DEVELOPMENT OF SPERM VITRIFICATION PROTOCOLS FOR FRESHWATER FISH (EURASIAN PERCH, *Perca fluviatilis*) AND MARINE FISH (EUROPEAN EEL, *Anguilla anguilla*)

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Abstract

Vitrification was successfully applied to the sperm of two fish species, the freshwater Eurasian perch (*Perca fluviatilis*) and marine European eel (*Anguilla anguilla*). Sperm was collected, diluted in species-specific non-activating media and cryoprotectants and vitrified by plunging directly into liquid nitrogen without pre-cooling in its vapor. Progressive motility of fresh and vitrified-thawed sperm was evaluated with computer-assisted sperm analysis (CASA). Additional sperm quality parameters such as sperm head morphometry parameters (in case of European eel) and fertilizing capacity (in case of Eurasian perch) were carried out to test the effectiveness of vitrification. The vitrification method for Eurasian perch sperm resulting the highest post-thaw motility (14 ± 1.6%) was as follows: 1:5 dilution ratio, Tanaka extender, 30% cryoprotectant (15% methanol + 15% propylene-glycol), cooling device: Cryotop, 2 µl droplets, and for European eel sperm: dilution ratio 1:1, with 40% cryoprotectant (20% MeOH and 20% PG), and 10% FBS, cooling device: Cryotop, with 2 µl of sperm suspension. Viable embryos were produced by fertilization with vitrified Eurasian perch sperm (neurulation: 2.54±1.67%). According to the ASMA analysis, no significant decrease in head area and perimeter of vitrified European eel spermatozoa were found when compared to fresh spermatozoa.

Keywords: spermatozoa vitrification, ultra-rapid cooling, fish sperm cryopreservation, perch, eel

List of abbreviations: ASMA = Computer automated sperm head morphometry analysis; BSA = Bovine Serum Albumin; CASA = Computer-assisted sperm analysis; CP = Cryoprotectant; EG = Ethylene glycol; FBS = Foetal Bovine Serum; hCG =

Human chorionic gonadotropin; MeOH = Methanol; PG = Propylene glycol; STR = straightness (%); VCL = curvilinear velocity (µm/s)

1. Introduction

Cryobiology is an area of science that studies the survival of live cells and tissues at extreme low temperatures. Cryopreservation has been applied to the storage of sperm, oocytes and embryos of numerous species. Two main methods have been developed for the cryopreservation of cells and tissues without the formation of ice crystals: slow-equilibrium programmable freezing and ultra-rapid non-equilibrium freezing (vitrification). The two procedures differ from each other in cryoprotectant (CP) concentration and cooling/warming rates. Programmable freezing requires expensive equipment and laboratory conditions during the cooling process. On the other hand, ultra-fast cooling or vitrification, which does not require any special equipment or conditions, has attracted increasing interest in recent years (Vajta et al, 2006).

Vitrification is the solidification of a liquid into an amorphous or glassy state which can be attained at very fast cooling rates (10^6-10^{10} °C/s; Franks, 1982). The success of the vitrification principally relies on achieving ultra-fast cooling and thawing rates and on the determination of appropriate (usually high) CP concentrations in cryoprotective media, in order to prevent ice formation during the process. Although high concentrations of CPs lower the temperature of ice formation, they can be toxic to cells. Consequently, CP concentration has to be reduced and the cooling rate has to be enhanced. For this reason, the material and capacity of the cooling device is very important to achieve fast heat transfer and avoid creation of ice crystals (Tsai et al, 2015).

Recently, several studies have been published on sperm vitrification of different fish species: channel catfish (*Ictalurus punctatus*; Cuevas-Uribe et al., 2011/a), green swordtail (*Xiphophorus hellerii*; Cuevas-Uribe et al., 2011/b), rainbow trout (*Onchorynchus mykiss*; Varela et al., 2009, Merino et al., 2011, Figueroa et al., 2013), Russian sturgeon (*Acipenser gueldenstaedtii*; Andreev et al. 2009), spotted seatrout (*Cynoscion nebulosus*), red snapper (*Lutjanus campechanus*), red drum (*Sciaenops ocellatus*; Cuevas-Uribe et al, 2013), Atlantic salmon (*Salmo salar*; Figueroa et al., 2015) and tambaqui (*Colossoma macropomum*; Varela et al., 2015). A potential further application for fish sperm vitrification is the cryopreservation of the spermatozoa of small laboratory model fish species, such as the zebrafish. One male individual of this species can produce approximately 1 microliter of sperm (obtained with stripping), which is ideal for vitrification, contrary to slow freezing in straws.

In general, sperm of marine fish retains a higher quality after cryopreservation compared to that of freshwater species (Drokin et al, 1998, Suquet et al, 2000, Herrarez et al, 2012). Kopeika et al (2008) suggest that sperm of marine fish species shows better adaptation to osmotic changes upon release into the aquatic environment. Alternatively, the same authors postulate that the composition of the sperm membrane in marine species is different from that of freshwater fish. Better adaptation of the sperm of marine fish to higher osmotic pressures can explain their survival following exposure to high CP concentrations during vitrification (Cuevas-Uribe et al, 2013).

According to the published results on fish sperm vitrification, the efficient CP concentrations and vitrifying media compositions are quite diverse. The volume of vitrified sperm suspension varies between 10-250 µl, while the CP concentrations vary between 0-40%. The type of the cooling device also shows high variability. Our

aim was to test devices with different cooling capacities (2-250 µl) in combination with different vitrifying media in sperm vitrification of the Eurasian perch and European eel.

2. Materials and methods

2.1. Broodstock

2.1.1. Eurasian perch

Eurasian perch broodstock [27 males (TL: 19.5 ± 4 cm; W: 101 ± 67 g) and 14 females (W: 106 ± 57g)] was caught in the wild, transported to and maintained at the hatchery of the Department of Aquaculture, Szent István University, Gödöllő, Hungary. Experiments were carried out from October 2014 until April 2015.

2.1.2. European eel

Seventy European eel males (W: 115 ± 8 g) were kept at the Polytechnical University of Valencia, Spain. Fish were supplied by Valenciana de Acuicultura, S. A. (Puzol, Spain). Fish were distributed in three 96-l aquaria and fasted during the experiment. Vitrification trials were performed in November and December of 2014.

2.2. Gamete collection and quality assessment

2.2.1. Eurasian perch

After drying the genital area, Eurasian perch sperm was collected into 1.5-ml Eppendorf tubes by applying gentle abdominal pressure to anaesthetized (with MS-222, 150 mg L^{-1}) males. The fish were sampled 6 days after hormonal injection (250

IU kg^{-1} of hCG (human chorionic gonadotropin, Ferring, Saint Prex, Switzerland)). Experiments were carried out according to the license number XIV-I-001/2299-4/2012 issued by the Directorate for Food Chain Safety and Animal Health of the Government Office of Pest County, Budapest, Hungary. Fish were not sacrificed during the experiments. Following each gamete sampling in anesthesia the individuals were placed into anesthetic-free water for recovery. Sperm was kept on ice until analysis (up to 30 minutes).

Progressive motility of fresh sperm samples was evaluated with computer assisted sperm analysis (CASA, Sperm VisionTM v. 3.7.4., Minitube of America, Venture Court Verona, USA), after activation with Modified Lahnsteiner's activating solution (75 mM NaCl, 2 mM KCl, 1 mM $MgSO_4 \bullet 7H_2O$, 1 mM $CaCl_2 \bullet 2H_2O$, 20 mM Tris, pH 8 (Lahnsteiner et al, 2011)) with the addition of 0.01 g/mL BSA (Bovine Serum Albumin). Progressive motility, curvilinear velocity (VCL) and straightness (STR) (WHO, 2010) values were used to characterize sperm quality in all CASA measurements. Samples with higher progressive motility than 80% were selected for experiments.

To evaluate the effectiveness of the improved vitrification protocols, fertilization tests were carried out with vitrified sperm samples. After the use of a single hormonal injection (hCG, 500 IU/g fish), females were checked daily by ovarian biopsy in order to predict the accurate time of ovulation. One day before the planned ovulation the genital pores were sutured to avoid spontaneous spawning into the tank. After removing the suture and drying the genital area the eggs were stripped into a dry dish.

2.2.2. European eel

European eel sperm samples were collected into 50 ml plastic centrifuge tubes after drying the genital area with paper (to avoid contamination of samples with sea water or mucus), by applying gentle abdominal pressure to anaesthetized males (benzocaine, 60 mg/l). As the eels stop feeding at the silver stage and throughout sexual maturation, they were not fed during the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 on protection of animals used for scientific purposes (BOE 2013). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Polytechnical University of Valencia (UPV) and the local government, Generalitat Valenciana (Permit Number: 2014/VSC/PEA/00147). All efforts were made to minimize animal suffering and stress. The fish were previously treated with weekly hormonal injections (hCG, 1.5 IU/g fish). Sperm was stripped between the 8^{th} and 10^{th} week of the weekly hormonal treatments, when the quality of the sperm is the most appropriate (Pérez et al., 2000). Eels were sampled 24 h after the hormonal injection. Samples were kept on ice until analysis (up to 30 minutes).

Progressive motility of fresh sperm samples was evaluated with a CASA system (ISAS, Proiser, Valencia, Spain). For activation, artificial seawater (354.7 mM NaCl, 52.4 mM $MgCl_2$, 9.9 mM $CaCl_2$, 28.2 mM Na_2SO_4, 9.4 mM KCl; pH 8) was used mixed with FBS (Foetal Bovine Serum) in 1:1000 dilution (sperm:activating solution). Samples with progressive motility values higher than 70% were selected for experiments.

2.3. Experimental design

2.3.1. Experiment 1: Effect of cryoprotectants and extenders on the motility rates of vitrified Eurasian perch sperm

For the vitrification of Eurasian perch spermatoza, sperm was diluted in modified Tanaka extender (137 mM NaCl and 76.2 mM $NaHCO_3$ (Szabó et al, 2005)) to the final ratio of 1:5 (including CPs). Methanol (MeOH) and propylene glycol (PG) were used in three combinations: 10% MeOH + 10% PG (20% total CP concentration), 15% MeOH + 15% PG (30% CP) and 20% MeOH + 20% PG (40% CP). All CP concentrations were calculated as a v/v concentration relative to the final mixture of sperm, extender and CPs. Chemicals were purchased from Reanal (Budapest, Hungary) and Sigma-Aldrich (Budapest, Hungary). For vitrification experiments, inoculating loops (10 µl), straws (250 µl, Minitüb, Tiefenbach, Germany) and Cryotops (Kitazato-Dibimed, for 2 µl of solution, Fig. 1.) were used as cooling devices. In case of innoculating loops, 10 µl sperm suspension was pipetted into the loop laid out in a dry petri-dish. Straws were loaded with sperm suspension with a pipette inserted into the plugged end of the straw. In case of Cryotops, 2 µl of sperm suspension was pipetted onto one side of the device. Devices containing the sperm suspension were plunged directly into liquid nitrogen without pre-cooling in its vapor. After at least 2 hours (range: 2-48 hours) of storage in liquid nitrogen, samples vitrified in inocculating loops or Cryotops were thawed directly (within 1 second after removing from liquid nitrogen) on a Makler Chamber containing activating soultion at room temperature. Samples in straws were thawed in a 40°C water bath for 5 seconds. Motility parameters of vitrified-thawed samples were determined with the CASA system.

2.3.2. Experiment 2: Effect of the volume of vitrified sperm on fertilization rates in the Eurasian perch

For fertilization tests, approximately 100 eggs were used in each sample. Sperm was vitrified in the presence of CPs at a final concentration of 30% (15% MeOH + 15% PG). Vitrified Cryotops were thawed directly into 10 ml of Modified Lahnsteiner's activating solution in a petri dish containing the eggs. Fresh sperm was used for control. Fertilized eggs were incubated at 13 °C in a floating system (polystyrene boards with filters on a tank).

Three trials were conducted to find the most appropriate number of Cryotops per egg batch. We used 1, 6 and 18 Cryotops for the fertilization of one portion of eggs (2 µl of diluted sperm per one Cryotop contained approximately 0.33 µl of sperm). Fertilization ratios were counted under a stereomicroscope 15 days following fertilization (neurula stage).

2.3.3. Experiment 3: Effect of cryoprotectants and extenders on the motility rates of vitrified European eel sperm

Sperm was diluted with modified Tanaka extender supplemented with 10% FBS to final ratio of 1:9 (including CPs). Firstly, combinations of MeOH, EG (ethylene glycol) and PG were tested in various concentrations (20-40%) and straws (250 µl) and Cryotops were tested as devices. For all methods, the sperm suspension was plunged directly into liquid nitrogen without pre-cooling in its vapor. Straws were thawed in a 40 °C water bath for 5 s, while the Cryotops were thawed into the activating media.

Additionally, we checked the possible effect of different dilution ratios in the 40% CP group (highest motility from previous trial). Sperm was diluted in the same extender to a final ratio of 1:1 and the cryopreservation procedure was the same as previously described.

2.3.4. Experiment 4: Effect of cryoprotectants and extenders on the morphometry parameters of vitrified European eel sperm

In case of European eel, sperm quality could not be measured through fertilization success because of the reproductive characteristics and inavailability of eggs in this species. Therefore we used computer automated sperm head morphometry analysis (ASMA, Sperm Class Analyzer, Morfo Version 1.1, Imagesp, Barcelona, Spain) as an additional sperm quality characteristic since it was shown that these parameters affect fertilization success (Rurangwa et al., 2004). Head morphometry analyses were conducted simultaneously with motility assessment. To measure the damage caused by vitrification of the sperm, pre- and post-cryopreservation, a fraction of sperm samples was diluted 1:20 (v/v) with 5% glutaraldehyde in saline solution. Sperm morphology was analyzed using the ASMA software, 110 spermatozoa were analyzed in each sample (Fig. 2). Morphological parameters examined were average head perimeter and area.

2.3.5. Statistical analysis

To analyze the results of motility, morphometry and fertilization tests, the statistical software STATISTICA v 1.2 (Statsoft, Tulsa, OK, USA) was used. Percentage parameters (motility parameters and fertilization rates) were arcsine transformed before statistical analysis. To calculate differences in general, One-way

ANOVA followed by Tukey post hoc test was used at the significance level of $p \leq 0.05$. All values are presented as mean ± SD.

3. Results

Generally, higher motility percentages were observed by vitrifying lower volumes of diluted sperm (2 µl) with the use of 30-40% total CP content, in case of both species.

3.1. Experiment 1: Effect of cryoprotectants and extenders on the motility rates of vitrified Eurasian perch sperm

Sperm vitrification caused a decrease in sperm motility parameters compared to the control. Cryoprotectant concentrations had a significant effect on progressive motility ($F_{(2, 130)} = 12.57$, $p < 0.001$). Tukey's HSD showed a significant decrease in progressive motility in the 40% CP group compared to the vitrified groups with 20% CP and 30% CP ($p < 0.05$; Fig. 3). CP concentrations had a significant effect on both VCL ($F_{(2, 130)} = 4.01$, $p = 0.02$) and STR ($F_{(2, 130)} = 5.44$, $p < 0.01$). In both parameters, a significant difference between CP 30% and CP 40% groups was observed (Tukey's HSD; $p < 0.05$). Control motility parameters were as follows: progressive motility: 76 ± 17%; VCL: 136.4 ± 22 µm/s; STR: 0.8 ± 0.1%.

3.2. Experiment 2: Effect of vitrified sperm volume on fertilization rates in the Eurasian perch

There were no significant differences among the tested batches of eggs ($F_{(2, 13)} = 0.87$, $p = 0.44$; Fig. 4). Control sperm displayed a fertilization rate of 76 ± 14.5%.

3.3. Experiment 3: Effect of cryoprotectants and extenders on the motility rates of vitrified European eel sperm

Sperm vitrification caused a decrease in sperm motility parameters compared to the control. Two-way ANOVA on progressive motility displayed a significant effect of devices ($F_{(1, 22)} = 7.8$, $p < 0.05$) while the effects of CPs and the interaction between CPs and devices were not significant. There were no significant differences between the experimental groups (Tukey's HSD; $p > 0.05$; Table 1). Similar observations were made for VCL and STR where only the devices used had a significant effect ($F_{(1, 22)} = 8.9$, $p < 0.01$ for VCL and $F_{(1, 22)} = 9.19$, $p < 0.01$ for STR).

In order to improve motility data, we checked the possible effect of dilution ratios in the 40% CP group. Sperm vitrified with a 1:1 dilution ratio had a significantly higher progressive motility ($F_{(1, 9)} = 10.74$, $p < 0.01$) and VCL ($F_{(1, 9)} = 10.67$, $p < 0.01$), while there were no differences in the STR values. Control motility parameters were as follows: progressive motility: 26.4 ± 4%; VCL: 140 ± 14 µm/s; STR: 48 ± 3%. No progressive motility was observed after thawing the vitrified samples with any of the tested protocols with 250-µl straws as cooling devices.

In summary, vitrification of eel sperm resulting in the highest progressive motility after thawing was carried out with using the following protocol: dilution ratio 1:1, with 40% CP (20% MeOH and 20% PG), and 10% FBS using Cryotops as a cooling device. The measured progressive motility of 5±1 % was significantly more effective than the other protocols (Table 1.) resulting in motile spermatozoa.

1. Table. Effectiveness of the tested protocols for the vitrification of European eel sperm (N=16).

Cryoprotectant	Device	Ratio	Progressive motility
10% MeOH + 10% PG	Cryotop	1:9	0.6±0.49%[a]
10% MeOH + 10% PG	straw	1:9	0%
10% MeOH + 10% PG + 10% EG	Cryotop	1:9	0%
10% MeOH + 10% PG + 10% EG	straw	1:9	0%
20% MeOH + 20% PG	Cryotop	1:9	1.25±1.64%[a]
20% MeOH + 20% PG	straw	1:9	0%
20% MeOH + 20% PG	Cryotop	1:1	5±0.81%[b]
20% MeOH + 20% PG	straw	1:1	0%

3.4. Experiment 4: Effect of cryoprotectants and extenders on the morphometry parameters of vitrified European eel sperm

According to the ASMA analysis, no significant decreases in head area and perimeter of vitrified spermatozoa were found when compared to fresh spermatozoa (Fig 5A and 5B).

4. Discussion

In this study we demonstrated for the first time the feasibility of vitrification of Eurasian perch and European eel sperm. The motility rates in case of both species were low compared to the average motility rates following conventional cryopreservation, but these motility values are similar to the results reported by other authors (Cuevas-Uribe et al, 2011a, 2011b, Figueroa et al, 2013). It is a general observation that the sperm in vitrification solutions has a tendency toward local motility (vibration) rather than progressive motility (Cuevas-Uribe et al, 2011b). The reason of this vibration is not clear, it could be caused by cellular damage or the high viscosity of the solution. Nevertheless, fertilization of perch eggs with vitrified sperm resulted in developing embryos in case of Eurasian perch, thus vitrified sperm with low progressive motility rates could be able to preserve genetic material.

In addition to the motility results, the feasibility of the vitrification of eel sperm was evidenced through the absence of decreases in head area and perimeter of vitrified European eel spermatozoa when compared to fresh spermatozoa. In this species, several studies concentrated on the head morphometry of spermatozoa (Marco-Jiménez et al, 2006, Asturiano et al, 2006) and the morphological damage caused by cryopreservation protocols (Peñaranda et al, 2009). As a consequence of osmotic stress, significant morphomteric alterations could be observed in eel spermatozoa (Asturiano et al, 2007). The head regions of dead speratozoa suffer a greater decrease than that of living cells (Peñaranda et al, 2009), thus our vitrification protocols were less harmful for eel sperm. In this experiment, total CP concentrations below 40% were tested, based on the results of our preliminary experiments showing that membrane integrity (viability) of European eel spermatozoa decreases significantly when 50% total CP is used for vitrification. Contrarily, after thawing the samples vitrified with 30% and 40% of total CP, no significant decrease was observed in viability parameters (Kása et al, 2014).

Our results demonstrate that for fish sperm vitrification, devices for low volumes of sperm (in the range of microliters) are needed. The efficiency of vitrification with inoculating loops was less successful compared to Cryotops, and the use of 250 µl straws was not suitable for vitrification in any of our experimental species. When the thickness of the solution layer in these devices is around 0.07 cm, the calculated cooling rate of approximately 720,000 K/min (Isachenko et al, 2003) is fast enough to prevent ice formation.

Cryoprotectants used at concentrations high enough for successful vitrification have a toxic and hypertonic effect on spermatozoa (Yavin & Arav, 2007). Fish spermatozoa can tolerate high CP concentrations when the proportion of the

chemicals is appropriate (Cuevas-Uribe et al., 2011a, b), and it is possible to decrease the toxicity by combination of at least two different CPs and reducing the equilibration time to the minimum (below 1 minute). In combination, the effectiveness for vitrifying the solution is higher and the toxicity is lower than the use of a single CP (Ali & Shelton, 2007). In our experiments, the equilibration time was below 1 minute in every case, and for the most feasible protocols 2 or 3 different CPs were used. According to our results, CP concentrations above 40% are too harmful for the cells, while CP concentrations below 30% do not inhibit the formation of ice crystals entirely.

Another crucial point of vitrification is the recrystallization occuring during thawing. In our experiments, samples were thawed directly into the activating solution without paying attention to its temperature. A previous study on vitrified green swordtail sperm showed that there were no significant differences between thawing in solutions at 24 °C or 37 °C, therefore both methods were fast enough to prevent ice formation (devitrification or recrystallization) during thawing (Cuevas-Uribe, 2011b). However, another study on vitrification of mouse oocytes described that survival could be enhanced using laser-induced ultra-rapid thawing (Jin et al, 2014).

5.Conclusion

In summary, the present study demonstrated that vitrification is a feasible alternative sperm cryopreservation method in either marine- or freshwater species. Successful vitrification of Eurasian perch and European eel sperm was conducted for the first time. Motile spermatozoa were recovered following vitrification in case of both species, and fertilization of eggs with vitrified sperm resulted in developing embryos in case of Eurasian perch.

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Figure legends

Fig. 1. *Different devices used for vitrification experiments. From top to bottom: Cryotop (2 µl), straw (250 µl), inoculating loop (10 µl).*

Fig. 2. Picture of measured spermatozoa for morphometry analysis

Fig. 3. Progressive motility (A), curvilinear velocity (VCL, B) and straightness (STR, C) measured in sperm vitrified with 20%, 30% and 40% CP (MeOH and PG in 1:1 ratio). Columns represent the mean±SD of data from 3 experiments, 3 vitrified samples in 4 replicates. Different letters mean significant differences ($p<0.05$).

Fig. 4. Fertilization results of Eurasian perch eggs fertilized with vitrified sperm in control (fertilized with fresh sperm) groups and vitrified sperm with 1/6/18 Cryotops per egg batch. Columns represent the data from 3 test groups.

Fig. 5. Average head perimeters (A) and areas (B) of European eel spermatozoa vitrified at various cryoprotectant (CP) concentrations, dilution ratios and devices measured with ASMA. Fresh: control, 1: 20% CP, 1:9 dilution ratio, Cryotop, 2: 20% CP, 1:9 dilution ratio, straw, 3: 40% CP, 1:1 dilution ratio, Cryotop, 4: 40% CP, 1:9 dilution ratio, Cryotop, 5: 40% CP, 1:9 dilution ratio, straw.

Fig 1

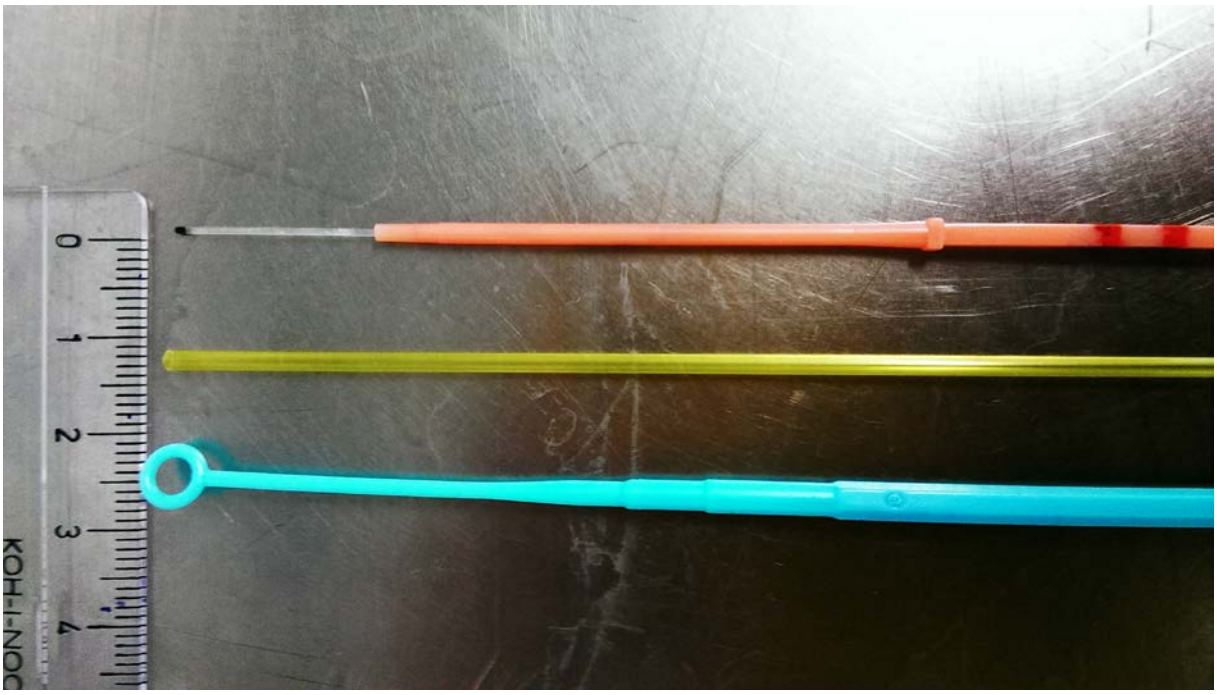
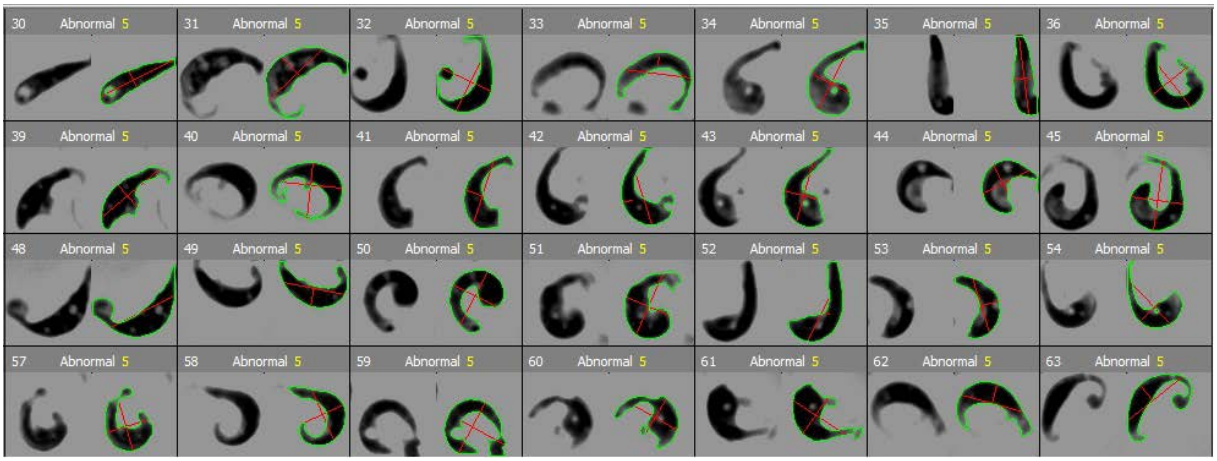


Fig. 2



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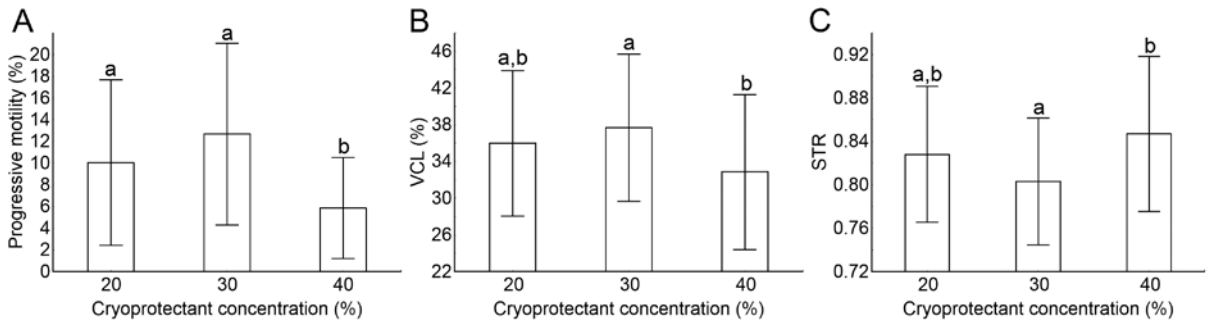

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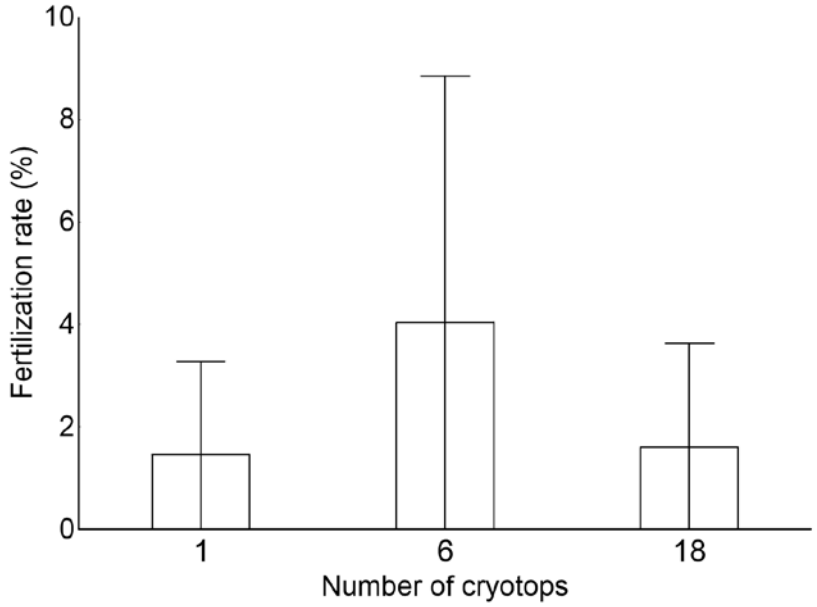

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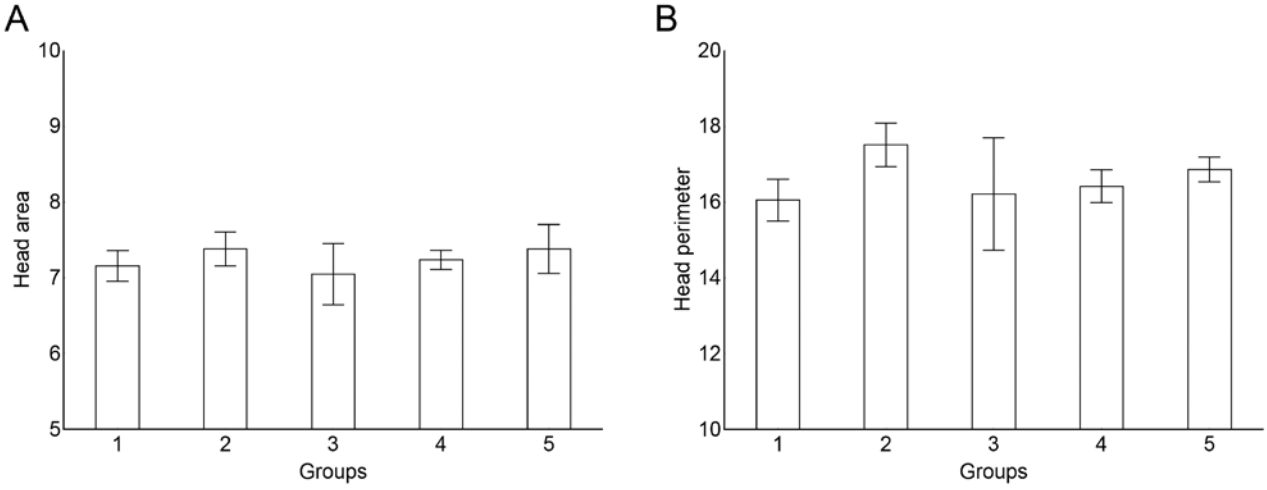
510 Fig. 5



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