



Artificial insemination of African catfish (*Clarias gariepinus*) using cryopreserved sperm

Tamás Müller^{a,*}, Tamás Szabó^a, Tímea Kollár^a, Balázs Csorbai^a, Zoran Marinović^a,
László Horváth^a, Balázs Kucska^b, Ádám Bodnár^a, Béla Urbányi^a, Ákos Horváth^a

^a Department of Aquaculture, Faculty of Agricultural and Environmental Sciences, Szent István University, Gödöllő, 2100, Hungary

^b Department of Aquaculture, Faculty of Agricultural and Environmental Sciences, Kaposvár University, Guba S. u. 40, 7400, Kaposvár, Hungary

ARTICLE INFO

Article history:

Received 30 March 2018

Received in revised form

13 September 2018

Accepted 26 September 2018

Available online 27 September 2018

Keywords:

Induced spawning

Gene preservation

Ovarian lavage

ABSTRACT

In this study, we aimed to develop a practical protocol for using cryopreserved sperm for induced/wild/tank spawning of fish species with external fertilization. Experiments were carried out on African catfish (*Clarias gariepinus*) as a model species. Sperm was collected for cryopreservation and diluted with the cryomedium (266 mM fructose, 20% methanol) at a ratio of 1:1 with a final methanol concentration of 2.47 M pH7.73. Diluted sperm was loaded into 0.5-ml straws and cryopreserved by conventional protocol. Samples were prepared for insemination 24 h later, by thawing for 13 s in a 40 °C water bath, and centrifuged at 500 × g for 10 min at 20 °C. The seminal plasma, extender and external cryoprotectant were removed from the concentrated spermatozoa. The pellet was then resuspended in common carp (*Cyprinus carpio*) seminal plasma to reconstitute the lost volume. Sperm samples were then injected by a catheter into the ovarian cavity through the oviduct of the experimental females by the so-called ovarian lavage method in parallel with the intramuscular hormonal administration (5 mg carp pituitary/kg bw). Inseminated females (n = 9) were monitored for 10 h and ovulated eggs and spermatozoa stored in the ovary were stripped. Stripped gamete samples were divided into two batches: (1) the first batch contained only the previously injected spermatozoa and was activated by aerated water (WA) immediately after stripping; (2) in case of the second batch additional, freshly stripped sperm was added as positive control to the stripped eggs before water activation (PC). Furthermore, five females were propagated by using the dry fertilization method (in vitro fertilization) as negative control (NC). All sperm and hormone injected females produced fertilised eggs with a hatching rate of 17.7 ± 13.2%, 12.5 ± 9.3%, and 61 ± 11.5% for WA, PC and NC respectively. These results indicate that artificial insemination based on using cryopreserved sperm with ovarian lavage can be a viable alternative to in vitro fertilization in a catfish species. Thus, we describe a proof of principle for a practical protocol for the induced/wild/tank spawning of an externally fertilising fish species with economical importance and propose that the protocol could be also applied to endangered marine or fresh fish species.

© 2018 Published by Elsevier Inc.

1. Introduction

Cryopreservation of germplasm allows for both short- and long-term storage of valuable genetic resources in genetic selection programs, biodiversity preservation and assisted reproduction [1]. At temperatures applied during cryopreservation physical and chemical reactions are significantly limited, thus enabling long-

term quality preservation of the frozen cells. Researchers have been working on the improvement of fish sperm cryopreservation methods for the last 60 years. Sperm cryopreservation is generally used for laboratory work and in practice in cultured marine and freshwater fish species as it is a valuable tool for preservation of genetic resources or it can make the synchronization of spermiation and ovulation unnecessary [2,3]. It is also an indispensable tool in preventing loss of genetic diversity, inbreeding depression, accumulation of deleterious mutations, bottleneck effect and/or population concentration in cultured aquatic species or *ex situ* conservation of endangered fish.

In the case of fish species with external fertilization, testing of

* Corresponding author. Department of Aquaculture, Faculty of Agricultural and Environmental Sciences, Szent István University, Páter Károly str. 1, 2100, Gödöllő, Hungary.

E-mail address: muller.tamas@mkk.szie.hu (T. Müller).

cryopreserved/thawed sperm is conducted through the in vitro fertilization method. Post-thawed sperm samples must be used for fertilization immediately after thawing, due to the cellular toxicity caused by cryoprotectants, which in turn, protect the cells from the harmful effects of freezing. Cryoprotectants are usually solutes with small molecular weight that promote cellular dehydration, but also stabilise membranes and proteins. Most permeable cryoprotectants, such as methanol (MeOH) and dimethyl sulfoxide (Me₂SO) are toxic at room temperature, however, they are not harmful to the cells at low temperatures. Apart from them, non-permeable cryoprotectants with high molecular weight (e.g. bovine serum albumin, egg yolk lipo-proteins, dextran, etc.) also play an important role in stabilizing the plasma membrane and dehydrating the cell [4]. One of the obstacles to applying cryopreserved sperm is that there is not yet a method described, which could be applied for induced/wild/tank spawning of fish species with external fertilization. Several economically important marine and freshwater farmed fish (e.g. *Sparus aurata* [5], *Morone* species [6]), endangered fish (e.g. *Umbra krameri* [7]) or model fish (*Danio rerio* [8], *Oryzias latipes* [9]) etc. are produced primarily by captive breeding/tank spawning, or by induced spawning for a number of considerations. For instance: the arduous process of inducing and detecting ovulation, which occurs within a narrow window of time so that the fertile ova can be collected for the in vitro fertilization [6]; large fluctuations in response times to maturation-inducing steroids [10], small size etc. In these cases, it is difficult to implement genetic selection programs, biodiversity.

In contrast, viable larvae were produced from cryopreserved sperm in live-bearing (viviparous) fishes (*Xiphophorus helleri* and *X. couchianus*) [11,12]. These authors developed a method to avoid the toxic effects of cryoprotectants by centrifugation of post-thawed sperm thus separating the seminal plasma and cryoprotectants from the spermatozoa. Subsequently, spermatozoa were resuspended in artificial seminal plasma (distinct ionic composition, and with addition of glucose and/or fetal bovine serum (FBS)) and inseminated, which induced fertilization of the ovulated eggs within the ovary.

Müller et al. [13] has recently demonstrated a novel propagation method, the so-called ovarian lavage, in common carp (*Cyprinus carpio*). In this case, collected sperm samples were introduced to the ovary lobe of females with the help of a catheter 12 h before ovulation, which was induced through hormonal administration. In another experiment, a mixture of sperm and carp pituitary extract (CPE) was injected into the ovarian lobes of African catfish (*Clarias gariepinus*). The absorbed CPE induced ovulation and all females produced good quality eggs that developed normally after activation of gametes and fertilization [13,14]. In neither experiments did the ovarian fluid activate the spermatozoa, which maintained their biological activity for up to 12 h. These proof of principle experiments demonstrated the potential of these methods for routine use as an alternative to induced spawning, in which synchronising of the production of ovulated eggs with the availability of sperm may be limited or where induction of egg release and or stripping is risky or technically unfeasible.

We hypothesized that by combining the two methods (cryopreservation of sperm and artificial insemination of sperm using a catheter) can be applied to wild/tank bred species. In support of this hypothesis, our aim was to combine these two methods and test them on *C. gariepinus*, thus to develop a simple method for the use of cryopreserved/thawed sperm in artificial spawning of fish species with external fertilization.

2. Material and methods

Protocols for fish sperm cryopreservation and fish propagation

and the template informed consent forms contained in the Appendix (Scientific Ethics Council for Animal Experimentation; XIV-001-2299-4/2012, XIV-001-2306-4/2012) were reviewed and approved by the National Food Chain Safety Office, Animal Health and Animal Welfare Directorate of Government Office of Pest Country with respect to scientific content and compliance with applicable research subject regulations.

2.1. Selection and maintenance of spawners

Sixteen-month old African catfish were used as broodstock and reared outdoors in 9-m diameter (50 m³) circular, concrete tanks supplied with running well water at 23 °C (min-max: 20–27 °C; fish farm: V'95 Ltd., Nagyatád, Halastó, Hungary). Randomly selected fish (n = 26) were transported to the Fish Laboratory of the Szent István University. African catfish males and females were sorted and kept separately in the same recirculation system during the trial (9 males and 17 females in 3000 L tanks). Carp males (n = 12) were selected from the own reared stock of the Fish Laboratory, for sperm stripping and semen collection.

2.2. Induction of final maturation of males

Hormonal induction of all African catfish males was carried out with a single intraperitoneal injection of carp pituitary extract (3.0 mg CPE/1.0 mL 0.9% NaCl solution/kg BW) 24 h prior to sperm collection. Four African catfish males (sperm cryopreservation: n = 2, 1340 and 1466 g and for collecting seminal plasma n = 2, 1100 and 1298 g, respectively) and two carp males (506 and 543 g, respectively) were used for an investigation of the short storage of cryopreserved/thawed sperm at room temperature for 24 h (Experiment 1., Fig. S1). Five experimental African catfish males (sperm cryopreservation: n = 4, BW: 743.5 ± 202.2 g and one control male fertilization with additional sperm BW: 1420 g) were treated in the Experiment 2.

Carp males (n = 2, BW: 489–506 g in Experiment 1. and n = 10, BW = 578 ± 207 g in Experiment 2.) were treated with 4.0 mg CPE/kg BW (dissolved in 1.0 mL 0.9% NaCl solution) 24 h before stripping sperm. Fish were anaesthetized with 2-phenoxyethanol (0.4 mL/L). Prior to stripping, the urogenital area was wiped dry to prevent contamination with water or mucus. Sperm was collected with an automatic pipette and distributed into plastic test tubes. Subsequently, 1.5 mL microcentrifuge tubes were centrifuged at 10,000 × g for 10 min at 20 °C.

2.3. Sperm cryopreservation

Sperm was extracted from African catfish testes, which were surgically removed from the body cavity of the fish after terminal anaesthesia by 2-phenoxyethanol and decapitation. Cryopreservation was carried out as previously described [15,16]. In short, sperm was diluted at a ratio of 1:1 with the cryomedium containing 266 mM fructose, 20% methanol (final methanol concentration of 2.47 M, pH 7.73). Sperm was loaded into 0.5-ml straws (Minitüb, Germany) which were subsequently placed 3 cm above the liquid nitrogen surface on a polystyrene frame. After 3 min, the straws were plunged into liquid nitrogen and stored in canister storage dewars (Bio 10, Statebourne, UK). 24 h after the cryopreservation, the African catfish sperm samples were thawed in a 40 °C water bath for 13 s. All samples were pooled and sperm quality parameters were measured by Computer-assisted sperm analysis (CASA). Following sperm quality measurements, samples were split into 1.5 mL microcentrifuge tubes (n = 2) and were centrifuged at 500 × g for 10 min at 20 °C in order to reduce toxicity by cryoprotectant.

2.4. Preparation of sperm

Seminal plasma, extender and external cryoprotectant were removed from the concentrated spermatozoa by centrifugation as previously described. In parallel with the thawing of the African catfish sperm samples, African catfish centrifuged seminal plasma (only Experiment 1, Fig. S1.) and common carp centrifuged plasma (both experiments, Fig. S1, S2.) were added to the centrifuged African catfish spermatozoa pellet. The amount of native common carp and African catfish seminal plasma added to the spermatozoa was similar to the amount of post-thawed African catfish plasma removed previously. Sperm parameters were measured by a CASA system. The samples were stored at 20 °C for 24 h. Sperm quality was measured at the following storage times: 0 h, 5.5 h and 24 h in the Experiment 1. and immediately after thawing in the Experiment 2. A Bürker-Türk type chamber was used for determination of sperm concentration after dilution in Experiment 2. (3.51×10^9 spermatozoa/mL).

2.5. Measurement of sperm motility

As described above, sperm motility parameters were measured with a CASA system (Sperm Vision™ v. 3.7.4., Minitube of America, Venture Court Verona, USA connected to an Olympus BX 41 microscope using 20× negative phase contrast objective). Key motility parameters assessed in this study were progressive motility (%), average path velocity (VAP; $\mu\text{m/s}$), curvilinear velocity (VCL; $\mu\text{m/s}$), linearity (LIN; %) and path straightness (STR; %). A commonly used activating solution in cyprinids containing 45 mM NaCl, 5 mM KCl, 30 mM Tris (pH = 8) was used during all sperm quality measurements [17]. Pooled samples were activated twice for as biological replication. Summary CASA outputs of each measurement were used in statistical analysis.

2.6. Hormonal induction of females and ovarian lavage/artificial insemination of sperm (Experiment 2.)

Hormonal induction of African catfish females was carried out by injection of CPE (5 mg CPE / 1 mL 0.9% NaCl solution / kg BW) intramuscularly. Cryopreserved/thawed African catfish sperm samples with additional carp seminal plasma were injected into the ovarian cavity of African catfish females through the genital papilla as described by Müller et al. [12]. Injections were carried out by inserting a silicone catheter (Feeding tube, 400 mm length, size: CH 4, outer diameter 1.3 mm, inner diameter 1 mm, Galmed, Poland) coupled to a 2 mL syringe directly into the ovary lobes (approximately 10–15 cm deep into the oviduct depending on the size of fish) via the genital opening. Two mL of post-thawed sperm/kg female BW was split equally between each of the two ovaries 10 h before the gamete stripping. After the treatment, females were transferred back into the holding tank (Fig. S2.).

2.7. Fertilization

2.7.1. Control group (NC)

To evaluate induced egg production, the pseudo-gonadosomatic index [PGSI % (weight of stripped egg mass/BW before stripping) $\times$ 100] was calculated. The stripped gamete batches were fertilised using aerated tap water ($n = 5$ females, BW = 630.8 ± 116.5 g, min-max = 522–828 g). Five minutes later 3 egg samples were collected from each batch and incubated in Petri dishes ($\varnothing = 100$ mm, average egg number/Petri dish = 64.2) at 24 °C.

2.7.2. Artificial inseminated/ovarian lavage group

All females ($n = 9$, BW = 596 ± 148.4 g, min-max: 356–832 g) were successfully stripped and egg batches were measured for calculation of PGSI values. The stripped gamete batches were divided into two parts. One batch was fertilised using aerated water (WA). In order to test the potential capacity for fertilization of eggs, a second egg batch from each female (about 15 g/egg batch) was also mixed with additional (freshly collected) milt (0.5 mL sperm for each egg batch, PC, Fig. S1.). Five minutes later, three egg samples (average egg number/Petri dish: 55.5) were collected from each batch and incubated in Petri dishes as described above. Every 2 h the water was changed in the Petri dishes until determination of the fertilization rate. Egg quality was determined by calculating embryo survival rate at the somitogenesis stage (12 h post fertilization) and hatching (36 h post fertilization).

2.8. Water parameters

The water temperature and dissolved oxygen parameters were measured in the Experiment 2. with the WTW FDO 70× IQ sensor. Water temperature and dissolved oxygen contents were constant during the experiment (25.3–25.4 °C and 7.7 mg/L-1, respectively).

2.9. Statistical analysis

The measured and calculated parameters were carried out by independent *t*-test in SPSS v22 for Windows. Treatment means were compared using $p = 0.05$ for significance.

3. Result

3.1. Experiment 1

The aim was to investigate the key sperm parameters of prepared post-thawed sperm samples during the short term storage. Progressive motility, VCL, VAP and LIN were lower in the post-thawed samples compared to fresh samples. Values of all parameters decreased during the short-term storage at 20 °C, however, even 24 h after thawing approximately 10% of motile sperm still remained (Fig. 1.). Interestingly, progressive motility of centrifuged African catfish spermatozoa was higher when diluted in common carp seminal plasma than in African catfish plasma in the Experiment 1. (Fig. S1.). Accordingly, centrifuged post-thawed African catfish spermatozoa were mixed with freshly collected and centrifuged carp seminal plasma. Sperm samples prepared in this way were then artificially inserted into both ovarian lobes of females.

3.2. Experiment 2

The fertilization ability of prepared post-thawed sperm samples were tested by using ovarian lavage/artificial insemination method. No statistically significant differences in PGSI values were observed between the two treatment groups (control: $10.5 \pm 4.2\%$; ovarian lavage: $12.4 \pm 3.1\%$; $p < 0.05$) indicating that the ovarian lavage did not negatively affect the ovulation success. Additionally, no significant differences were observed in body weight, ovulation rate (100%) nor latency time (10–11 h) between the ovarian lavage treated and control groups (Table 1.). All sperm-injected females produced fertilised eggs, which developed normally. Both fertilization and hatching rates were significantly lower in the ovarian lavage groups when compared to the control, ($p < 0.05$), however, there were no statistical differences within the ovarian lavage groups ($p > 0.05$, Table 1). All females survived and started feeding one week after the insemination procedures.

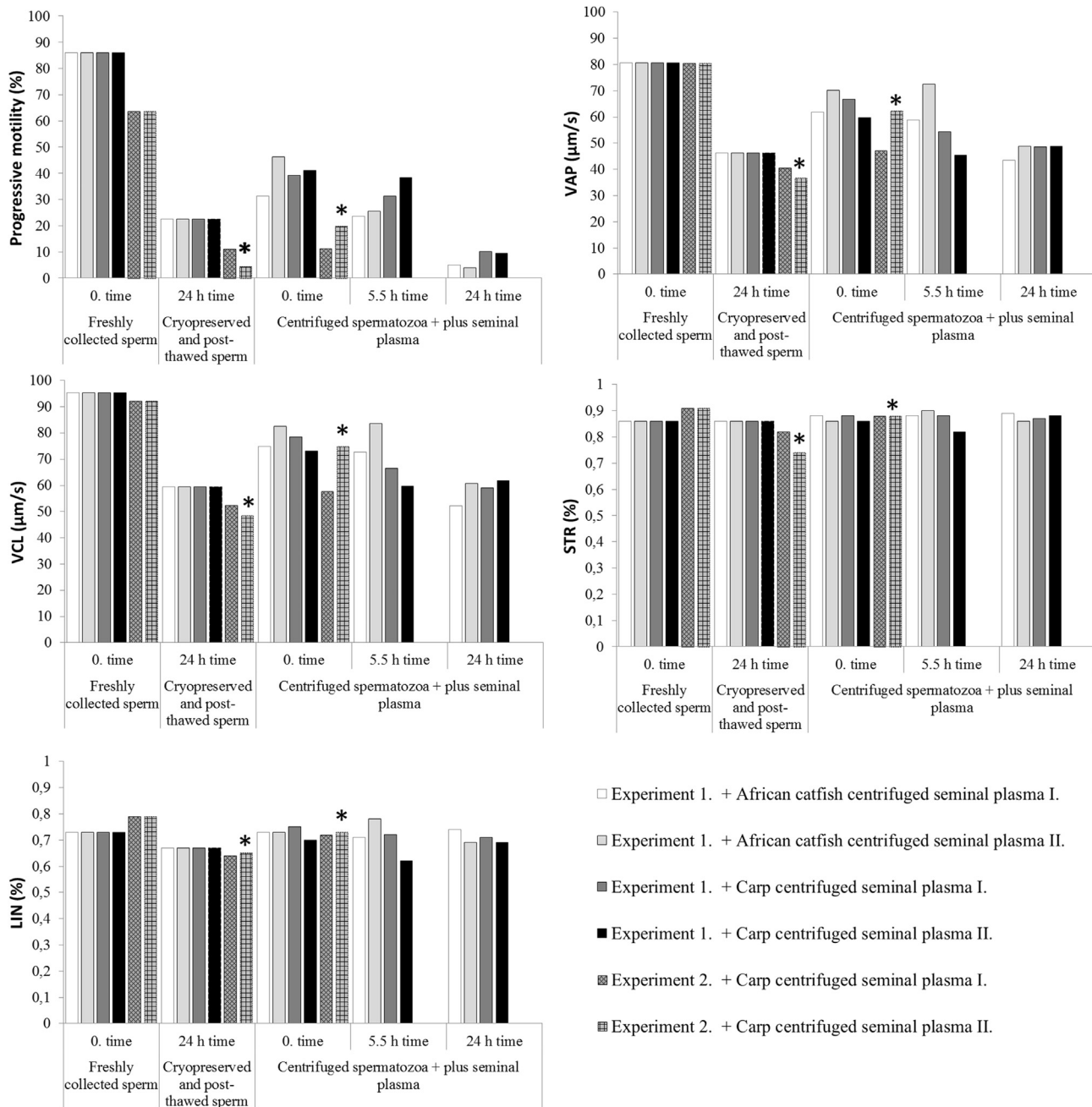


Fig. 1. Summarised sperm parameters of Experiment 1. ($n = 2$ African catfish, $n = 2$ common carp) and Experiment 2. ($n = 4$ African catfish, $n = 10$ common carp). All columns represent cumulative average values (two replicates) per pooled sperm samples. * activation with tap water in 30 min.

Table 1

Summarised data of the main reproduction trial. Data are presented as means and standard deviations ($\pm$ SD). Abbreviations: BW: body weight, PGSI: pseudogonadosomatic index. Different letters in the superscript indicate significant differences ($p \leq 0.05$, independent sample t -test) within the experimental groups.

		Fresh sperm control (NC)	Post-thaw of cryopreserved sperm (WA)	Post-thaw of cryopreserved with additional samples: PC)
Fertilization rate (%)	Control ($n=5$) mean $\pm$ SD (min-max)	71 $\pm$ 14.4 ^b (50.7–93.9)	—	—
	Ovarian lavage ($n=9$) mean $\pm$ SD (min-max)	—	23.5 $\pm$ 16.1 ^a (3.1–64)	17.6 $\pm$ 13.7 ^a (2.5–50.8)
Hatching rate (%)	Control ($n=5$) mean $\pm$ SD (min-max)	61 $\pm$ 11.5 ^b (43.7–77.4)	—	—
	Ovarian lavage ($n=9$) mean $\pm$ SD (min-max)	—	17.7 $\pm$ 13.2 ^a (1.8–51.2)	12.5 $\pm$ 9.3 ^a (2.5–35.3)

4. Discussion

Artificial insemination is a necessary step to test the fertility of cryopreserved sperm in fish species with external fertilization, where in vitro fertilization method is not practical or difficult. In case of live-bearing (viviparous) fish (*X. helleri* and *X. couchianus*), a practical protocol including sperm cryopreservation, thawing, and post-thaw sample preparation for artificial insemination, which has produced viable larvae has previously been described [11,12]. Concentration of sperm and removal of cryoprotectants through centrifugation, and the addition of FBS in the extender was recommended for insemination trials with cryopreserved samples. In our method we opted for an alternative solution based on our previous experiences [13,14], and applied pooled carp seminal plasma on the centrifuged pellet of spermatozoa to extend its biological activity in the ovarian lobe until water activation (approx. 10 hrs). Further experiments are needed to reveal the optimal extender for biological activity during artificial insemination of post-thawed sperm samples.

Several studies on African catfish sperm cryopreservation, which focused on optimizing cryopreservation parameters and in vitro fertilization techniques have been published [15–26]. These publications described in vitro fertilization by using several types of media/extendors and cryoprotectants with different ratios and sperm dilution conditions. Post-thaw storage of cryopreserved sperm for 24 h at 4 °C resulted in higher fertilization rates with a higher percentage of cells with an intact membrane than those that were used immediately after thawing [16]. This indicates that spermatozoa undergo a slow recovery process following thawing. During the short-term post-thaw storage, methanol might not cause damage to spermatozoa as was suggested previously. Based on these considerations, we hypothesized that apart from the surplus methanol which was in the seminal plasma and later eliminated by centrifugation, residual methanol penetrating the spermatozoa membrane does not cause significant fertility reduction, if a recovery process was facilitated in natural seminal plasma. Additionally, methanol content in the extender might cause cell damage within the ovary. Related observation in the Experiment 1. was that the cryopreserved/thawed spermatozoa resuspended in carp seminal plasma displayed higher motility values than the normal cryopreserved/thawed African catfish sperm. However, the exact causes of this phenomenon are beyond the scope of the current study. In previous experiments, seminal plasma was absorbed during the long latency time (10–12 h) and it did not display any adverse effects on the ovary [13,14]. Different artificial seminal plasmas (ASPs) were not tested in this study since this setting would require different experimental cycles to reveal the biological effects of ASP on the ovarian cells, particularly on the ovulated ova within the ovarian lobe.

The addition of freshly collected sperm (PC) did not increase the fertilization rate of the artificially injected egg batches (WA). We supposed that the sperm density was extremely high in the ovarian lobes and that substantial quantities of “dead” or DNA-damaged spermatozoa near or within the micropyle region physically blocked or reduced the chance of fertilization for the freshly added sperm. In Experiment 1. with African catfish by using artificial sperm injecting, the addition of the freshly added sperm did not significantly increase the fertilization rate ($p < 0.05$; fertilization rate without additional sperm $74.7 \pm 18.4\%$; with additional fresh sperm $75.1 \pm 11.5\%$, $n = 7$) [13,14].

This is the first report, which provides a possible method to use cryopreserved sperm via an artificially spawned propagation method for fish species with external fertilization. This has important practical implications for economically important fish species where the main propagation technique is via induced

spawning in tanks, ponds and where there is egg collection after spawning (e.g. *Sparus aurata*, Tuna fishes, Tilapia, etc.). Selective breeding programs can be improved by using cryopreserved sperm samples (without changing the main techniques of the propagation method) but allowing directed and planned genetic lineages to be produced. Our method could be a solution for breeding programmes of *ex situ* conservation of fish species, with lack of available induced-spawning technology or in case of paired-spawning of fish (e.g. aquarium spawning the endangered *U. krameri* [7]). Our protocol could facilitate the increase of genome diversity in such species by relying on cryopreserved sperm from gene banks. Horie et al. [27], Okamura et al. [28], Biase et al. [10] found that the success of fertilization and hatching in group spawning eel species (*Anguilla japonica*, *A. anguilla*) were higher after spontaneous spawning than by in vitro fertilization/artificial insemination. Similarly to the paired-spawned fish, genome diversity could be increased by using mixed cryopreserved sperm from several males. Further development of routine technology based on our proof of principle approach is needed to improve the fertilization and hatching rate.

4.1. Conclusion

Cryopreserved sperm samples could be effectively used only through in vitro fertilization so far. This proof of principle experiment demonstrates the potential method to use cryopreserved sperm via an artificially spawned propagation/wild/tank spawning method for fish species with external fertilization. Breeding programs including genetic base increasing can be started in economically important fish species, which are propagated through induced spawning by using selected high-quality cryopreserved sperm samples independently on spawning behavior (paired or group spawned fish). In case of *ex situ* conservation, fish breeding in captivity is a widely used management practice where the aim is to restore wild populations of endangered species. By using selected sperm samples, several criticisms regarding loss of genetic diversity, inbreeding depression, accumulation of deleterious mutations and bottleneck effect can be avoided.

Author roles

All authors contributed to this paper. TM, TSz, BK, ÁH conceived and designed the experiments. TM, TSz, TK, BCs, LH, BK, ÁB, BU, ÁH performed the experiments. TM, BK, LH, ZM, BCs, ÁH analyzed the data. TM, TSz, TK, BCs, LH, BK, ÁB, BU, ÁH contributed reagents/materials/analysis tools. TM, TSz, ZM, BK, ÁH wrote the paper.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgement

We thank Mr. Bence Schmidt-Kovács and Attila Boros for providing the experimental fish for this study. The work was supported by the EUROSTARS project (E9390 TRANSANDER and NEMZ_15-1-2016-0016), the European Fisheries Fund Fisheries Operative Programme III. axis, European Fisheries Fund for Renewable Fisheries provided by the EU and Hungary, the Department for Angling and Fisheries, Ministry of Agriculture and the EFOP-3.6.3-VEKOP-16-2017-00008 project, the Higher Education Institutional Excellence Program (1783–3/2018/FEKUTSRAT) awarded by the Ministry of Human Capacities within the framework of water related researches of Szent István University, the European Regional and Development Fund and the Government of

Hungary within the project GINOP-2.3.2-15-2016-00025, the Hungarian Scientific Research Fund (NKIF(OTKA)- 105393 as well as the Stipendium Hungaricum Scholarship Programme (106360). The project is co-financed by the European Union and the European Social Fund.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.theriogenology.2018.09.034>.

References

- [1] Martínez-Paramo S, Horváth Á, Labbe C, Zhang T, Robles V, Herráez P, Suquet M, Adams S, Viveiros A, Tiersch TR, Cabrita E. Cryobanking of aquatic species. *Aquaculture* 2017;472:156–77.
- [2] Yang H, Tiersch TR. Current status of sperm cryopreservation in biomedical research fish models: zebrafish, medaka, and Xiphophorus. *Comp Biochem Physiol C Toxicol Pharmacol* 2009;149:224–32.
- [3] Cabrita E, Sarasquete C, Martínez-Paramo S, Robles V, Beirão J, Pérez-Cereales S, Herráez MPP. Cryopreservation of fish sperm: applications and perspectives. *J Appl Ichthyol* 2010;26:623–35.
- [4] Muchlisin ZA. Review: current status of extenders and cryoprotectants on fish spermatozoa cryopreservation. *Biodiversitas* 2005;6(1):66–9.
- [5] Wahbi OM, El-Greisy ZAB, El-Sayed HS. Reproductive performance of Sea bream (*Sparus aurata*) induced with two different hormone protocols with respect to the effect on gonadotrophic cells. *J Biol Sci* 2017;17:278–87.
- [6] Salek SJ, Godwin J, Sullivan CV. Courtship and tank spawning behavior of temperate basses (Genus Morone). *Trans Am Fish Soc* 130:833–847.
- [7] Tatár S, Bajomi B, Specziár A, Tóth B, Müllerné Trenovszki M, Urbányi B, Csányi B, Szekeres J, Müller T. Habitat establishment, captive breeding and conservation translocation to save threatened populations of the Vulnerable European mudminnow *Umbra krameri*. *Oryx* 2017;51:718–29.
- [8] Nasiadka A, Clark MD. Zebrafish breeding in the laboratory environment. *ILAR J* 2012;53:161–8.
- [9] Ishakawa Y. Medakafish as a model system for vertebrate developmental genetics. *Bioessays* 2000;22:487–95.
- [10] Di Biase A, Casalini A, Emmanuele P, Mandelli M, Lokman PM, Mordenti O. Controlled reproduction in *Anguilla anguilla* (L.): comparison between spontaneous spawning and stripping-insemination approaches. *Aquacult Res* 2016;47:3052–60.
- [11] Yang H, Hazlewood L, Heater SJ, Guerrero PA, Walter RB, Tiersch TR. Production of F-1 interspecies hybrid offspring with cryopreserved sperm from a live-bearing fish, the swordtail *Xiphophorus helleri*. *Biol Reprod* 2007;76:401–6.
- [12] Yang H, Hazlewood L, Walter RB, Tiersch TR. Sperm cryopreservation of a live-bearing fish, *Xiphophorus couchianus*: male-to-male variation in post-thaw motility and production of F1, hybrid offspring. *Comp Biochem Physiol C Toxicol Pharmacol* 2009;149:233–9.
- [13] Müller T, Horváth L, Szabó T, Ittész I, Bognár A, Faidt P, Ittész Á, Urbányi B, Kucska B. Novel method for induced propagation of fish: sperm injection in oviducts and ovary/ovarian lavage with sperm. *Aquaculture* 2017;482:124–9.
- [14] Müller T, Kucska B, Horváth L, Ittész Á, Urbányi B, Blake C, Gutí Cs, Csorbai B, Kovács B, Szabó T. Successful, induced propagation of African catfish (*Clarias gariepinus*) by ovarian lavage with sperm and hormone mixture. *Aquaculture* 2018;485:197–200. 2018.
- [15] Miskolczi E, Mihálfy S, Patakiné Várkonyi E, Urbányi B, Horváth Á. Examination of larval malformations in African catfish *Clarias gariepinus* following fertilization with cryopreserved sperm. *Aquaculture* 2005;247:119–25.
- [16] Kovács É, Müller T, Márián T, Krasznai Z, Urbányi B, Horváth Á. Quality of cryopreserved African catfish sperm following post-thaw storage. *J Appl Ichthyol* 2010;26:737–41.
- [17] Saad A, Billard R, Theron MC, Hollebecq MG. Short-term preservation of carp (*Cyprinus carpio*) semen. *Aquaculture* 1988;71:133–50.
- [18] Steyn GJ, Van Vuren JHJ, Schoonbee HJ, Chao NH. Preliminary investigations on the cryopreservation of *Clarias gariepinus* (Clariidae: pisces) sperm. *WaterSA* 1985;11:15–8.
- [19] Steyn GJ, Van Huren JHJ. The fertilizing capacity of cryopreserved sharptooth catfish, *Clarias gariepinus* sperm. *Aquaculture* 1987;6:187–93.
- [20] Steyn GJ. The effect of freezing rate on the survival of cryopreserved African sharptooth catfish (*Clarias gariepinus*) spermatozoa. *Cryobiology* 1993;30:581–90.
- [21] Urbányi B, Horváth Á, Varga Z, Horváth L. Effect of extenders on sperm cryopreservation of African catfish, *Clarias gariepinus* (Burchell). *Aquacult Res* 1999;30:145–51.
- [22] Horváth Á, Urbányi B. The effect of cryoprotectants on the motility and fertilizing capacity of cryopreserved African catfish *Clarias gariepinus* (Burchell, 1822) sperm. *Aquacult Res* 2000;31:317–24.
- [23] Rurangwa E, Volckaert FAM, Huyskens G, Kime DE, Ollevier F. Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilization in African catfish (*Clarias gariepinus*). *Theriogenology* 2001;55:751–69.
- [24] Viveiros AT, So N, Komen J. Sperm cryopreservation of African catfish, *Clarias gariepinus*; Cryoprotectants, freezing rates and sperm: egg dilution ratio. *Theriogenology* 2000;54:1395–408.
- [25] Oyeleye OO, Omitogun OG. Evaluation of motility of the short-term cryopreserved sperm of African giant catfish (*Clarias gariepinus*). *Ife Journal of Agriculture* 2007;22:11–6.
- [26] Bozkurt Y, Yavaş İ. Effect of extender compositions, glycerol levels and thawing rates on motility and fertility of cryopreserved wild African catfish (*Clarias gariepinus*) sperm. *Isr J Aquacult Bamidgah* 2017;69(1357):1–7.
- [27] Horie N, Utoh T, Mikawa N, Yamada Y, Okamura A, Tanaka S, Tsukamoto K. Influence of artificial fertilization methods of the hormone-treated Japanese eel *Anguilla japonica* upon the quality of eggs and larvae. *Nippon Suisan Gakk* 2008;74:26–35 [in Japanese with English abstract].
- [28] Okamura A, Horie N, Mikawa N, Yamada Y, Tsukamoto K. Recent advances in artificial production of glass eels for conservation of Anguillid eel populations. *Ecol Freshw Fish* 2014;23:95–110.