Trehalose and Dimethyl Sulfoxide As Cryoprotective Solution Inclusions in a Static Liquid Nitrogen Vapour Vitrification Method for African Catfish (*Clarias gariepinus* Burchell, 1822) Sperm

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Abstract

Captive breeding of *Clarias gariepinus* (Burchell, 1822) entails stripping of mature females, while males must be killed or testes partially excised to obtain spermatozoa. Cryopreservation could significantly improve the reproductive potential of male catfish. In this study, Ginzburg fish Ringer (GFR) was the common extender in all protocols with or without 0.3 M trehalose. Dimethyl sulfoxide (DMSO) and methanol were tested to determine the preferred equilibration period for the complex before cryofreezing. The cryopreservation method and sperm fertilisation capacity were assessed in terms of hatchability rate. A feasible semen rationing protocol for maximised fertilisation was also evaluated. Semen equilibration with cryoprotectants was allowed for 30 minutes at 4 °C. Liquid nitrogen (LN_2) vapour-induced freezing rates ranged from -2.7 to -5 °C.min^{-1}, and cryovials/straws were immersed in LN_2 when -41 °C was reached after 12 min. Straws showed significantly improved cryopreservation efficacy over cryovials in terms of fertilisation. Optimal fertilisation occurred with trehalose and DMSO-treated cryostored sperm, comparable to fresh semen. Only 0.05 mL diluted semen was required to fertilise 35g eggs, compared with a similar volume efficacy for undiluted sperm. Trehalose enhanced egg fertilisation for both diluted fresh sperm and DMSO-treated thawed sperm. All methods produced healthy larvae to 21 days post-hatch. The validated method can yield 60 straws (0.5 mL) from 3 mL semen, fertilising approximately 2.1 kg eggs (~162 × 10^4 eggs). The LN_2 vapor vitrification method was shown to be both as effective as and more cost efficient than controlled rate freezing equipment.

Keywords: cryopreservation, DMSO, methanol, trehalose, vapour vitrification

Introduction

The African catfish *Clarias gariepinus* (Burchell, 1822) flourishes in diverse environments across a wide geographical range, from sub-tropical to tropical regions. It has unique respiratory capabilities that contribute to its adaptability to diverse water quality conditions. It consumes a diverse range of natural prey and is highly fecund. These biological characteristics make it ideal for captive breeding, and the African catfish is widely considered the most suitable species for aquaculture (Hecht et al., 1996). Furthermore, it can potentially be produced at super-intensive high stocking densities (>400 kg.m^{-3}) within a suitably designed RAS (Baßmann et al., 2023).

Sexually mature *C. gariepinus* broodstock undergoes continuous gametogenesis under controlled (28 °C; 24 hours of darkness) photothermal conditions (Fouché, pers. comm., 2023; El-Nasser et al., 2001). After that, the broodstock is treated with spawning inducers, and the females release eggs readily after hand stripping. Males, however, do not exhibit typical reproductive behaviour or release sperm, either spontaneously or after stripping (Steyn et al., 1985). Consequently, conditioned male breeders must be sacrificed (Steyn et al., 1985), or a portion of their testes must be excised (Bart and Dunham, 1990). Also, the maximum quantitative egg fertilisation capacity of diluted thawed cryopreserved African catfish sperm must still

be determined to justify the economic cryo-storage. Cryopreservation techniques could be useful to address this significant broodstock management challenge (Viveiros et al., 2000). Some of the cryobiological implementations in aquaculture have been outlined by Bozkurt (2023): a) Cryostorage of sperm cells for routine fertilisation processes, b) Utilisation of all the gametes for large-scale production, c) Transportation of gametes or embryos among the farms, d) Marketing of well-characterised gametes, e) Hybridisation of species, f) Reduction of the synchronisation treatments, g) Year-round supply of broodstock gametes.

Extensive research has already been conducted into sperm cryopreservation as a tool to address the endangered status of wild salmonid stocks. This research has yielded improved sperm cryopreservation protocols using various extenders and cryoprotectants, as well as freezing and thawing methods (Sarvi et al., 2006). While much progress has been made, factors such as oxidative stress, cryoprotectant toxicity, and ice crystal formation (affected by freezing rate) still provide significant challenges, inflicting damage to spermatozoa form and function (Erraud et al., 2021).

Several studies have investigated sperm cryopreservation methods and recommended treatments for African catfish (Steyn et al., 1985; Steyn and van Vuren, 1987; Steyn, 1993; van der Walt et al., 1993; Urbanyi et al., 1999; Viveiros et al., 2000; Omitogun et al., 2010). Prior to 2000, the extender-cryoprotectant of choice was glucose-glycerol. Post-2000 saw the use of alternative extender-cryoprotectant complexes viz. GFR extender + DMSO or methanol (Urbanyi et al., 1999; Viveiros et al., 2000; Omitogun et al., 2010). Several freezing rates and a range of endpoints have been evaluated. Viveiros et al. (2000) also demonstrated that African catfish sperm could be cryopreserved with post-thaw egg fertilisation efficacy similar to fresh sperm (10 % methanol with GFR as diluent; post-thaw diluted 1:200) using controlled rate freezing, as well as an endpoint freezing of -41 to -45 °C post initiation (prior to LN_2 immersion).

The inclusion of trehalose - a disaccharide - improved post-thaw spermatozoa motility and fertility in common carp semen by providing an energy source, maintaining diluent osmotic pressure and having cryoprotectant properties (Bozkurt et al., 2016). Because monosaccharides and disaccharides cannot diffuse across the membrane, the osmotic pressure induces cell dehydration and reduces the incidence of intracellular ice formation (Agca et al., 2002; Fuller, 2004). The cryoprotective efficacy of sugars is influenced by storage temperature, the molecular weight of the sugar, and the type of buffer utilised in the extender (Yıldız et al., 2000).

Testing a disaccharide like trehalose on cryofreezing of African catfish sperm may also improve post-thaw spermatozoa motility and fertility.

African catfish semen at 0.035 mL provides the best egg hatchability for 2041 ± 90.9 eggs (Ayoola, 2009). Sperm density is a function of reproduction status and testicular hydration (hormone-induced). Knowing the quantity of sperm required per egg will maximise both egg quantity per unit of sperm storage and economical farm use of sperm cryo-storage reserves. Therefore, a sperm count required per egg will be more accurate. About 24×10^3 sperm.egg^{-1} is required according to Viveiros et al. (2000), although 15×10^3 sperm.egg^{-1} was indicated by Rurangwa et al. (1998).

This study selected Ginzburg fish Ringer (GFR) as the common diluent (extender) in all protocols with or without 0.3 M trehalose. Two cryoprotectants (DMSO and methanol) were tested to assess the preferred equilibration period for the mixed sperm and cryoprotectant complex at 4 °C before cryofreezing. The egg hatchability rate was used to assess the cryopreservation method and thawed sperm fertilisation capacity compared to the control. The ratio of semen volume to quantified egg fertilisation capacity was also determined to develop a reliable and feasible semen rationing protocol for maximised egg fertilisation. Finally, the efficacy of LN_2 vapour sperm vitrification was assessed as a cost-effective alternative to the more expensive controlled rate freezing equipment.

Materials and Methods

Ethical approval

The experiment was approved by the Ethics Committee of the University of Stellenbosch, South Africa (ACU-2018-7285), following the guidelines of the South African National Standards (SANS10386:2008) regarding the care and use of animals for experimental and scientific purposes.

Husbandry of broodstock

The brood stock of the African catfish, *C. gariepinus*, originated from the Gariep Dam in South Africa (30.6008°S, 25.4945°E), and has been bred for two generations in the hatchery of the Gariep ATDC (30.62°S, 25.49°E). Mature males and females were kept together under constant temperature (28 °C) and darkness (0L:24D) in closed circular tanks (2.5 kL) connected to a recirculating system. The flow rate was 41.6 L.min^{-1}. Fish were fed catfish pellets (SA Feeds, Hermanus, South Africa) at a level of 1.1 % of body weight daily. The nutritional composition of the feed was 33 % protein, 10 % fat and 20 % carbohydrate. A week before the spawning event, the broodstock was fed a paste feed to improve gonad development before spawning induction. The paste feed consisted of 10 % sodium carboxymethyl cellulose (binder), 20 % chicken liver, 7 % fish oil, 2 % lecithin granules, 2 % spirulina,

1 % mineral (zinc and selenium included) and vitamin mix, 0.5 % Vitamin C; 0.3 % Vitamin E; 15 % chicken egg yellows. About 42 % of the mix consisted of water.

Sperm cryopreservation

Males (×2) and females (when required), weighing 2300–5000 g were injected with Ovaprim® (Syndel, Canada) at a dosage of 0.3 mL.kg^{-1} body weight, in the evening (2200 h) and kept in hanging bags for easy removal the following day, as well to keep the fish apart (aggression increases after injection). The following day at 0900 h, the males were anaesthetised with 1 mL of 2-phenoxyethanol per 3.3 L water (300 ppm) and then sacrificed by spinal transection. Testes were removed by dissection and cut along the median section, between the posterior-distal lobular part and vas deference, and the semen was collected in a petri dish by gentle squeezing. Motility was determined subjectively by mixing one drop of fresh semen with two drops of tap water and observing under a microscope at a magnification of 200×. Only samples with more than 80 % motile spermatozoa were experimentally frozen.

For each trial, GFR (Table 1) with a pH of 7.6 was used as an extender and 0.4 mL mixed with 0.05 mL methanol or DMSO (Merck, Germany) in a 2 mL cryovial. Then 0.05 mL of semen was added, and the cryovial was gently shaken. The procedure was repeated but with 0.3 M trehalose added to the GFR, totalling a sum of 4 trials. Twelve replicate samples were prepared respectively for each of the four trials, and six of these replicates were used to fill 0.5 mL straws. The marked straws and cryovials per trial were respectively packed in perforated toothbrush holders with inner and terminally mounted stainless-steel nut (15 mm) weights and with tagged string connections (Fig. 1). The holders were placed horizontally in a refrigerator (4 °C). The equilibration time between mixing spermatozoa with cryoprotective solution and freezing was approximately 30 min.

Table 1: Ginzburg fish Ringer (GFR) composition.

Ingredient	Mass (g)
NaCl	7.00
KCl	0.28
$CaCl_2$	0.33
$NaHCO_3$	0.23
Trehalose (trial-specific)	102.70
Distilled water	Up to 1000 mL

After about 25 min of semen and cryoprotectant solution equilibration time, the styrobox freezing chamber (Fig. 1) was prepared by installing the two equally spaced and vertically positioned (~10 cm above the floor) slide-fit rods (2 mm) through the longer sides of the box, onto which the toothbrush holders with samples were horizontally mounted. The LN_2 was then carefully poured into the box from a 2 L Dewar until the liquid level reached the microjet tube adaptor (slightly

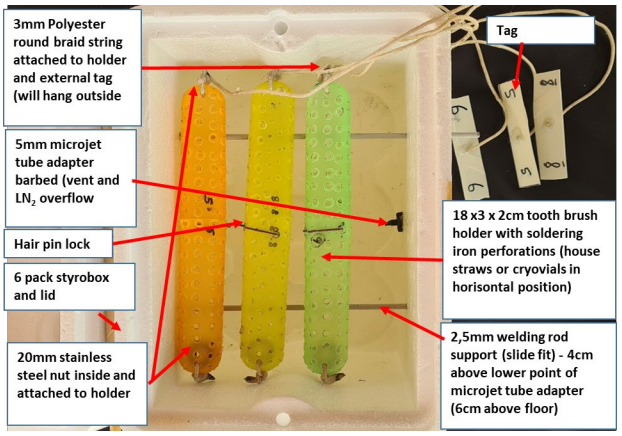

Fig.1. Styrobox with toothbrush holders suspended by slide-fit welding rods (2.5 mm).

overflowing) and with an approximate liquid depth of 6 cm. The LN_2 level was 4 cm spaced from the suspended rod level. The chamber height from the rod to the box's upper end was about 8 cm. Box length and width were 26 cm and 19 cm respectively. The lid of the box was 6.5 cm high. The needle of a digital thermometer (MT 600, Major Tech, South Africa) with -50 °C to 200 °C range was inserted through the box wall and at the mid-level of the toothbrush holder position. The samples in the holders were then transferred from the refrigerator (~30 min equilibration time) to the open styrobox and mounted onto the rods using cryo-gloves. The LN_2 level was finally checked and topped up if required.

Closing of the box commenced when the temperature reached 0 °C, and a timer was then activated. The temperature was recorded once per minute. If the freezing rate reached -5 °C.min^{-1}, the box was opened for a couple of seconds before closing again. After about 12 minutes, the recorded temperature was about -41 °C, and at this stage the rods were pulled out and the samples were plunged into the LN_2. The holders were equipped with fixed weights (20 mm OD stainless steel nuts) in the terminal ends to ensure complete immersion - since the holders tend to float on the LN_2, impeding the flash-freezing effort. Removal of the holders by its strings followed when no "boiling" of the LN_2 could be heard or seen. The holders were transferred to a 32 L Dewar filled with LN_2, with the string-attached tags hanging outside the closed Dewar.

Calculation of sperm volume and experimental egg quantity to be fertilised

According to Ayoola (2009) there are approximately 5.166 × 10^8 sperm cells per 0.035 mL of semen. Therefore, 148.2642 × 10^8 sperm cells per mL of undiluted sperm for males not injected with Ovaprim® thus, no hydration and endogenous sperm dilution. Ovaprim-injected males undergo testicular hydration to approximately 2.5 times testicular volume (Gbemisola and Adebayo, 2014). Based on this, an

estimate of 5.9×10^9 sperm.mL^{-1} of the hydrated testis (Ovaprim-induced males at 11 h latency time and at 28 °C, mass between 4 and 5 kg) was used to test the diluted sperm fertilisation efficacy of eggs. Hypothetically, if at least 11,300 spermatozoa are required per egg (Fouché, pers. comm., 2023), then 525,000 eggs could be fertilised with 1 mL of sperm (hydrated testis). A 10 % sperm dilution in a 0.5 mL mixture of cryo-solution equates to 0.05 mL of sperm, which should fertilise approximately 26,250 eggs. At 750 eggs.g^{-1} about 35 g of eggs should be fertilised with 0.05 mL of fresh sperm or successfully prepared and thawed cryopreserved sperm.

Artificial insemination and hatching of eggs

Two days after semen cryofreezing and cryostorage, two pre-conditioned female catfish and one male were injected with Ovaprim® at 0.3 mL.kg^{-1} body weight, and the females stripped 11 h later (at 28 °C). Eggs were kept at room temperature (24 °C) while being used for a maximum of 1.5 h. For each of the four trials and control, replicate aliquots (×4) of 35 g eggs and 70 g eggs were weighed respectively in marked 100 mm diameter Petri dishes (20 mm height). Fresh sperm of the sacrificed male was diluted by adding 0.05 mL semen to 0.045 mL GFR in four sterile cryovials (Control 1) and repeated but with GFR + 0.3 M trehalose (Control 2). Four cryovials and straws per trial were thawed in a temperature-controlled water bath at 28 °C for 5 min. The thawed semen in the remaining two cryovials/straws, as well as fresh sperm, were used to subjectively determine the percentage of motile sperm (200× magnification).

After thawing, spermatozoa from each cryovial or straw (previously diluted 1:10) were mixed with 2 mL saline (0.9 %) and immediately after that with the pre-weighed egg samples. Fertilisation was initiated by adding 10 mL (35 g egg batch) and 20 mL (70 g egg batch) tap water (dechlorinated, room temperature) and slow stir mixing for 40 s. About 5–10 mL of additional chlorine-free tap water was further added, and eggs were then transferred by spreading it evenly onto a polyester/cotton tulle net bag (0.7 mm mesh) attached to a floating 10 cm × 30 cm PVC pipe (20 mm OD) frame. The two control (1 and 2) egg batches were treated similarly. The eggs were incubated at 28 °C, and the tanks were connected to a recirculating system.

The number of fertilised ova was calculated as a percentage of total eggs exposed to spermatozoa 18 h after fertilisation, using 4 cm^2 square counts as sub-samples (n = 4). The same squared areas were used to determine hatching rates (number of dead/unhatched eggs remaining) 24 h after fertilisation.

Statistical analysis

For the quantified egg fertilisation capacity per trial-specific sperm treatment and controls, data from 4 replicates per treatment (4 samples of 35 g eggs, respectively treated per vial) were pooled to calculate the mean and standard deviation. All statistical analyses were done using the SPSS 11.5 statistical package (IBM Corporation, Armonk, New York, USA). Hatching rates from fresh and cryopreserved spermatozoa per fertilisation trial were tested for significant differences by ANOVA using the parametric General Linear Model procedure, followed by Duncan's Multiple Range Test. The residues from the different ANOVA models were tested for normal distribution using the univariate procedure. *P* values < 0.05 were considered to be significant.

Results

Table 2 indicates the freezing rate measured by the needle sensor at the level of samples approximately 4 cm above the liquid nitrogen level within the styrobox cooling chamber.

Table 2: Freezing rate in styrobox with microjet tube adaptor vent at 4 cm below rod platform of horizontally mounted plastic sample holders.

Minutes after closing the lid (± 1 min delay in the open position before start)	Temperature (°C)	Differential (°C)
1	-4.0	-4.0
2	-9.0	-5.0
3	-14.0	-5.0
4	-18.0	-4.0
5	-22.0	-4.0
6	-24.7	-2.7
7	-27.4	-2.7
8	-30.1	-2.7
9	-32.8	-2.7
10	-35.6	-2.8
11	-38.5	-2.9
12	-41.4	-2.9

The difference between fertilisation and hatching rates was not significant (*P* > 0.05). Hatching rates for the respective trial and control groups are presented in Table 3.

No significant difference (*P* > 0.05) in average hatching rate was observed when eggs were fertilised with either diluted fresh sperm or straws of DMSO cryo-protected post-thaw spermatozoa (with or without 0.3 M trehalose) in Trials 1 and 2 and when using 0.05 mL diluted semen to fertilise 35 g eggs (Table 3). Undiluted fresh sperm fertilised eggs with similar hatching rate

Table 3: Hatching rates as a percentage of total eggs in contact with spermatozoa of trial and control groups.

Trial no.	Sample ID	Hatching rates as a percentage of total eggs in contact with spermatozoa		Subjectively evaluated post-thaw sperm motility (%)
		0.05 mL semen per 35 g eggs at an assumptive calculation of 11500 sperm.egg^{-1}	0.05 mL semen per 70 g eggs at an assumptive calculation of 5750 sperm.egg^{-1}	
	Fresh sperm mixed with:			
	GFR (control 1)	82.6 ± 1.10	62.6 ± 2.6	>80
	GFR + trehalose (control 2)	92.8 ± 1.17	73.4 ± 2.2	>90
	Thawed sperm + cryoprotective solution			
1	GFR + DMSO vial	51.9 ± 2.10	33.4 ± 2.9	~50
	GFR + DMSO straw	82.4 ± 1.60	62.2 ± 1.9	>80
2	GFR + 0.3 M trehalose + DMSO vial	62.4 ± 1.80	41.8 ± 1.9	~60
	GFR+ 0.3 M trehalose + DMSO straw	92.6 ± 1.20	72.6 ± 1.4	>90
3	GFR+ methanol vial	30.3 ± 2.80	10.7 ± 3.4	<30
	GFR+ methanol straw	61.6 ± 1.30	40.8 ± 2.6	~60
4	GFR+ 0.3M trehalose + methanol vial	40.7 ± 3.20	21.2 ± 3.6	~40
	GFR+ 0.3M trehalose + methanol straw	72.3 ± 1.70	52.6 ± 2.7	~70

Mean ± SD. n = 4.
GFR: Ginzburg fish Ringer, DMSO: Dimethyl sulfoxide.

(P > 0.05). GFR + 0.3 M trehalose also increased hatching rates by about 10 % in both controls and all trials. Further dilution of the semen could fertilise approximately 20 % fewer eggs on average when the egg quantity was doubled (70 g). Vial-preserved semen fertilised approximately 30 % fewer eggs than straw-preserved semen across all trials (Table 3).

Vials and straws with 10 % methanol-treated semen (Trials 3 and 4) underperformed with a differential of approximately 20 % (P < 0.05) less egg-hatchability compared to DMSO-treated semen (Trials 1 and 2), with or without trehalose-inclusion.

Subjectively evaluated spermatozoa motility percentage in thawed semen of vials/straws for all trials is presented in Table 3. Trehalose increased sperm motility by approximately 10 % in controls and for the respective vial and straw-thawed inclusions of cryoprotectants used.

In all four trials, hatched larvae were allowed to grow for 21 days post-hatch and with no macroscopically or microscopically observed morphological deficiencies or growth rate differences compared to controls.

Discussion

Cryoprotectants

According to Tiersch et al. (1994), a longer time of equilibration before freezing can enhance the effectiveness of cryoprotectants that act more slowly than methanol. Viveiros et al. (2000) used methanol and DMSO as cryoprotectants in African catfish but only allowed an equilibration time of 2 minutes at 5 °C. The methanol acted quickly, but the DMSO was less effective. The current investigation demonstrated that DMSO is more effective than methanol when an equilibration time of 30 minutes was allowed since the thawed DMSO-included semen cryoprotective solution could fertilise significantly more eggs than the methanol-exposed cryo-protected sperm (Table 3). However, it is unclear if methanol became toxic and if exposure time must be more limited during the equilibration period. Further investigations are required in this regard.

With extender-added trehalose (0.3 M), an increased (~10 %) egg fertilisation capacity in all trials and the control was measured. This indicates that trehalose can act as an energy substrate for sperm cells during egg fertilisation (control + post-thaw sperm) and incubation (trials), further maintaining the osmotic pressure of the extender or even being an additional cryoprotectant (Yıldız et al., 2000).

Freezing rates and endpoints

According to Viveiros et al. (2000), several freezing rates have been tested for African catfish spermatozoa.

However, aside from Viveiros et al. (2000), the range of endpoints (in the first step of freezing) has been limited to -65, -70 and -80 °C (Steyn et al., 1985; Steyn and van Vuren, 1987; Steyn, 1993; van der Walt et al., 1993; Urbanyi et al., 1999). Viveiros et al. (2000) suggested that the nucleation point may have been passed and that improved hatching rates could have been obtained at other endpoint temperatures. Viveiros et al. (2000) obtained better freezing rates at -5 °C min^{-1}, suggesting that this is the best freezing rate for *Clarias gariepinus* spermatozoa. Their results were supported by Steyn (1993). Both authors used controlled rate freezing techniques. In this investigation apparent sperm protection was achieved with a non-controlled but spontaneous freezing rate between -2.7 and -5 °C min^{-1} and an endpoint of -41.4 °C (Table 2), using a styrobox and static LN_2 vapour vitrification. This was confirmed by the effectiveness of the best cryoprotective solution (DMSO + GFR + 0.3 M trehalose), with post-thaw semen (0.05 mL) volume, that fertilised 35 eggs with similar efficacy as for 0.05 mL fresh or undiluted fresh sperm (GFR + 0.3 M trehalose).

Post-thaw spermatozoa motility

Trehalose promoted thawed sperm motility by an approximate increase of 10 % in both vials and straw treatments for both cryoprotectants used, suggesting that this disaccharide prolonged and promoted spermatozoa activity as in carp (Bozkurt et al., 2016). Minimal sperm damage occurred during cryofreezing using DMSO with or without trehalose since spermatozoa motility percentages were comparable between thawed samples and the controls (Table 3). The efficacy of DMSO as cryoprotectant exceeded those of methanol since sperm motility percentages were lowest when using diluted methanol in 2 mL cryovials. However, relative motility and egg fertilisation capacity improved when cryostored in straws (Table 3). A shortened pre-freeze equilibration time for methanol will probably exclude its possible toxic effects (Viveiros et al., 2000).

Semen volume to egg fertilisation capacity ratio and the surgical re-use of selected males

According to Ayoola (2009), African catfish semen at 0.035 mL provides the best egg hatchability for 2041 ± 90.9 eggs. It will then equate to 2916 eggs (3.88 g) fertilised with 0.05 mL semen (post-thawed or fresh). However, the current investigation demonstrated that 35 g eggs could be fertilised with 0.05 mL post-thawed sperm and with efficacy similar to fresh sperm (diluted or non-diluted). Since 1 g equals about 750 eggs (Baidya and Senooi, 2004), the actual fertilisation capacity is about nine times more than that determined by Ayoola (2009). More importantly, this high fertilisation capacity rate justifies the validity of sperm storage and its routine use in African catfish. Therefore, the currently developed method can produce 60 straws (0.5 mL) from 3 mL semen, with the capacity to fertilise about 2.1 kg eggs (~162×10^4 eggs). The semen used was from 4–5 kg conditioned males injected with Ovaprim® at 0.3 $mL.kg^{-1}$, with an expected testes hydration factor of about 2.5 times testicular volume (Gbemisola and Adebayo, 2014). About 3 mL of semen could be extracted per testis, which doubles the fertilisable egg capacity potentially to 4.2 kg (~324×10^4 eggs), and at a potential straw count of 120 per donor male.

Sacrificing good-quality males can be prevented by surgical removal of the distal portion of testes (75 % portion) aseptically for testicular regeneration and re-use within 3–6 months. The postsurgical survival of *C. gariepinus* is 100 %, indicating the efficiency of the surgical procedure. There is also no significant difference in sperm production, percentage fertilisation, hatchability and survival of the larvae using sperm derived from regenerated testes of 75 % gonadectomised *C. gariepinus* (Adebayo et al., 2012). In this way, good-quality males can be saved and used as repetitive sperm cryopreservation donors.

Cryovials vs straws as preferred sperm cryostorage vessels

Although straws and cryovials are considered equally effective in sperm cryofreezing (Lorenzoni et al., 2011), the efficacy of the cryovial usage in the current investigation could have been reduced by oxidative stress on sperm cells since only 25 % of the 2 mL vial was filled with semen. Radical scavengers such as glutathione may be required for the cryoprotectant solution (Gorbani et al., 2016). It is also possible that the temperature gradient to the middle core of semen + cryoprotectant solute was more efficient and at an improved dehydration rate in straws than in cryovials, with a deeper volumetric core. Therefore, the controlled rate freezing method may be more advantageous for cryovial usage.

Larval condition and duration of cryostorage

None of the 21 DPH larvae observed displayed any macroscopic or microscopic anatomical deficiencies or slower growth rates compared to controls for all four tested trials. It must also be noted that sperm was only cryostored for two days before thawed use in the current investigation, thus longer term storage should be tested by further investigation.

Practical and economic considerations for using static LN_2 vapour sperm vitrification above controlled rate freezing

A fish farm with access to LN_2 resources can cryopreserve African catfish semen with the static LN_2 vapour vitrification described in the current paper and at a very low cost, compared to controlled rate freezing, with high apparatus cost (>800 times more expensive). However, occupational health and safety

procedures must be followed when working with LN_2.

Conclusion

The currently developed sperm cryopreservation method can be implemented without sophisticated equipment and combined with the re-use of surgically treated males to enhance mass cryostorage of genetically selected African catfish sperm. The developed method is simplified enough to facilitate standard operating procedures such as the year-round supply of broodstock gametes or its requirement in genetic improvement breeding programs. The frequency of sacrificing males as sperm donors can also be drastically reduced when a cryostorage source of good genetic-quality sperm is accessible.

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Conflict of interest: The authors declare that they have no conflict of interest.

Author contributions: Chris Fouche: Study conceptualisation and design, data collection and analysis, original draft. Mark Goodman: data collection and manuscript editing.

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