender showed the highest significant values of total motility and progressive motility (73.52±1.35% and 18.37±0.61%, respectively) as compared to the three molarities of glycerol (0.5, 1.0 and 2.0 M) in TCAYE extender. It is encouraging to compare this finding with that found by Pan *et al*. (2008) who found that Ringer extender and 10% methanol was the best combination maintained the highest post-thawed motility (65.00±5.00%), fertilisation (90.47±3.67%) and hatching rate (88.00±4.00%). Other finding by Viveiros *et al*. (2000) showed highest hatching rates were obtained by sperm frozen of African catfish, *Clarias gariepinus*, in 10% methanol in Ginzburg fish ringer and post-thawed diluted to 1:200. Previous study by Rurangwa *et al*. (2001) on frozen-thawed sperm of African catfish suggested Mounib's extender provided the best

cryoprotection to the sperm for all post-thawed sperm quality measurements and at all freezing durations. To cryopreserve sperm for long-term, diluents were usually used to supply the sperm cells with sources of energy, protect the cells from temperature-related damage, and maintain a suitable environment for the sperm to survive temporarily (Purdy, 2006). Various extenders with different ion concentrations, osmolality and pH have been successfully used for cryopreservation of different freshwater fish sperm (Linhart *et al*., 2000; Ji *et al*., 2004). Prior studies noted that ringer extender is used for diluting sperm of freshwater fish (Li *et al*., 1994), Kurokura-1 extender is better for sperm Chinese carps, and D-15 extender is known to be very efficient for freezing sperm of Grass carp, *Cicnopharyngodon idellus,* and Silver carp, *Hypopthalmichihys molitrix,* (Chen *et al*., 1992). A finding by Kumar (1988) has shown that egg-yolk citrate produced the highest post-thawed motility and fertilising ability of cryopreserved Indian major carp sperm. Similar result was found in cryopreservation of *Cyprinus carpio* sperm when using Tris-egg yolk (Lakra *et al*., 1997).

Cryoprotectant protects sperm cells from damaging during the process of freezing and thawing, but the extent of damage varies according to the species. The effectiveness of each cryoprotectant such as DMSO, Glycerol and methanol vary in different animal species (Horvath *et al*., 2003; Zhang *et al*., 2003; Velasco-Santamaria *et al*., 2006). In the present study, 10% DMSO gave a better result for sperm motility characteristics of African catfish. The observed 10% DMSO showed higher total motility of sperm and relatively gave the best results, this could be attributed to the fast penetration into sperm and by its interaction with the phospholipids of the sperm membrane (Ogier de Baulny *et al*., 1996). Glycerol with molarity of 0.5 M also showed the moderately good sperm total motility which obtained 32.27±2.05%. An implication of this is the possibility that glycerol was found to be less harmful *in vitro* in many fish species at concentrations

greater than 20% and for longer equilibration times (Stoss *et al*., 1983; Leung *et al*., 1991; Linhart *et al*., 1993).

The pattern of correlations among sperm motility characteristics showed a similarity between glycerol with molarity of 1.0 and 2.0 M. It is likely, therefore, that such connections exist between 1.0 and 2.0 M glycerol. However, a slightly different pattern of correlations among sperm motility characteristics for 0.5 M glycerol in comparison of 1.0 and 2.0 M glycerol was shown in which it differs between correlations of total motility and VAP; total motility and VSL; total motility and VCL; progressive motility and VAP; progressive motility and VSL; progressive motility and VCL; rapid and VAP; rapid and VSL; rapid and VCL; slow and VAP; slow and VSL and slow and VCL. As for 10% DMSO in FRE extender, it showed similarities with 0.5, 1.0 and 2.0 M glycerol, except it differs from those of the three molarities of glycerol among correlations of medium and ALH and static and STR.

5.5 GENERAL DISCUSSION

5.5.1 Overall Findings of This Study

This is believed to be the first successful study on sperm cryopreservation of African catfish (*Clarias gariepinus*) using TCAYE and FRE extenders reported in Malaysia. The findings of this study showed that FRE extender produced more efficient freezability than the TCAYE extender. One of the main problems of African catfish sperm is the presence of lipid globules in the semen that interfere with the freezing process. However, satisfactory procedures developed in this study were able to overcome the problems somewhat, especially for the FRE extender freezing protocol. Some of the successful approaches carried out in this study were to determine the sperm frozen-thawed effects of type and molarity of cryoprotectants, equilibration duration, vapour temperature and

vapour exposure duration. Consequently, after analysing the frozen-thawed sperm using the IVOS 73%, 32% total motility was obtained for the FRE and TCAYE extenders, respectively.

5.5.2 Constraints and Suggestions for Future Improvement

In the present study, there are a lot of constraints and limitations which were faced during the sperm cryopreservation of African catfish. Among the constraints was the limited supply of male African catfish broodstocks, which were bought from a local fish farm. The problem was worsened by the necessity to sacrifice the male broodstocks for each collection of fresh semen. This is in contrast, for an example, in red tilapia fish, stripping is applied to collect the semen. In African catfish, hand-stripping is impractical due to the body shape of this fish is not flat as in red tilapia. Apart from that, the testis of African catfish is located rear of gut and fats; when massage the abdomen, the pressure is applied to the guts and fats instead of testis. This causes blockage of semen from flowing out. Thus, it is highly recommended that a new approach such as surgical technique can be applied for semen collection of African catfish rather than sacrificed technique to ensure that the male of African catfish broodstocks can be kept as brooders for other experiments.

Other constraint faced during the present study was the viscosity and insolubility of African catfish semen with some types of extender that resulted interference with the freezing process of the sperm. From the present study, it was found that TCAYE extender insoluble with African catfish semen, when mixed, it tends to form sperm egg yolk agglutination. Sperm egg yolk agglutination becomes worse when the sperm-extender mixture was activated with water affecting the reading of automated semen analyzer (IVOS; Hamilton-Thorne, USA) failed to analyse the sperm motility characteristics. Understanding the basis of seminal fluid composition may help to improve the suitability

of extender-sperm for freezing technique. A further study should be carried out to design a suitable extender that is similar to the environment of semen.

IVOS assessment is a useful method since it has the added advantage of providing accurate data on additional parameters such as path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN) that cannot be validated by subjective assessment. IVOS has been used widely and proven, especially in the field of andrology for human semen assessment which provide results that reproducible within and between laboratories, and eliminate the subjective human error as well as reduce time-consuming. In the present study, the same concept was applied in fish breeding for determining the parameters affecting fertility in fish. Although IVOS is not the promising method for the success of reproduction of African catfish using frozen-thawed sperm, but it can give a preliminary prediction and assists the selection of high fertility broodstocks. It is recommended that in future a fertilisation trial should be carried out in order to ensure that the frozen-thawed sperm can produce fingerlings.

Besides this, the quality of sperm among individual fish may vary according to their age and maturity of the testis during collection. However, in order to get better results, a maturity age of male catfish should be further studied. In the present study, the age of fish was estimated according to the rough information obtained from the local farmers that supply the fish. It was found that one of the factors that can be regarded as an indicator of maturity age of male catfish is through the size of active testis. The mature male catfish produces a bigger size of testis which formed a whitish-like lobules colour as opposed to the inactive or immature that looks small in size and appears translucent. In future, a study on the effects of testis size gives a clear view in understanding the maturity age of male catfish. Also, the feeding regime may also affect the spermatogenesis of

African catfish. African catfish is unknown for high feed intake; therefore, a sufficient amount of pellets is required for enhancement of the spermatogenesis process.

From the results of the present study, it is obvious that a lot of factors during the freezing process could affect the survivability of African catfish sperm. The effects of each factor are different from one another. Factors such as molarity of glycerol, type of extender and cryoprotectant, equilibration duration and vapour temperature, have shown to play important roles in survivability of frozen-thawed sperm of African catfish sperm. Further research should address practical problems such as increasing frozen-thawed sperm motility characteristics in TCAYE extender, reducing the occurrence of egg yolk agglutination in TCAYE extender so that the IVOS assessment can analyse the data of frozen-thawed sperm. Similarly, for FRE extender, refined experiments should be conducted in certain areas of external factors such as toxicity study of various types and concentrations of cryoprotectants as well as temperature and rates of freezing and thawing. In addition, inherent biological factors of the fish such as age, body size and species as well as external factors such as micro- and macro-environment and nutrition would improve the freezability of African catfish sperm. Therefore, with the increase in understanding the biology and factors affecting the survival of sperm during freezing process, it will facilitate to design experiments on factors involved in optimising and developing a practical and simple freezing protocol of African catfish so that it can be used efficiently and routinely both by the scientists, conservationists and entrepreneurs.

Chapter 6

CONCLUSIONS

Chapter 6

6.0 CONCLUSIONS

a) African catfish (*Clarias gariepinus*) sperm were successfully frozen using TCAYE and FRE extenders.

b) TCAYE and FRE extenders comprise of glycerol and DMSO as cryoprotectants.

c) Equilibration duration of 120 minutes for TCAYE extender gave the highest sperm total motility after frozen-thawed, while 140 minutes for FRE extender.

d) Vapour temperatures of -90 and -100^{o}C for TCAYE and FRE extenders, respectively, gave the highest sperm total motility after frozen-thawed.

e) Exposure vapour durations ranging from 5 to 15 minutes for both TCAYE and FRE extenders were suitable for African catfish sperm freezing.

f) The FRE extender produced higher frozen-thawed sperm motility characteristics than TCAYE extender.

g) The optimal frozen-thawed sperm motility characteristics in African catfish using TCAYE extender was obtained with combination of 0.5 M of glycerol, 140 minutes equilibration duration, -90^{o}C vapour temperature and 5 to 15 minutes exposure vapour duration.

h) The optimal frozen-thawed sperm motility characteristics in African catfish using FRE extender was obtained with combination of 120 minutes equilibration duration, -100^{o}C vapour temperature and 5 to 15 minutes exposure vapour duration.

i) Therefore, it is recommended that FRE extender using DMSO as cryoprotectant apparently is suitable to cryopreserve the sperm of African catfish (*Clarias gariepinus*). However, further studies are needed in future so that it could be used routinely for the industry as well as conservation purposes.

j) The importance of IVOS giving more parameters that will assist the understanding sperm cryobiology and also may solve the fertility problems in the male broodstock with reference to sperm cryopreservation.

k) Using the IVOS, satisfactory frozen-thawed sperm motility characteristics of African catfish using FRE extender when compared with those of fresh semen.

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APPENDIX

APPENDICES

(The following material was adopted from FAO, 1996)

APPENDIX 1 CHARACTERISTICS OF AFRICAN CATFISH (*Clarias gariepinus*)

Appendix 1.1 Description of the Genus and Species

In general, there are three types of catfish species which are similar physically, they are; Asian catfish, *Clarias batrachus* (native to South East Asia including Malaysia, Thailand, Eastern India, Sri Lanka, Bangladesh, Myanmmar, Indonesia, Singapore and Borneo), African catfish, *Clarias gariepinus* (native to middle east and throughout of Africa) and broadhead catfish, *Clarias macrocephalus* (native to tropical Asian countries such as Thailand, Vietnam, China, Malaysia, Guam and Philipines). The main distinguishing feature of the species is the shape of occipital process of skull (Appendix Figure 2.1).

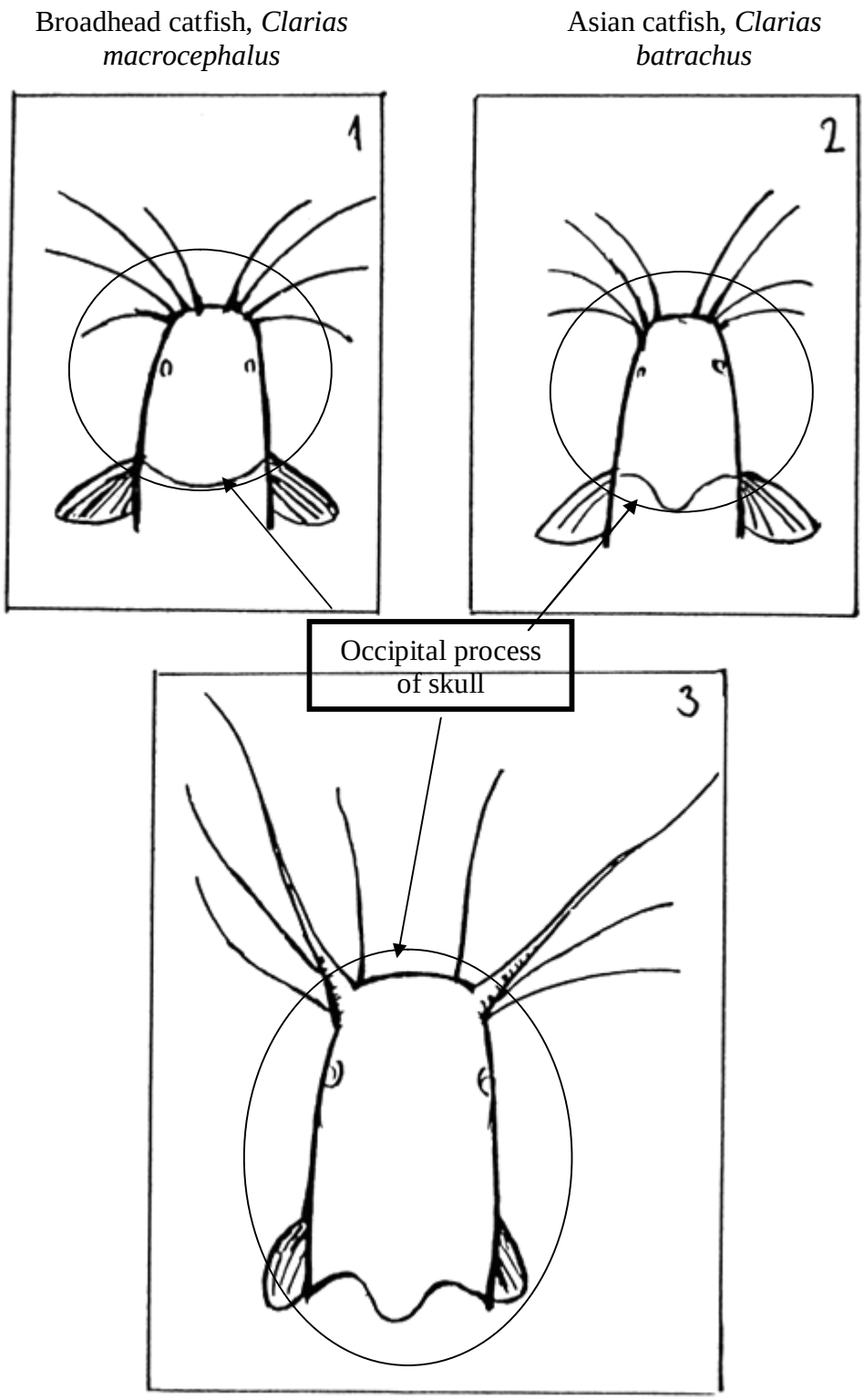
The catfish genus can be defined as displaying an eel shape, having an elongated cylindrical body with dorsal and anal fins being extremely long (nearly reaching or reaching the caudal fin) both fins containing only soft fin rays (Appendix Figure 2.2). The outer pectoral ray is in the form of a spine and the pelvic fin normally has six soft trays. The head is flattened, highly ossified, the skull bones (above and on the sides) forming a casque and the body is covered with a smooth scaleless skin. The skin is generally dark pigmented on the dorsal and lateral parts of the body. The colour is uniform marbled and changes from grayish olive to blackish according to the substrate. On exposure to light, the skin colour generally becomes lighter. They are very well-known for their ability to “walk” on land for long distances, especially during or immediately following rainfall (Axelrod *et al*., 1971; Courtenay *et al*., 1974; Hensley and Courtenay, 1980; Liem, 1987; Shafland, 1994).

Catfish species possesses a large accessory breathing organ which enables them to breathe atmospheric oxygen. A supra-branchial or accessory respiratory organ, composed

of a paired pear-shaped air-chamber containing two arborescent structures is generally present. These arborescent or cauliflower-like structures located on the second hand forth branchial arcs, are supported by cartilage and covered by highly vascularised tissues which can absorb oxygen from atmospheric air (Moussa, 1956). The air chamber communicates with the pharynx and with the gill-chamber. The accessory air-breathing organ allows the fish for many hours out of the water or for many weeks in muddy marshes.

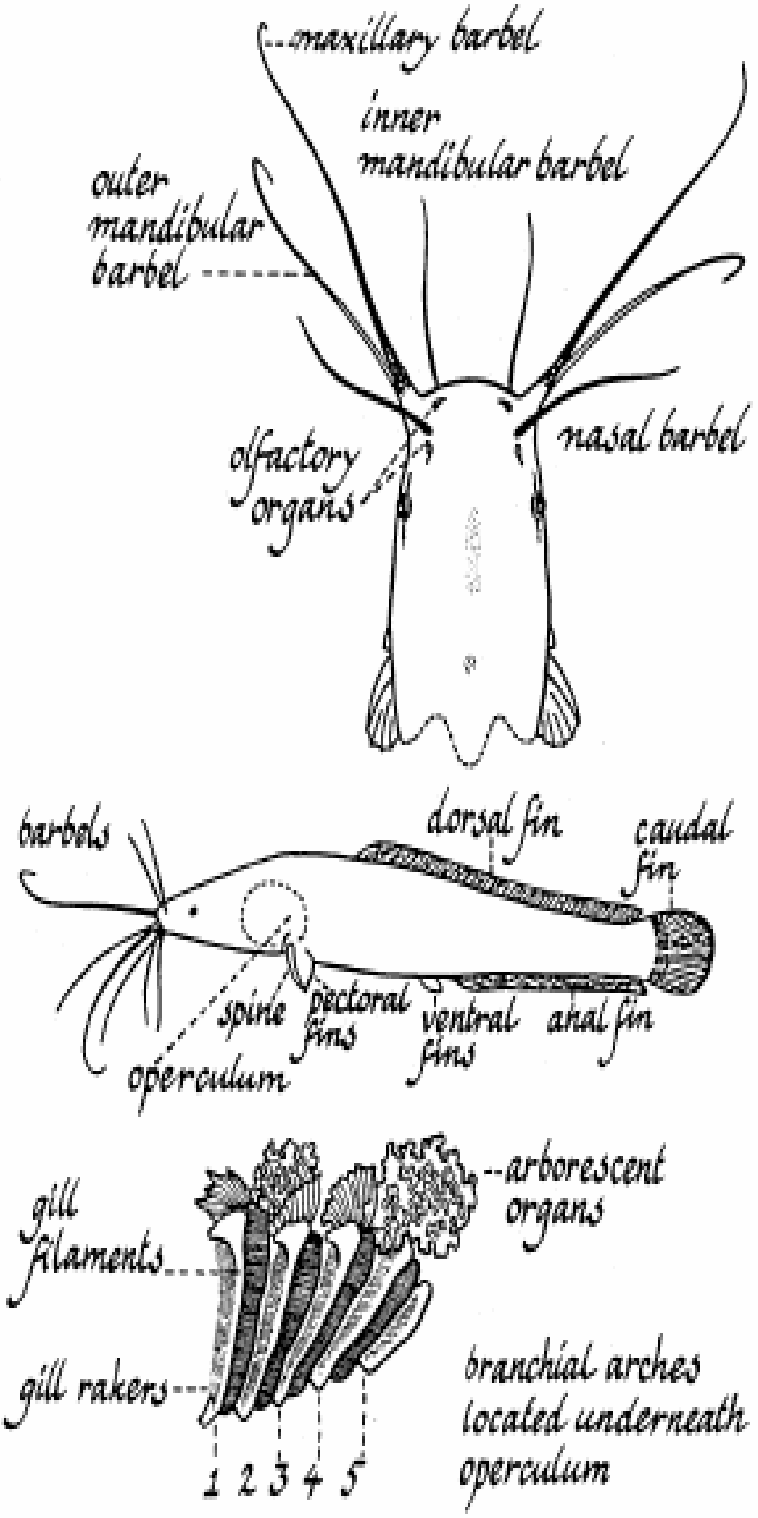
They have four pairs of unbranched barbells, one nasal, one maxillar (longest and most mobile) on the vomer and two mandibulars (inner and outer) on the jaw. Tooth plates are present on the jaws as well as on the vomer. The major function of the barbels is prey detection.

The sex of the African catfish (*Clarias gariepinus*) can be easily distinguished as the male has a distinct sexual papilla, located just behind the anus. This sexual papilla is absent in females (Appendix Figure 1.1).

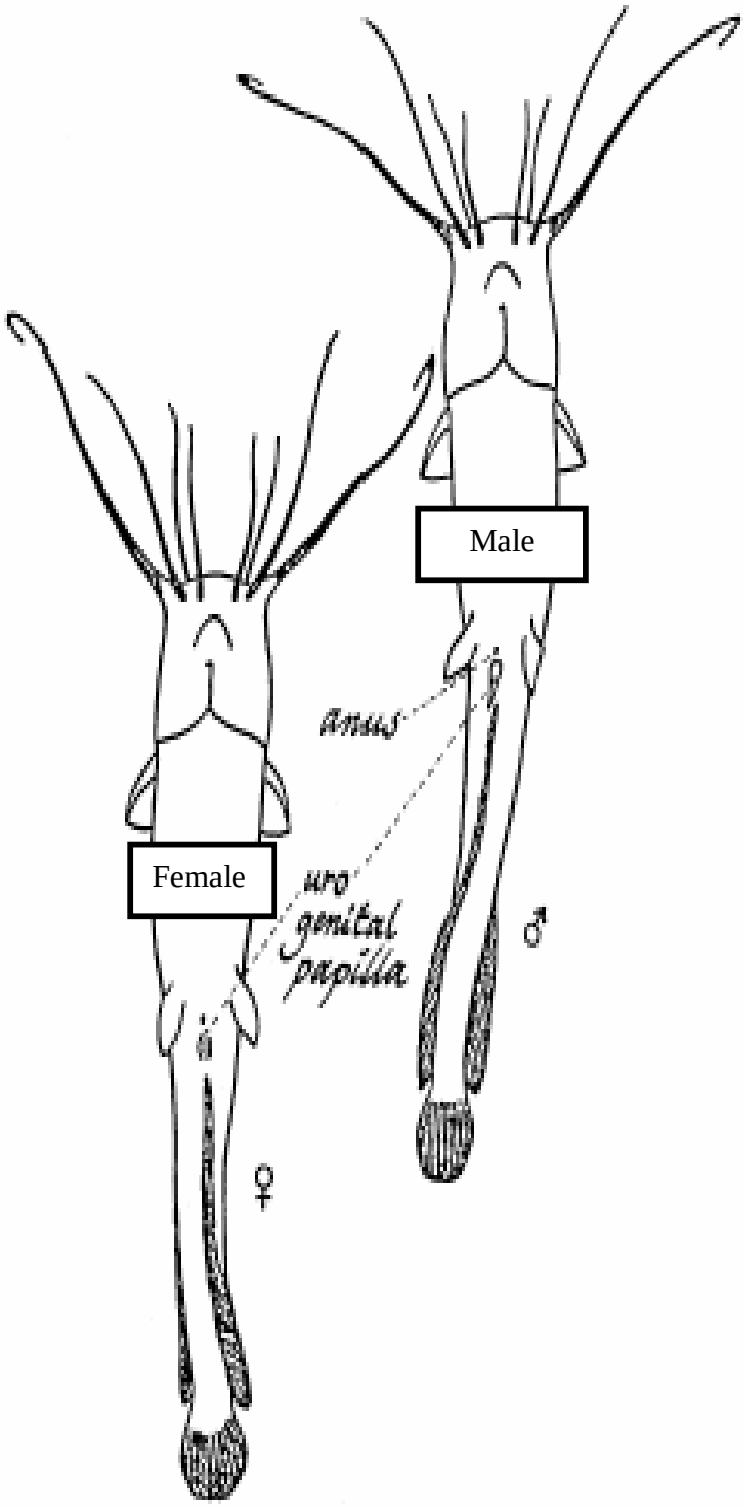


African catfish, *Clarias gariepinus*

Appendix Figure 1.1: Occipital process of *Clarias macrocephalus* (1), *Clarias batrachus* (2) and *Clarias gariepinus* (3) (Srisuwahtach and Tangtrongpiros, 1985; Viveen *et al.*, 1985).



Appendix Figure 1.2: Morphological characteristics of *Clarias* spp. (FAO, 1996).



Appendix Figure 1.3: Sexual characteristics of *Clarias gariepinus* (FAO, 1996).

Appendix 1.2 Habitat

Clarias spp. inhabits calm waters from lakes, streams, rivers, swamps to floodplains, some of which are subject to seasonal drying. The most common habitats frequented are floodplain, swamps and pools in which the catfish can survive during the dry seasons due to the presence of the accessory air breathing organs (Bruton and Clay, 1979).

Appendix 1.3 Temperature Tolerance

The walking catfish is a tropical species with a moderate tolerance to colder waters. Shafland and Pestrak (1982) reported a lower lethal temperature of 9.8^{o}C, based on which they placed Gainesville as the northern limit to its potential range expansion. During cold dry months, walking catfish burrow into sides of ponds and streams where they remain dormant until the spring rains initiate (Courtenay *et al*., 1974). Courtenay (1970) reported winter-kills of walking catfish during January of 1970, when the temperature in northern Broward County dropped to -1.67^{o}C. However, deeper warm waters served as refuges, allowing many walking catfish to survive the brief cold spell.

Appendix 1.4 Salinity Tolerance

Clarias spp. occurs in fresh, brackish as well as marshy, muddy waters over its native range (Sen, 1985). Courtenay *et al*. (1970) reported walking catfish to occur in intercostals waterways of up to 18 ppt.

Appendix 1.5 Distribution

There are still some questions regarding the natural range of *Clarias* spp. (Talwar and Jhingran, 1991). Walking catfish are considered native to Sri Lanka, Eastern India,

Pakistan, Bangladesh, Myanmmar, Ceylon, Malaysia, Singapore, Philipines, Borneo, Java and Thailand (Axelrod *et al*., 1971; Jayaram, 1981; Sen, 1985; Talwar and Jhingran, 1991).

Appendix 1.6 Trophic Interaction

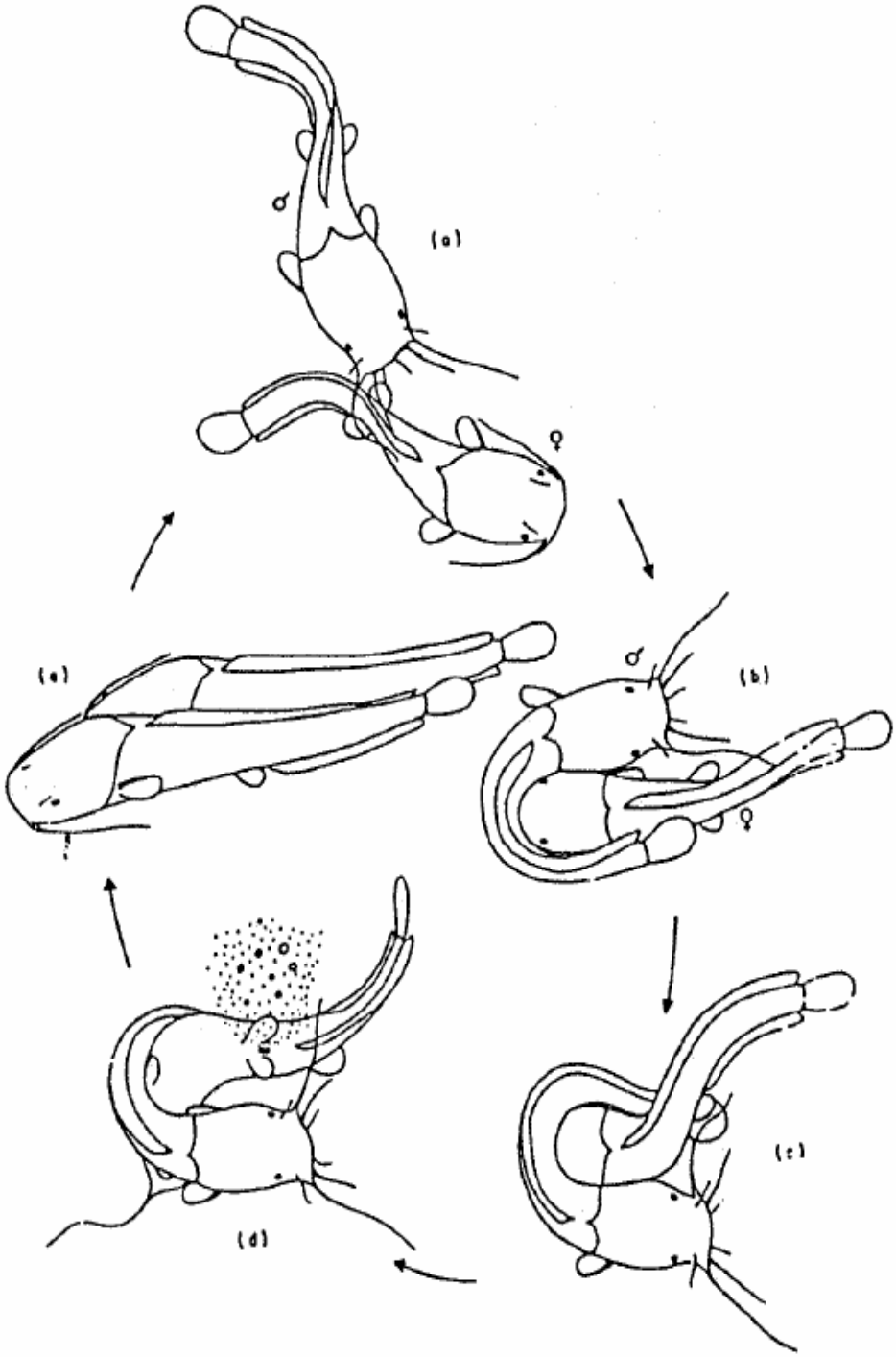
Walking catfish are voracious, opportunistic feeders. They are mainly active at night. Major prey items include attached periphyton for the young, insect larvae, insects such as *Haliplus* sp., dystiscid beetles, mayflies, and dragonflies, fish larvae, attached fish eggs, fish such as *Fundulus, Gambusia,* and *Lepomis* and occasionally they may take plant material (Courtenay, 1970; Courtenay *et al*., 1974). Courtenay and Miley (1975) and Courtenay (1978) reported walking catfish to kill large bass, without consuming them afterwards. During periods of drought, large numbers of individuals may congregate into isolated pools, and quickly consume most other species present (Courtenay *et al*., 1974). This species can remain dormant through periods of drought, and go several months without eating. Courtenay (1970) reported keeping several individuals deprived of food for eight months, without any ill effects observable, except minor weight loss.

Appendix 1.7 Natural Reproduction

Clarias spp. engages in mass spawning migrations in late spring and early summer (Courtenay *et al*., 1974). Inundated paddy fields have been reported as favored spawning grounds over its native range (Talwar and Jhingran, 1991). Adhesive eggs are laid in a nest or on submerged vegetation (Hensley and Courtenay, 1980). Males guard the nests (Hensley and Courtenay, 1980). Juveniles 50 mm appear in late summer, and late larval stages as well as early juveniles, have been collected until the first week of November in Broward County, Florida (Courtenay *et al*., 1974). Sexual maturity is attained at the end of the first year (Talwar and Jhingran, 1991).

Spawning usually takes place at night in the shallow inundated areas of the rivers lakes and streams. Courtship is preceded by highly aggressive encounters between males. Courtship and mating takes place in shallow waters between isolated pairs of males and females. The mating posture, a form of amplexus (the male lies in a U-shape curved around the head of the female) is held for several seconds (Figure 2.4). A batch of milt and eggs is released followed by a vigorous swish of the female's tail to distribute the eggs over a wide area. The pair usually rest after mating (from seconds and up to several minutes) and then resume mating.

There is no parental care for ensuring the survival of the catfish offspring except by the careful choice of suitable site. Development of eggs and larvae is rapid and the larvae are capable of swimming within 48-72 hours after fertilization at 23-28^{o}C.



Appendix Figure 1.4: The courtship ritual of catfish (Bruton, 1970).