



Review Article

Semen cryopreservation in catfish species, with particular emphasis on the African catfish

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Abstract

This paper reviews current knowledge concerning the cryopreservation of semen from catfish species, with emphasis on the African catfish, *Clarias gariepinus*. The majority of the fish species reared in captivity exhibit some form of reproductive dysfunction, leading to reduced semen production which may or may not be of low quality. Reproductive hormones have successfully been used to induce spermiation and increase semen volume in several species. However, in most catfishes, the response of the male remains poor and semen is not released by hand-stripping. In practice, males are sacrificed, testes are macerated and intratesticular sperm is spread over the eggs. To overcome this problem, fish semen preservation has become an indispensable alternative in fish selection and synchronisation of gamete availability. However, the treatment of sperm prior to storage and the cryopreservation conditions reduce its ability to fertilise eggs. Semen from some catfish species has been frozen in cryotubes, cryovials, straws or as pellets, in salt or sugar solutions combined with an internal cryoprotectant (glycerol, dimethyl sulphoxide (DMSO), dimethyl acetamide (DMA), or methanol), at slow or fast cooling rates. Many of these freezing protocols yield high post-thawed motility and/or hatching rates and are discussed in this review.

Keywords: Semen preservation: Cryoprotectants: *Clarias gariepinus*: African catfish: Freezing techniques: Frozen semen: Cooling

1. Introduction

Since the first attempt to preserve semen [1], there have been numerous improvements in conservation technology (e.g. discovery of cryoprotectants, development of semen extender, progress of reproductive technology) with a number of detailed reviews of cryopreservation of both mammalian [2–4] and fish spermatozoa [5].

The importance of preserving genetic resources for the future is widely recognised, and the conservation of semen would be a major contribution with great potential applications in agriculture, biotechnology, species conservation and clinical medicine [4]. In aquaculture, the major benefits resulting from fish gamete preservation include [6]:

- Long-term storage for regular or future use;
- Genetic improvement through selective breeding programmes;
- Production of reference stocks for culture or research;
- Production of hybrids;
- Reduction of the cost and labour of maintaining broodstock;
- Elimination of the need for precise synchronisation of males and females;
- Repeated spawning of specific males;
- International shipment and use;
- Germplasm conservation and development of germplasm repositories;
- Use in conjunction with interspecific androgenesis to restore endangered or extinct species.

It is estimated that semen from 200 fish species has been cryopreserved, including that from several endangered species [6–10]. There has also been considerable research on the development of gene banks for aquatic species [5]. However, the use of information in many published protocols for cryopreservation of fish sperm is difficult. The reported post-thaw viability, even for the same species, is highly heterogeneous, some reports are incomplete, and given that only positive results are usually published, the true variability of results remains unknown. Because of these difficulties, development of reliable cryopreservation protocols for fish sperm is often practised on a species-by-species basis. To assist in sharing knowledge, emphasis should be placed on standardisation of each step in a procedure: semen collection, dilution ratio, extenders, cryoprotectants, equilibration time, cooling rates, thawing rates, and sperm:egg ratio in fertilisation tests [6]. Before any protocol is adopted, methods for evaluation should be carried out for each step. Motility tests are mostly used to assess sperm viability after thawing. However, during our experiments with African catfish [11], we have observed that post-thawed samples with very few (sometimes none) motile sperm cells yielded high hatching rates. This might be surprising to those familiar with mammalian sperm, where fertilising capability is highly correlated with motility, but the same phenomena observed in African catfish was reported in the Mekong giant catfish *Pangasius gigas* [6]. Therefore, fertility or hatching rates should be used to evaluate post-thaw sperm viability. On the other hand, when fertilisation is included in the post-thaw evaluation, sperm:egg ratios must be optimised. Using an excess of sperm cells for fertilisation obviously masks the quality of cryopreserved semen, making comparison of protocols difficult.

To develop an efficient freezing protocol, some background information on cryobiology is needed, especially the possible causes of cryoinjuries. It is well known that cryoinjuries can be caused by slower- or faster-than-optimal cooling and thawing rates, and that the level of cryoinjury can be affected by the choice of cryoprotectants and the composition of the freezing medium (extenders).

2. Cryoinjuries and cooling rates

A complete freezing programme combines cooling rate, the number of steps involved during cooling (programme type) and temperature at which plunging into liquid nitrogen (LN₂) occurs. The combination of these three elements determines whether or not viable cells can be frozen to temperatures that permit indefinite storage. A summary of the literature on freezing programmes for catfish semen is given in Table 1.

Some background information on cryobiology is needed to understand the possible causes of cryoinjuries and how to prevent them. For more details on cryobiology, refer to Mazur [12,13]. Cooling, either

slowly or too rapidly, tends to be damaging. When cells are cooled between -5 and -15°C, ice forms in the external medium, but cell contents remain unfrozen and supercooled. The latent heat of crystallisation raises the temperature to the freezing (or melting) point of the medium, e.g. to -5°C. As part of the water exits the extracellular medium as it becomes ice, the residual solute concentrates. This residual brine is hyperosmotic to the unfrozen cells. The supercooled water in the cells then flows out. The subsequent physical events in the cell depend on the cooling rate. If cooling is sufficiently slow, the cell is able to lose water rapidly, dehydrate and shrink. However, prolonged exposure of the unfrozen cells to the increasing hyperosmotic residual medium will lead to excessive dehydration, shrinkage and a very high intra- and extracellular solute concentration. The effects of these events is referred to as “solution effects” [12]. On the other hand, if the cell is cooled too fast, it is not able to lose water fast enough to maintain equilibrium. Water becomes increasingly supercooled and eventually freezes internally, forming intracellular ice. During thawing, these ice crystals may recrystallise to larger crystals and disrupt cell membranes. A clear line between damage caused by intracellular ice and solution effects cannot be drawn, mainly because solution effects can happen throughout the entire freezing process. The optimal cooling rate, then, is slow enough to prevent intracellular freezing but yet rapid enough to minimise the length of time during which cells are exposed to solution effects [13]. Because different cells have different volume-to-surface area ratio and permeability to water, the optimal cooling rate varies over a wide range, from -1.6°C/min for marrow stem cells to about -4000°C/min for the human erythrocyte [12]. The optimal cooling rate for sperm cells varies from -1-10°C/min for human semen [14] to -100°C/min for bull semen [3].

The cooling rate should be neither too fast nor too slow, as described above and the number of steps involved during cooling (type of programme) depends on the cooling rate. When semen is submitted to a very rapid cooling rate by plunging directly into LN₂ (1-step programme), the post-thawed survival is low. African catfish [15] and Indian catfish *Heteropneustes fossilis* [16] sperm were frozen in straws or vials directly in LN₂ and the post-thawed survival rate was 0% in both studies. The absence of survival after thawing indicates that cells did not have time to dehydrate and died due to intracellular ice formation, either during cooling or during rewarming. A possibility for increasing sperm survival of samples frozen directly in LN₂, is increasing the thawing rate in order to block recrystallisation of the small ice crystals into larger ice crystals (see next section).

In order to reduce intracellular freezing, 2-step programmes have been used. In general, this programme consists of an initial slow cooling period followed by rapid cooling as samples are plunged into LN₂ for final storage. In 2-step programmes, cells have more time to dehydrate before intracellular freezing, compared to the 1-step programme. Catfish

Table 1. Literature review on cooling programmes tested on semen cryopreservation of catfish species.

Catfish species	Cooling rates	Plunging temperature (°C)	Number of steps	Recommended cooling programme	Thawing rate	Refs
<i>Clarias gariepinus</i>	Plunging directly into LN ₂ ; at LN ₂ vapour for 2 min; slower rates	+20 to -80	1, 2, 3, 4	-7°C/min to -65°C; LN ₂	25°C water bath	[15]
	-4 or -6.5°C/min to -5°C then -11°C/min	-70	3, 4	-11°C/min from -5°C to -70°C; LN ₂	25°C water bath	[33]
	-8°C/min	Not described	3	Not described	25°C water bath	[41]
	-2, -5, -8, -11, -14 and -17°C/min	-70	2, 3, 4	-4°C/min to and held at -5°C for 2 min, then -5°C/min to -70°C; LN ₂	25°C water bath	[17]
	-4°C/min to -5°C then -5°C/min	-70	4	-4°C/min to and held at -5°C for 2 min, then -5°C/min to -70°C; LN ₂	25°C water bath	[38]
	-4°C/min to -4°C then -11°C/min	-80	3	-4°C/min to -4°C then -11°C/min to -80°C; LN ₂	40°C water bath for 5 s	[22] [20]
	-2, -5 and -10°C/min	-25 to -70	2, 3	-5°C/min to and held at -40°C for 5 min; LN ₂	27°C water bath for 5 min	[11]
	-8°C/min	-80	4	-8°C/min to -80°C with a 5-min hold at -5°C; LN ₂	25°C water bath for 2 min	[21]
	-2, -5 and -10°C/min	-30 to -65	2, 3	-5 or -10°C/min to and held at -40°C for 5 min; LN ₂	27°C water bath for 3 min	[18]
<i>Clarias batrachus</i>	Directly to -70°C	+25; stored at -70°C	1	directly to -70°C and stored at -70°C	25°C water bath for 2 min	[16]
<i>Heteropneustes fossilis</i>	Directly to -70°C or to LN ₂	+25 and -70; stored at -70°C or at LN ₂	1, 2	directly to -70°C and stored at -70°C	25°C water bath for 2 min	[16]
<i>Heterobranchius longifilis</i>	3 cm above LN ₂ level	Not described	3	20 min at 3 cm above LN ₂ level; LN ₂	25°C water bath	[42]
<i>Ictalurus furcatus</i>	6.5 cm above LN ₂ level	-66	3	15 min at 6.5 cm above LN ₂ level; LN ₂	Air-thawed at 25°C	[34]
<i>Ictalurus punctatus</i>	-16°C/min	-140	2	-16°C/min to -140°C; LN ₂	40°C water bath for 7 s	[25]
<i>Mystus nemurus</i>	-40°C/min	-80	3	-40°C/min to and held at -80°C for 20 min; LN ₂	40°C water bath for 120 s	[23]
<i>Pangasius gigas</i> (<i>Pangasianodon gigas</i>)	Varying heights above LN ₂ level	Not described	2	127 mm above LN ₂ level; LN ₂	4°C water bath	[43]
<i>Pangasius hypophthalmus</i> (<i>P. sutchi</i>)	At LN ₂ vapour (-80°C)	-75	2	4 min at -80°C; LN ₂	40°C water bath for 7 s	[24]
	Crushed ice for 5 min + dry ice for 5 min	Not described	3	Crushed ice for 5 min + dry ice for 5 min; LN ₂	40°C water bath for 5 min	[36]
	-10; -12; -20 and -40°C/min	Not described	Not described	-12°C/min	50-, 70-, 80°C water bath for 20-140 s	[6]
	-10°C/min; -4°C/min to -4°C then -11°C/min	-80	2, 3	-10°C/min to -80°C; LN ₂	Air-thawed at 25°C for 40 s	[26]
	2 cm or more above LN ₂ level	Not described	2	Post-thawed semen coagulated into a soft jelly-like mass	29°C water bath	[44]
<i>Silurus glanis</i>	0 to 20 mm above LN ₂ level	-50 to -105	2	10 min at 2 mm above LN ₂ level ($\cong$ -85°C); LN ₂	30°C water bath for 10 s	[19]
	3 cm above LN ₂ level	Not described	3	20 min at 3 cm above LN ₂ level; LN ₂	37°C water bath for 10 s	[35]

semen has been frozen at several cooling rates in 2-step programmes. Some studies have evaluated the effects of different cooling rates on post-thawed sperm viability. One of the first studies on freezing African catfish semen is described by Steyn *et al.* [15]. The authors tested seven cooling rates: plunging samples directly into LN₂; at LN₂ vapour (-80°C) for 2 min then plunged into LN₂; and slower rates by adding known quantities of dry ice to isopropanol. The most successful cooling rate tested was a relatively slow rate of -7°C/min to -65°C, then plunging into LN₂. Later on, the same authors used a computerised freezing apparatus to freeze catfish semen at the rates of -2, -5, -8, -11, -14 and -17°C/min. Samples were plunged into LN₂ until it reached -70°C and sperm motility was analysed 24 h later. The highest sperm motility (60%) was observed in samples frozen at -5°C/min [17]. In another study of the African catfish, a programmable freezer was used to freeze semen at -2, -5 and -10°C/min in combination with different temperatures at plunging into LN₂ (-25 to -70°C). Hatching rates equal to unfrozen semen were obtained by samples frozen at -5°C/min to -45 to -50°C and at -10°C/min to -55°C [11]. The same researchers repeated this experiment measuring both freezer and semen temperature during the freezing programme. It was observed that cells cooled at a rate of -2-5°C/min survived plunging at a semen temperature of -38°C, while cells cooled at -10°C/min did not. At -10°C/min, high post-thaw sperm survival was obtained only when plunging occurred at a lower temperature of -51°C. It appears that, at a cooling rate of -10°C/min, and certainly at faster rates, cell dehydration lags behind and osmotic equilibration is not reached or approached [18]. Based on these results, one can conclude that at slower cooling rates, the use of a 2-step programme is beneficial when cryovials or cryotubes are used as containers. However, good results were obtained when the pellet technique (which produces a very fast cooling rate), in combination with a 2-step programme, was used to freeze semen. In the European catfish, pellets were formed by dripping semen onto aluminium discs placed at 0, 2, 4 or 20 mm above the LN₂ level. After 10 min, the pellets were plunged into LN₂. Highest sperm motility was obtained in samples frozen at 2 mm (-80 to -85°C) above LN₂ level [19].

At fast rates, the use of a 3-step programme is more appropriate. This programme consists of an isothermal holding period added to the end of the slow cooling period and before plunging. There are three interesting studies on African catfish that describe when a 3-step programme improves post-thaw sperm survival and when it does not, compared to a 2-step programme. Firstly, a computerised freezing apparatus was used to freeze semen at the rates of -2, -5, -8, -11, -14 and -17°C/min. Half of the samples were plunged into LN₂ until they reached -70°C and the other half remained at -70°C for 2 min before plunging. The authors concluded that there was a clear cellular specificity for the cooling rate of -5°C/min regardless of the programme type and, depending on the medium composition and cryoprotectants, the optimal rate may vary from -5 to -11°C/

min. Furthermore, the incorporation of the 2-min holding period before plunging led to a remarkable improvement in sperm survival when the freezing medium was composed of DMSO and glucose [17]. Secondly, semen was frozen at the same rate of -5°C/min to -35 and -40°C in a programmable freezer and plunged into LN₂. The hatching rates produced by these samples were 0%, but when samples were subjected to a 5-min holding period at -35 or -40°C before plunging, the hatching rates increased to values similar to unfrozen semen [11]. Finally, in a subsequent study, the same authors measured both freezer and semen temperature during several freezing programmes. It was observed that when the freezer temperature was -35°C, semen temperature was still at -13°C, but decreased to -33°C after a 5-min holding period. Similarly, during the 5-min holding period at -40°C, semen temperature fell from -21°C to -38°C. The decrease in semen temperature during the holding period before plunging was followed by an increase in the hatching rate of these samples after thawing. During the holding period, while the freezer temperature remained constant, the semen temperature continued decreasing until equilibrium with the freezer temperature was achieved or until plunging occurred. Consequently, extra time was provided for water efflux and the elimination of spatial temperature gradients within the semen sample. When a faster cooling rate of -10°C/min was used in combination with a 3-step programme, semen could be plunged into LN₂ at a semen temperature of -36°C instead of -51°C (2-step programme) to produce high hatching rates [18]. For the sake of clarity, the use of a holding period is not always beneficial. At the slowest cooling rate tested, -2°C/min, the addition of holding periods was negative for sperm viability. It seems that at such a slow rate, sperm cells were already in osmotic equilibrium with the surrounding medium. Remaining at a constant low temperature would expose the cells for too long to a highly concentrated solute and may force excessive dehydration and shrinkage.

The temperature at which plunging into LN₂ should occur, as observed by Viveiros *et al.* [18], is one point that merits special attention. While most of the research on semen cryopreservation focuses on freezing injuries caused by fast cooling (the intracellular ice formation), the other source of injuries caused by slow cooling has received less attention. In fact, the formation and/or dissolution of intracellular ice is lethal and no hatching is obtained when semen is frozen too fast or is plunged into LN₂ before dehydration has reached the level necessary to preclude intracellular freezing. This is the reason why most of the protocols use a very low plunging temperature for semen, not only for African catfish (-65°C [15]; -80°C [20–22]), but also for channel catfish (-80°C [23,24]), blue catfish (-140°C [25]), European catfish (-80 to -85°C [19]), striped catfish *Pangasius hypophthalmus* (-80°C [26]), carp (-80°C [27]), the marine ocean pout (-95°C [28]) and also for bulls (-140°C [29]). However, in the African catfish, decreasing hatching rates were obtained when semen temperature at plunging was below -50°C. The authors

speculated that, below -50°C and at relatively slow cooling rates, sperm cells were damaged by overlong exposure to high concentrations of electrolytes and/or continued osmotic shrinkage. According to these results, catfish semen should be cooled at a rate of -2 , -5 or $-10^{\circ}\text{C}/\text{min}$ to a freezer temperature of -40°C and plunged into LN_2 as soon as the semen temperature reaches -38°C [18]. One should keep in mind, however, that the suggested protocol is valid only when 1-ml cryovials are used. Cryovials are shorter and wider, and consequently have a smaller volume-to-surface area ratio, than straws. While cooling occurs more uniformly among sperm cells displayed inside a straw, different temperatures may be possible within the cryovial. Lower temperatures are found among the cells displayed closer to the cryovial wall, while higher temperatures are found at the centre (Viveiros and Komen, unpublished observation). Similar research on the optimal plunging temperature should be carried out with semen of other catfish species and using other semen containers such as 0.5- and 0.25-ml straws.

3. Thawing rates

The warming rate during thawing of the semen is also of importance. The effects of warming rate on cell survival will depend on the cooling history. Cells cooled much too rapidly will have large intracellular ice crystals and will be dead. Cells cooled more slowly, but still relatively fast could have very small intracellular ice crystals. During slow warming these crystals could grow in a process called recrystallisation, which could damage the cells. These cells could therefore be rescued by rapid thawing. When cells are cooled very slowly the intracellular ice formation could be insignificant, and a high warming rate may not be needed. Actually, these cells are probably very much dehydrated and shrunk and could be damaged by rapid thawing because of a too rapid rehydration [30]. In general, cells frozen at fast cooling rates, as pellets, 0.25- and 0.5-ml straws, should be very rapidly thawed in a warmer water-bath, compared to those cells frozen at slower rates in vials or tubes containing a larger semen volume. Frozen catfish semen has been mostly thawed in a 25 - to 40°C -water bath (Table 1).

4. Cryoprotectant agents and extenders

Even though the survival of the cells during freezing and thawing can be maximised by optimising the freezing and warming protocols, the post-thaw recovery will be unacceptably low if cryoprotectants are not added to the freezing medium. The discovery that glycerol could prevent the damaging effects of slow cooling and thawing on spermatozoa and red blood cells [31], renewed interest in banking living cells and tissues at low temperatures in a state of anhydrobiosis or cryptobiosis. Since then, many other compounds have been found to protect living cells against some of the damage caused by freezing. A range of permeating cryoprotectants has been used

but the most common ones for protecting fish semen during freezing are: dimethyl sulphoxide (DMSO), glycerol, methanol, dimethyl acetamide (DMA), ethylene glycol and propylene glycol. These cryoprotectants depress the freezing point of the extracellular medium, ameliorate the detrimental effects of ice crystals and regulate the rate of cell dehydration, reducing the damages caused by high solute concentration during slow cooling rates (the solution effects). However, at high concentration and/or during prolonged equilibration time (period of time between adding the cryoprotectant to the cells and freezing), cryoprotectants denature cellular proteins and this can reduce pre-freezing viability [32].

Methanol has the smallest molecule size and thus the fastest cellular permeation when compared to other permeating compounds such as DMSO, DMA and glycerol. However, whether to be protective, these compounds have to permeate a cell, is still unclear. Experiments using different ways of blocking cryoprotectant permeation (such as addition of copper ions in the medium, a very short equilibration time of 30 seconds, or equilibration at temperatures that a cell is not permeable to the cryoprotectant anymore) have yet demonstrated good post-thaw cell survival. Perhaps, the ability of a cell to survive freezing depends more on protection of the cell surface than on protection of the cell interior [12]. Non-permeating compounds like sugars (glucose, trehalose, sucrose) and proteins (milk powder, egg yolk, glycoproteins) are frequently added to the freezing medium and are known for their membrane-stabilising activity [29].

In a preliminary experiment (Viveiros and Komen, unpublished observation) carried out at Wageningen University (Netherlands), fresh African catfish semen was incubated (4°C) with DMSO, glycerol or methanol at a concentration of 0–25% and for a period of 0–60 min prior to fertilisation. DMSO could be used at a maximum of 15% for 5-min equilibration and glycerol at 5% and for 5-min equilibration, without reducing sperm fertilising ability. Longer equilibration periods or higher DMSO or glycerol concentrations affected sperm viability and significantly decreased the hatching rates. Similar African catfish sperm sensitivity to glycerol was observed by Horváth *et al.* [20] who found that 5% glycerol in combination with only 2 minutes of equilibration significantly reduced post-thawed sperm viability. Contrary to these results, glycerol showed good freezing protection to sperm of the same fish species, at a concentration of 5–11% and an equilibration period as long as 60 minutes [15,17,33]. In the Wageningen study, fresh semen incubated with methanol at concentrations up to 20% for 60 min, did not affect the sperm fertilising capacity. Only when methanol was used at 25% did the sperm fertilising capacity decrease. When experiments on semen cryopreservation followed this preliminary part, it was confirmed that methanol was a more suitable cryoprotectant for freezing African catfish semen compared to DMSO [11]. However, Hungarian researchers have observed in the same species, that the highest post-

thawed motility rates were yielded by semen frozen in DMSO compared to those samples frozen in DMA, glycerol, methanol, ethylene glycol and propylene glycol [20].

The same contrasting results have been demonstrated in other catfish species (Table 2). In the blue catfish, semen frozen in DMSO produced higher post-thawed fertilisation rate compared to samples frozen in methanol [34]. However, fresh semen of the same catfish species exposed to methanol for 30 min yielded the highest sperm motility rates compared to samples exposed to glycerol, DMSO or DMA. Based on these results, methanol was chosen for use in subsequent cryopreservation trials [25]. In the channel catfish, methanol proved to be the best cryoprotectant yielding the highest post-thawed sperm motility, when compared to DMSO, glycerol, sucrose and polyvinylpyrrolidone [24], and to DMA [23]. In the European catfish, semen frozen in glycerol produced the highest post-thawed sperm motility, compared to those samples frozen in DMSO or ethylene glycol. Furthermore, ethylene glycol was so toxic to sperm cells at the range of 5–30% that no sperm motility was observed even before freezing [19]. In another study with the same catfish species, DMA was the best cryoprotectant compared to DMSO, glycerol, methanol or propylene glycol [35]. In the striped catfish *Pangasius hypophthalmus*, semen samples frozen in DMSO produced the highest post-thawed fertilisation rate, compared to those samples frozen in DMA, ethylene glycol or methanol [26]. In the bagrid catfish *Mystus nemurus*, methanol proved to be the best cryoprotectant, compared to ethanol, glycerol or DMSO when post-thawed sperm motility was evaluated [36].

The difference in catfish sperm sensitivity to cryoprotectants observed in the literature (Table 2), may be due to different protocols used to incubate semen (such as extender composition, semen:freezing medium dilution ratio or temperature during equilibration) or different methods to assess sperm motility or fertility (such as spermatozoa:egg ratio). Furthermore, it is possible that differences in seminal plasma composition exist between wild and domesticated catfish, and among domesticated catfish reared under different conditions and that these differences may influence sperm sensitivity to cryoprotectants.

The efficacy of cryopreservation is also dependent on the pre-freezing sperm quality. Semen should be collected under hygienic conditions in order to avoid contamination by fish urine, mucus or water. In freshwater teleosts, sperm is held within the testis in an immotile state by the ionic concentration in the seminal plasma. Sperm immotility is attributed to the presence of factors such as K^+ , Na^+ , Ca^{++} and Mg^{++} ions. Sperm motility is initiated when semen is diluted in water (or in another hypotonic solution), by a decrease in the osmolality of the extracellular medium and in the K^+ ion concentration, and an increase in the concentration of free Ca^{++} ions [37]. Thus, semen contamination by urine or water

induces sperm motility and decreases the quality of frozen sperm. In the European catfish, it is a common practice to strip semen directly onto an immobilising Saad solution, as urine contamination is highly inevitable [19]. The osmolality of the freezing medium should be similar to the seminal plasma. A 244-mOsm but not a 220-mOsm saline extender (Ginzburg fish Ringer; Viveiros and Komen, unpublished observation) and a 355-mOsm 6% fructose [22] were suitable media for freezing African catfish semen. Hanks' balanced salt solution is a 290–295-mOsm extender and extensively used as a freezing medium for the blue catfish [25], channel catfish [23,24] striped catfish *P. hypophthalmus* [6] and Mekong giant catfish *P. gigas* [6] semen. Catfish semen has been diluted with the freezing medium at different ratios. Some researchers use a low 1:1 ratio [17,20,22,38], while others prefer a higher 1:10 [11,19] or even 1:20 [36] semen:extender-plus-cryoprotectant dilution ratio.

Several researchers have reported the effect of pH on sperm motility. In many species, pH is involved in the control of flagellar movement and the optimal pH values seem to be species-specific according to the seminal plasma pH. Seminal plasma pH has been determined in the African catfish (7.73 [39]), bagrid catfish *M. nemurus* (8.0 [36]), striped catfish *P. hypophthalmus* (8.3 [6]) and Mekong giant catfish *P. gigas* (8.2 [6]). As a consequence, most of the extenders suitable for freezing catfish semen are adjusted to a pH between 7.0 and 8.4 (Table 2).

Semen is mostly stored in LN_2 at $-196^\circ C$ and, at this temperature, cell viability can be maintained in a genetically stable form and is affected only by background radiation [40]. The importance of preserving genetic resources for the future is widely recognised, and the conservation of semen would be a major contribution with great potential application in agriculture, biotechnology, species conservation and clinical medicine. As males are killed for artificial reproduction in most catfish culture, semen cryopreservation offers a unique tool for genetic preservation. Conservation of genetic variability of the wild catfish population is also of importance, as domestication could lead to the formation of genetically altered strains with reduced genetic variability in a very short period of time. Van der Walt *et al.* [38] reported that the use of limited numbers of broodstock without adding genes from unrelated fish to successive generations, is responsible for the occurrence of genetic drift and the founder effect in most domesticated African catfish populations. Based on this statement, it is recommended to cryopreserve the excess intratesticular semen collected when a mature catfish male is sacrificed for reproduction purpose. For example, a 2-kg African catfish male can produce at least 7 ml of intratesticular semen. For immediate use, 1 ml of fresh semen can be diluted 2000 times in 0.9% NaCl. This 1 ml of semen is enough to effectively fertilise 5 kg of eggs (from about 10 females). The remaining 6 ml can then be diluted 10 times and cryopreserved in 120 vials, according to the methods described by Viveiros *et al.*

Table 2. Literature review on extenders and cryoprotectants tested on semen cryopreservation of catfish species.

Catfish species	Extenders	Cryoprotectants	Recommended cryodiluent	Refs
<i>Clarias gariepinus</i>	5% glucose; Mounib solution ^a ; milk + fish saline	DMSO; glycerol; methanol	5% glucose + 5% glycerol	[15]
	4% glucose; extender # 1 ^b	DMSO; glycerol; methanol	4% glucose + 11% glycerol	[33]
	4% glucose	DMSO; glycerol; methanol	4% glucose + 11% glycerol	[41]
	4% glucose (pH 8.1); extender # 1 ^b (pH 8.3)	DMSO; glycerol; methanol	4% glucose + 9% glycerol	[17]
	4% glucose	glycerol	4% glucose + 9% glycerol	[38]
	333 mmol/l glucose; 333 mmol/l fructose; 292 mmol/l sucrose; 171 mmol/l NaCl; 174 mmol/l KCl; artificial seminal plasma ^c (all pH 7.7)	DMSO	6% fructose + 10% DMSO	[22]
	6% fructose (pH 7.7)	DMA; DMSO; glycerol; methanol; ethylene glycol; propylene glycol	6% fructose buffered with NaHCO ₃ -CO ₂ + 10% DMSO	[20]
	Ginzburg fish Ringer ^d (pH 7.6)	DMSO; methanol	Ginzburg fish Ringer ^d + 10% methanol	[11]
	4% glucose; Mounib solution ^a + 10% egg yolk	DMSO; glycerol	Mounib solution ^a + 10% egg yolk + 8% DMSO	[21]
<i>Clarias batrachus</i>	cryodiluent B ^e ; cryodiluent C ^f ; 0.6% NaCl; glycerol	glycerol	0.6% NaCl + 10% glycerol	[16]
	Mounib solution ^a + egg yolk	DMSO; glycerol	Mounib solution ^a + 10% egg yolk + 5% DMSO + 5% glycerol	[42]
<i>Heterobranchius longifilis</i>	cryodiluent B ^e ; cryodiluent C ^f ; 0.6% NaCl; glycerol	glycerol	0.6% NaCl + 10% glycerol	[16]
<i>Heteropneustes fossilis</i>	HBSS ^g	DMSO; methanol + skim milk	HBSS ^g + 10% DMSO	[34]
<i>Ictalurus furcatus</i>	0.5% NaCl; modified Cortland solution ^h ; Truscott and Idler's Hfx # 1 ⁱ ; among others	DMSO; glycerol; propylene glycol	Truscott and Idler's Hfx # 1 ⁱ + 5 or 10% DMSO	[43]
<i>Ictalurus punctatus</i>	Modified HBSS ^j	DMSO; glycerol; methanol; sucrose; polyvinylpyrrolidone	Modified HBSS ^j + 5 or 10% methanol	[24]
	Modified HBSS ^j ; glucose-free modified HBSS ^j	DMA; methanol	Modified HBSS ^j + 5% methanol	[23]
	0.7% NaCl (pH 6.0); 0.8% NaCl + 0.5% glucose (pH 8.4); Ringer ^k (pH 7.9)	DMSO; ethanol; glycerol; methanol	Ringer ^k + 10% methanol	[36]
<i>Mystus nemurus</i>	calcium-free HBSS ^l ; 0.9% NaCl; bicarbonate buffer	DMSO; propylene glycol	0.9% NaCl + 10% DMSO	[6]
<i>Pangasius gigas</i> (<i>Pangasianodon gigas</i>)	Modified HBSS ^j ; calcium-free HBSS ^l ; 0.9% NaCl	DMA; DMSO; ethylene glycol; methanol	0.9% NaCl + 12% DMSO	[26]
<i>Pangasius hypophthalmus</i> (<i>P. sutchi</i>)	extender 251 ^m ; extender 189M ⁿ	DMSO; glycerol	Post-thawed semen coagulated into a soft jelly-like mass	[44]
	200 mM NaCl + 30 mM Tris (pH 7.0)	DMSO; glycerol; ethylene glycol	200 mM NaCl + 30 mM Tris + 12 or 15% glycerol	[19]
<i>Silurus glanis</i>	Mounib solution ^a + 10% egg yolk	DMA; DMSO; glycerol; methanol; propylene glycol	Mounib solution ^a + 10% egg yolk + 10 or 15% DMA	[35]

^a Mounib solution (g/l): reduced glutathione 2.0; KHCO₃ 10.0; sucrose 42.0. ^b Extender # 1 (g/l): KCl 1.0; CaCl₂ 0.15; NaCl 3.97; NaHCO₃ 3.07; MgCl₂ 0.06; glucose 0.13; hens egg albumen 1.38. ^c Artificial seminal plasma (mmol/l): glucose 278; NaCl 12.5; KCl 1.23; CaCl₂ 0.135; MgCl₂ 0.064. ^d Ginzburg fish Ringer (g/l): NaCl 6.5; KCl 0.25; CaCl₂ 0.3; NaHCO₃ 0.2. ^e Cryodiluent B (g/l): KCl 1.85; KHCO₃ 10.0; sucrose 102.0; egg yolk 100 ml. ^f Cryodiluent C (g/l): NaCl 6.0; NaHCO₃ 4.5; egg yolk 100 ml. ^g HBSS, Hanks' balanced salt solution (g/l): CaCl₂ 0.14; NaCl 8.0; KCl 0.4; MgSO₄·7H₂O 0.20; Na₂HPO₄·7H₂O 0.06; KH₂PO₄ 0.03; NaHCO₃ 0.17; glucose 1.0. ^h Modified Cortland solution (g/l): NaCl 1.88; CaCl₂·2H₂O 0.23; KCl 7.2; NaH₂PO₄·H₂O 0.41; NaHCO₃ 1.0; MgSO₄·7H₂O 0.23; glucose 1.0. ⁱ Truscott and Idler's Hfx # 1 (g/l): NaCl 5.16; KCl 1.64; NaH₂PO₄·H₂O 0.41; NaHCO₃ 1.0; MgSO₄·7H₂O 0.23; CaCl₂ 0.14; fructose 1.0. ^j Modified HBSS (g/l): CaCl₂·2H₂O 0.16; NaCl 8.0; KCl 0.4; MgSO₄·7H₂O 0.20; Na₂HPO₄·7H₂O 0.12; KH₂PO₄ 0.06; NaHCO₃ 0.35; glucose 1.0. ^k Ringer (g/l): NaCl 7.5; NaHCO₃ 0.2; KCl 0.2; CaCl₂·2H₂O 0.2; glucose 5.1. ^l Calcium-free HBSS (g/l): NaCl 8.89; KCl 0.44; MgSO₄·7H₂O 0.22; Na₂HPO₄·7H₂O 0.13; KH₂PO₄ 0.07; NaHCO₃ 0.39; glucose 1.1. ^m Extender 251 (g/l): NaCl 8.5; NaHCO₃ 5.0; vegetable lecithin 15.0. ⁿ Extender 189 M (g/l): NaCl 7.3; NaHCO₃ 5.0; fructose 5.0; vegetable lecithin 7.5; mannitol 5.0.

[18]. Vials should be thawed according to requirements, and the content of each vial will be enough to fertilise 25 g of eggs (ca. 15,000 eggs). Using this technique, semen from a single male can fertilise a total of 8 kg of eggs (ca. 4,800,000 eggs) and produce at least 2,400,000 larvae throughout the years.

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