Frozen-thawed Sperm Motility Characteristics of African Catfish (*Clarias gariepinus*) by Using Glycerol or DMSO Based Extender

Noor Azlina Kamaruding, Wan Khadijah Wan Embong, and Ramli Bin Abdullan

***Abstract*—The objective of this study was to evaluate the efficacy of Tris-Citrate Acid Yolk Extender and Fish Ringer Extender on sperm motility characteristics in African Catfish with special focus on equilibration durations (120, 140 or 160 min), vapor temperatures (-80, -90 or -100oC) and vapor exposure durations (5, 10 or 15 min) as well as the fish body weights. Combination of DMSO (10%) in FRE extender showed significantly the highest values of respective post-thawed total motility and progressive motility (73.52±1.35% and 18.37±0.61%) as compared to the three molarities of glycerol (0.5, 1.0 and 2.0 M) in TCAYE extender (32.27±2.05 and 3.75±0.41; 24.50±1.81 and 2.63±0.29; 26.74±2.14 and 2.45±0.37). It is suggested that FRE is a preferred extender to TCAYE to freeze the African Catfish sperm.**

***Index Terms*—Equilibration, extender, sperm cryopreservation, vapor exposure.**

I. Introduction

Fresh sperm motility assessment is a useful step to indicate the success of cryopreservation technique as well as giving the baseline information on the fecundity of African catfish. A considerable amount of literature has been published on factors affecting sperm quality in fish such as rearing photoperiod and temperature, nutrition, water and food contamination, stress, age of broodstock, breeding season, diseases of broodstock, hormonal induction and spermiation [1]-[3]. Age of broodstock could be apparently linked with their body weight and plays a major role in sperm maturation period. Until now, there is no study related to effect of body weight on sperm motility characteristics of African catfish.

To date, studies investigate the key parameters of sperm samples (e.g. ionic composition, osmolality [4], development of appropriate activation media, immobilization solutions, cryoprotective agents, equilibration time, cooling rates, sperm packaging unit, semen:extender ratio, storage vessel and thawing rates [5]-[7] have demonstrated a significant achievement in many freshwater fish species. These parameters are invariably different among and within fish species, and consideration of interaction factors should be assessed in order to develop successful freezing protocol. Protocols of sperm cryopreservation can be varied because of species-specific differences in sperm size, shape, and biochemical characteristics [8].

Extender acts as 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 cryoprotectant 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 [9],[10]. 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) [11].

Development of sperm cryopreservation protocols for African catfish in the present study was no simple task due to the seminal composition of this fish which consists of high lipid content. Therefore, 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, University of Malaya. Fish Ringer Extender (FRE) was a preliminary attempt in our laboratory for sperm cryopreservation of African catfish.

II. Materials and Methods

Adult catfish, *Clarias gariepinus*, 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. Routine management of fish includes periodical exchange the water with fresh clean water to ensure easy absorption of oxygen and disease prevention. The tap water was dechlorinated before being supplied to the fish. The broodstocks were hand-fed with "commercial finisher layer mash" twice a day, ad libitum and daily monitored.

Body weight of selected catfish was taken to quantify the dosage of hormone per body weight for each individual fish. Ovaprim (0.5 ml/kg body weight; Syndel, Vancouver,

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Noor Azlina Kamaruding is with the Department of Biological Sciences, Faculty of Science and Technology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, MALAYSIA (E-mail: noor.azlina@umt.edu.my).

Wan Khadijah Wan Embong and Ramli Bin Abdullah are with Animal Biotechnology-Embryo Laboratory (ABEL), Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, MALAYSIA (E-mail: wkhadi@um.edu.my; ramli@um.edu.my).

Canada) was injected intramuscularly into the dorsal muscle of catfish. 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. After receiving the hormone treatment, these males were isolated overnight in a separate tank to avoid aggressive interaction with other males and to maximize care during the experimental period.

A total of 15 fish were weighed individually to get an actual body weight (BW). The actual body weight was used to categorise three respective sizes, namely small BW (<1.0 kg), medium BW (1.0 -1.5 kg) and large BW (>1.5 kg). The testis of the sacrificed male African catfish was dissected out from the body cavity and was cleaned with tap water to rinse the blood. Then, the testis was gently perforated with needle to collect the milt. Precaution during perforation 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 fresh sperm motility by using IVOS. Total motility and progressive motility were used for assessing survivability rate of pre-freezing semen by using an automated semen analyzer (IVOS; Hamilton-Thorne, USA).

Collected semen from testis of sacrificed catfish was diluted with Fish-Ringer Extender (FRE) and Tris-Citrate Acid Yolk Extender (TCAYE), respectively, in a ratio of 1:10 using 0.5 ml or 0.25 ml French straws. FRE consists of NaCl (0.75 g), KCl (0.10 g), $CaCl_2$ (0.016 g), $MgSO_4$ (0.023 g), NaH_2PO_4 (0.041 g) and Glucose (0.10 g) per 100 ml. The molarity of cryoprotectant was fixed at 10% DMSO as described by [12]. TCAYE comprises of Tris, Citric Acid, Streptomycin, Egg Yolk and Penicillin [13],[14]. Three molarities of glycerol in TCAYE (0.5, 1.0 or 2.0 M) were evaluated. 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 min), 3 vapor temperatures (-80, -90 or -100^{o}C) and 3 vapor exposure durations (5, 10 or 15 min). Each of the combination treatments was replicated 3 times with 5 observations per replicate. Total motility and progressive motility were used for assessing survivability rate of post-thawed cryopreserved sperm using an automated semen analyzer (IVOS; Hamilton-Thorne, USA).

All the data were subjected to analysis of variance (ANOVA), followed by comparison of means using Duncan's multiple range test (DMRT). Data analysis was performed by SPSS (Statistical Package for Social Sciences) for windows, version 12.0.

III. Results

A. Effect of Individual Body Weight on Fresh Sperm Motility in African Catfish (Clarias gariepinus)

Evaluation of fresh sperm indicated that 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 lower in total motility compared with the other two groups. Progressive motility values 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 1).

TABLE I: Assessment of Fresh Sperm Motility of African Catfish in Different Body Weight Groups

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

N* = Number of fish.
^{ab}Means with different superscripts within a row were significantly different (P<0.05).

B. Effects of Different Extenders and Cryoprotectants on Frozen-thawed Sperm Motility Characteristics of African Catfish

TABLE II 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 significantly lower than 0.5 M of glycerol with a value of 32.27±2.05%. On the other hand, combination of DMSO (10%) in FRE extender showed the highest significantly highest 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 II: 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).

The velocity distribution of post-thawed cryopreserved sperm of African catfish using different types of extender and cryoprotectant is shown in TABLE III. 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. However, DMSO (10%) in FRE gave comparatively significantly the highest value of rapid and medium velocities (24.09±0.79% and 9.27±0.28%, respectively) as compared to 0.5, 1.0 and 2.0 M glycerol. 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 significantly higher value as compared 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 III: 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 IV demonstrates sperm motion characteristics of post-thawed cryopreserved sperm of African catfish by 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).

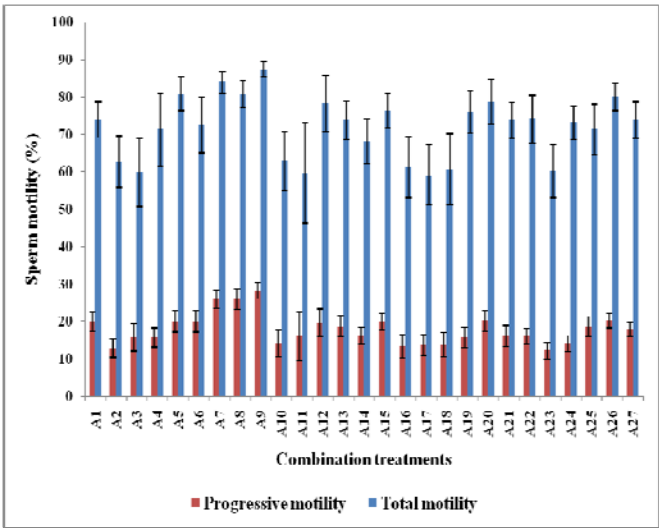
TABLE IV: SPERM MOTION CHARACTERISTICS (MEAN±SEM) OF POST-THAWED CRYOPRESERVED SPERM OF AFRICAN CATFISH (*CLARIAS GARIEPINUS*) USING DIFFERENT TYPES OF EXTENDER AND CRYOPROTECTANT

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

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

C. Effects of Combination Factors of Equilibration Duration, Vapor Temperature and Vapor Exposure Duration on Frozen-thawed Sperm Motility Characteristics of African Catfish (Clarias gariepinus) using FRE Extender

Fig. 1 shows total motility and progressive motility of post-thawed cryopreserved sperm of African catfish by using FRE extender. The highest values of total motility and progressive motility were obtained from combination factors of 120 min equilibration duration, -100oC vapor temperature and 15 min vapor exposure duration (87.44±2.07% and 28.22±2.16%, respectively). Combination of 140 min equilibration duration -100oC vapor temperature and 10 min vapor exposure duration showed the lowest values of total motility (59.27±8.00%), but the lowest value of progressive motility was observed at combination of 160 min, -90oC and 10 min (12.20±2.24%).

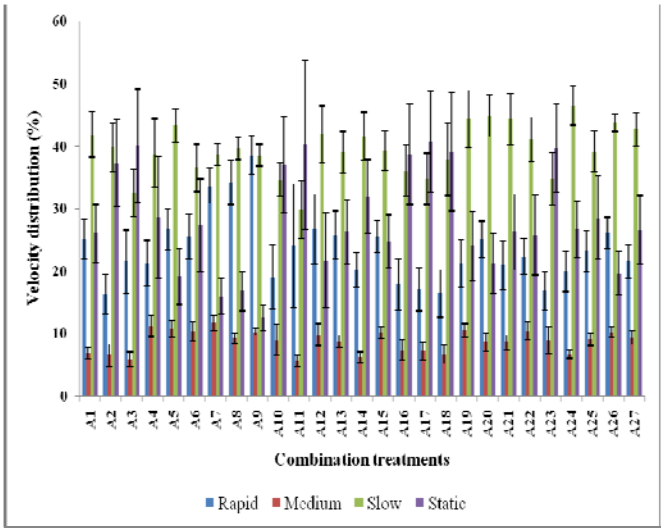


B1: 120 min, -80°C, 5 min; **B2**: 120 min, -80 °C, 10 min;
B3: 120 min, -80 °C, 15 min; **B4**: 30 min, -110 °C, 5 min;
B5: 30 min, -110 °C, 10 min; **B6**: 30 min, -110 °C, 15 min;
B7: 30 min, -120 °C, 5 min; **B8**: 30 min, -120 °C, 10 min;
B9: 30 min, -120 °C, 15 min; **B10**:45 min, -100 °C, 5 min;
B11: 45 min, -100 °C, 10 min; **B12**: 45 min, -100 °C, 15 min;
B13: 45 min, -110 °C, 5 min; **B14**: 45 min, -110 °C, 10 min;
B15: 45 min, -110 °C, 15 min; **B16**: 45 min, -120 °C, 5 min;
B17: 45 min, -120 °C, 10 min; **B18**: 45 min, -120 °C, 15 min;
B19: 60 min, -100 °C, 5 min; **B20**: 60 min, -100 °C, 10 min;
B21: 60 min, -110 °C, 15 min; **B22**: 60 min, -110 °C, 5 min;
B23: 60 min, -110 °C, 10 min; **B24**: 60 min, -110 °C, 15 min;
B25: 60 min, -120 °C, 5 min; **B26**: 60 min, -120 °C, 10 min;
B27: 60 min, -120 °C, 15 min

Fig. 1. Total motility and progressive motility in different combination treatments of equilibration duration, vapor temperature and exposure vapor duration of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*).

Fig. 2 shows velocity distributions of post-thawed cryopreserved sperm of African catfish using 10% DMSO in FRE extender for combination factors of equilibration duration, vapor temperature and vapor exposure duration. Combination of 120 min equilibration duration, -100oC vapor temperature and 15 min vapor exposure duration gave the highest value of rapid velocity (38.56±3.10%), while combination of 120 min, -80oC and 10 min gave the lowest value (16.33±3.21%). For medium, slow and static velocities, the highest value were attained by combination of 120 min, -100°C and 5 min (11.75±1.29%), 160 min, -80°C and 10 min (44.92±3.34%) and 140 min, -100°C and 10 min

(40.73±8.00%), respectively. Both the lowest values of medium and slow velocities were shown by combination of 140 min, -80°C and 10 min (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 min, -100°C and 10 min (40.73±8.00%), whereas the lowest value was combination of 120 min, -100°C and 15 min (12.56±2.07%).



B1: 120 min, -80°C, 5 min; **B2**: 120 min, -80 °C, 10 min;
B3: 120 min, -80 °C, 15 min; **B4**: 30 min, -110 °C, 5 min;
B5: 30 min, -110 °C, 10 min; **B6**: 30 min, -110 °C, 15 min;
B7: 30 min, -120 °C, 5 min; **B8**: 30 min, -120 °C, 10 min;
B9: 30 min, -120 °C, 15 min; **B10**:45 min, -100 °C, 5 min;
B11: 45 min, -100 °C, 10 min; **B12**: 45 min, -100 °C, 15 min;
B13: 45 min, -110 °C, 5 min; **B14**: 45 min, -110 °C, 10 min;
B15: 45 min, -110 °C, 15 min; **B16**: 45 min, -120 °C, 5 min;
B17: 45 min, -120 °C, 10 min; **B18**: 45 min, -120 °C, 15 min;
B19: 60 min, -100 °C, 5 min; **B20**: 60 min, -100 °C, 10 min;
B21: 60 min, -110 °C, 15 min; **B22**: 60 min, -110 °C, 5 min;
B23: 60 min, -110 °C, 10 min; **B24**: 60 min, -110 °C, 15 min;
B25: 60 min, -120 °C, 5 min; **B26**: 60 min, -120 °C, 10 min;
B27: 60 min, -120 °C, 15 min

Fig. 2. Velocity distribution in different combination treatments of equilibration duration, vapor temperature and exposure vapor duration of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*).

IV. Discussion

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 (<1.0kg) gave the lowest fresh sperm total motility (40.40±12.16%). It is difficult to explain this result, but it might be related to number 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 [15].

From the three molarities of glycerol studied in TCAYE extender, 0.5 M showed significantly the 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 [16], 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 [17], 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°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 [18]. Glycerol (10%) also proved effective for freezing Asian catfish, *Heteropneustes fossilis* and *Clarias batrachus* sperm, yielding 69 to 84% of control hatching rates [19]. However, glycerol was toxic to salmonid sperm, whereas DMSO could be used for cryopreservation [20]. Equilibration duration is another superior factor that gave significant differences in cryopreservation of African catfish sperm. However, the sperm total motility results for vapor temperature and vapor exposure duration using TCAYE extender were not statistically significant.

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 [21], 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 [22] 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 [23] 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 [24]. Various extenders with different ion concentrations, osmolality and pH have been successfully used for cryopreservation of different freshwater fish sperm [25],[26]. Prior studies noted that ringer extender is used for diluting sperm of freshwater fish [27], 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,* [28]. A finding by [29] 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 [30].

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 [31]-[33]. 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 [34]. 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 [35], [36].

Best cryopreservation of sperm in African sharptooth catfish, *Clarias gariepinus*, were obtained using a two-step cooling regime, including a cooling rate of 5°C / min [37], whereas, in channel catfish, a cooling rate of 45°C / min yielded higher post-thawed motility than did 3°C / min [38]. A cooling rate from 5 to 11°C / min was specified as optimal in cryopreservation of European catfish (*Silurus glanis*) [39]. The final temperature and its duration just before plunging the frozen sperm into liquid nitrogen were very important.

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, vapor temperature and vapor 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.

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 broodstock 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 color 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 result 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 vapor 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 be analyzed 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 optimizing 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.

V. Conclusion

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.

It is suggested that FRE is a preferred extender to TCAYE to freeze the African Catfish sperm.

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Noor Azlina Kamaruding received her Bachelor of Science (Biotechnology) from University of Malaya, Kuala Lumpur, Malaysia in 2002. After completing her undergraduate level, she then pursued her master`s degree in Biotechnology and graduated in 2010. This paper is part of her master's project. Presently, she is a tutor at the Department of Biological Sciences, Faculty of Science, Universiti Malaysia Terengganu. Currently, she is under study leave, pursuing her PhD in the area of reproductive maturation of shrimps, Macrobrachium rosenbergii.

Prof. Dr. Wan Khadijah Embong is currently a senior research fellow at University of Malaya. Her main research interests are animal and fish breeding concerning genetics aspect, goat improvement in tropics and animal embryo biotechnology. She obtained her Bachelor of Science and Master of Science specialisation in animal science and animal breeding and genetics, respectively from Louisiana State University, USA. She received her PhD from University of Malaya in animal breeding and genetics. She is actively conducting research in development of sperm cryopreservation, embryo transfer technique, In vitro fertilization and stem cells techniques from goat, caprine and monkey.

Prof Dr. Ramli Abdullah is professor at the University of Malaya. He received his undergraduate and postgraduate education from Louisiana State University, USA. His Bachelor of Science was in animal science and postgraduate level (master and PhD) were in reproductive physiology. His main research interests are in mammalian embryo and reproductive biotechnology, reproductive endocrinology, goat improvement in tropics and commercialisation of goats through reproductive biotechnologies.