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ABSTRACT BOOK

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Optimization of the Semen Cryopreservation Protocol for the Safeguard of Mediterranean Brown Trout

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An important purpose of developing fish sperm cryopreservation is to applicate it in restocking and conservation programs. In fact, germplasm cryobanking is an important tool to preserve the genetic resources of endangered fish species. A current problem in fish sperm cryopreservation is the lack of standardization during each step of the process. The goal of this study was to evaluate the effects of two basic extenders on the post-thaw quality and fertilizing ability of cryopreserved Mediterranean brown trout semen (*Salmo cettii*).

Semen was collected from thirty native males from Molise rivers. In total 6 pools were used. Each pool was split into two aliquots and diluted 1:3 in extender composed by 300 mM glucose (extender A) or 75 mM NaCl, 70 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 20 mM Tris (extender B) combined with 10% egg yolk and 10% DMSO. The diluted semen was packaged in 0.25 mL plastic straws and equilibrated at 4°C for 10 min, then the straws were frozen by exposure at 5 cm above the liquid nitrogen level for 10 min. Lastly, the straws were dipped in liquid nitrogen at -196°C. Semen samples were thawed at 30°C for 10 s. Sperm motility (CASA system), spermatozoa movement duration (SMD) and sperm viability (SYBR-PI) were assessed. Motility and SMD was

significantly higher in semen frozen with extender A(31.6±3.8 and 39.4±4.8). Fertilization trials were performed using three groups of eggs (around 100), one group was inseminated with fresh semen and another two groups with frozen semen using extender A or B.Fertilization and hatching rates were significantly higher in the fresh semen compared to the frozen one. No significant differences emerged when we compared the frozen semen using extender A or B, although, the higher percentage of fertilization and hatching rates were recorded in eggs fertilized with extender A (38.5±.3% and 34.8±8.3%) compared to extender B (27.5±4.1% and 22.7±2.7%). According to the results obtained extender A emerged as more effective for the semen cryopreservation in this native trout. The development of an effective freezing protocol will allow for the creation of a sperm cryobank to recover the original population of Mediterranean brown trout.

Keywords: Mediterranean brown trout, semen cryopreservation, semen cryobank, extender, fertilizing ability

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O101

Thawing rate effects on the cryosurvival of Mediterranean brown trout spermatozoa

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The Mediterranean trout, *Salmo cettii*, is listed in Annexe II of the EU Habitats Directive and is included as 'critically endangered' in the Italian freshwater fish Red List. One of the most effective tools to avoid the extinction is the semen cryobank and, in this regard, the development of a successful sperm cryopreservation protocol is needed. Therefore, the purpose of the present study was to evaluate the effects of two different thawing rates on the *in vitro* and *in vivo* sperm quality of *S. cettii* populations of Molise.

Native trout were captured by electro-fishing. Semen was collected from 40 males to obtain five total pools, each diluted 1:3 (v:v) with a freezing extender composed of 0.3M glucose, 10% DMSO and 10% egg yolk. The semen was loaded in 0.25 mL straws and equilibrated at 5 °C for 10 min. The straws were frozen through the exposure at 5 cm above the liquid nitrogen level for 10 min, dipped and stored in the liquid nitrogen. Straws were thawed at two different thawing rates 30 °C for 10 s and 10 °C for 30 s, using a water bath. The sperm parameters evaluated were: motility and movement duration, viability (SYBR-14, PI) and DNA integrity (Acridine Orange). Fertilisation trials were performed using three groups of eggs ($N \simeq 100$) inseminated with: (a) fresh sperm (control group); (b) sperm thawed at 30 °C for 10 s; (c) sperm thawed at 10 °C for 30 s. The data obtained *in vitro* showed that the freezing process impaired the post-thaw sperm quality compared to the fresh semen ($p<.05$). In addition, we also found significantly higher motility and viability ($p<.05$) in sperm thawed at 10 °C for 30 s than the 30 °C for 10 s.

The fertilisation and hatching rates were significantly higher in fresh sperm (73.26 ± 5.17 and 68.89 ± 5.51) respect to the frozen semen. However, the best eyed and hatched rates were found using semen thawed at 10 °C for 30 s (58.6 ± 2.8% and 54.5 ± 2.8%) than sperm thawed at 30 °C for 10 s (32.9 ± 4.9% and 29.9 ± 4.4%) ($p<.05$).

In conclusion, the use of low thawing rate (10 °C for 30 s) improved the semen fertilisation ability of *S. cettii*. Our findings provide an important contribution for the creation of a sperm cryobank aiming at the restoration of *S. cettii* in Molise. Natural reproduction of native trout occurs on the spawning grounds at the main springs of Volturno and Biferno rivers (T≃10 °C). Thus, the encouraging results at the thawing temperature of 10 °C, would facilitate the on-field artificial reproduction of wild breeders, using directly the spring water.

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P022

Biodiversity in local population of Mediterranean trout in Molise

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Freshwater fishes are particularly interesting due to the high number of endemic species described in Italy. Among these, Mediterranean trout *(Salmo cettii, syn. Salmo macrostigma)* has considerable economic significance for fisheries management, aquaculture and conservation biology. Unfortunately, especially in Italian rivers, due to anthropogenic disturbance as pollution, water depletion for irrigation, dam constructions for power production, habitat alteration and over-fishing, the native Mediterranean trout populations are decreased during the past decades. For this reason, alien trout as the domesticated strains of *Salmo trutta* have been introduced in freshwater basins for the implementation of fishing activities without considering the presence of wild autochthonous populations of the interfertile *Salmo cettii*, leading to the introgression by alien genomes. The restocking with non-native domesticated strains produces the 'founder effect' due to low genetic variability of spawners. The described scenario is also common to Molise watersheds in which no data of genetic variability has ever been reported. Here we present preliminary results of gene variation study to assess trout population structures in Biferno and Volturno rivers. A total of 300 samples in 30 different areas (15 for each river) were collected. Adipose fin tissue fragments were cut, immediately transferred into a tube containing 100% ethanol and, once in the lab, stored at –20 °C until DNA isolation (by Qiagen blood and tissue kit). PCR-RFLP analysis on nuclear gene LDH- C1* using the Ldhxon3F/Ldhxon4R primer pair and *Bsl*I restriction enzyme was carried out.

Preliminary results, carried out on a representative subset of samples, showed a different introgression level considering the two rivers. In the Volturno river drainage, the degree of homozygous specimens for the native allele (*S. cettii*) was 86.9% vs. 13.1% of heterozygous fishes. The Biferno river basin, instead showed 50.0% of specimens with homozygous native alleles vs. 41.2% of heterozygous fishes and 8.8% of specimens with homozygous alien alleles (*S. trutta*). Further investigations are required using all the samples and analysing also mitochondrial 16S rDNA.

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SCIENTIFIC REPORTS

OPEN

Semen cryopreservation for the Mediterranean brown trout of the Biferno River (Molise-Italy): comparative study on the effects of basic extenders and cryoprotectants

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This study was designed to optimize the semen freezing protocol of the native Mediterranean brown trout inhabiting the Molise rivers through two experiments: an *in vitro* analysis of the effects of two basic extenders combined with three cryoprotectants on post-thaw semen quality; and an *in vivo* test to assess the fertilization and hatching rate. Semen was diluted at a ratio of 1:3 in a freezing medium composed of a glucose extender (A) or mineral extender (B). Each basic component contained 10% dimethylsulfoxide, dimethylacetamide or methanol. The post-semen quality was evaluated considering motility, duration of motility, viability and DNA integrity. The basic extender and cryoprotectant were shown to have significant effects on these variables, and the best results were obtained using extender A or B combined with dimethylsulfoxide ($P < 0.05$). These freezing protocols were selected for fertilization trials *in vivo*. Fertilization and hatching rates were significantly higher in fresh semen. No significant differences were observed in frozen semen using extender A or B, although higher percentages of eyed eggs and hatching rates were recorded using extender A. According to our *in vitro* and *in vivo* results, the glucose-based extender and dimethylsulfoxide emerged as the best combination for an effective cryopreservation protocol for semen of this trout.

Cryobanking is a valuable tool to preserve the genetic resources of a wide range of fish species and cryopreservation has been extensively used in assisted reproductive technology, agriculture, and conservation programmes for endangered species playing an important role in genetic selection programmes, biodiversity conservation and restocking programmes[1–3].

In aquaculture, semen cryopreservation is widely used to provide gametes year-round for fertilization outside of the normal reproductive seasons or to promote alternative techniques for broodstock management. In the case of some catfish, the study of sperm cryopreservation is more extensive than studied concerning females because the availability male fish is often limited and their peak spawning occurs at different times of the year, thus sperm cryopreservation can be used strategically to improve the production of hybrid catfish[4]. In endangered wild aquatic species, gamete freeze preservation is a valuable tool for preserving the genetic material of native populations with a specific genotype[5]. Research on fish germplasm cryobanking has been carried out on different cell types, including sperm[2], somatic cells[6], fish oocytes and embryos[7,8], and more recently spermatogonia and primordial germ cells[9].

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In this regard, studies carried out so far on the cryopreservation of fish oocytes indicated that some initial promising results were obtained with early stage ovarian follicles, such as stage I and stage II follicles[2]. However, more work needs to be done in optimising the protocols for cryopreservation of fish ovarian follicles. Fish embryo cryopreservation has been a challenging objective for decades and has yet to be achieved. Therefore, successful fish embryo cryopreservation remains elusive[2]. Mainly due to the small size of sperm cells and relatively high resistance to chilling, sperm cryopreservation is more advantageous compared to the one performed on other cell types, thus resulting in this being the most established technique in aquatic species[2].

Salmonid sperm cryopreservation has been widely studied due to their high commercial value, both in the food sector and for recreational purposes such as fishing[2,10–13]. However, in salmonids many difficulties have been encountered because of the sperm's high susceptibility to cryopreservation-induced damage caused by the short duration of motility, low ATP production, high sensitivity to osmotic stress and large number of spermatozoa required to fertilize each egg[14].

Many authors have described damage caused by cryopreservation in trout spermatozoa as affecting motility, cell metabolism, and the structure of the plasma membrane, mitochondria, tail and chromatin[12,15,16]. The plasma membrane is one of the most susceptible structures since it is highly sensitive to cold-shock damage[17], osmotic stress[18], and the presence of reactive oxygen species (ROS) generated during the freezing/thawing processes. These agents alter lipid and protein composition, leading to a decrease in sperm quality after cryopreservation[3,19].

Cryopreservation involves several factors that need to be fine-tuned to improve sperm cryosurvival[13,20], including the quality of fresh semen, the composition of the basic extender, the type of cryoprotectant (CPA) and its concentration, and the freezing and thawing rate[10–13,21,22]. Among these factors a decisive role is played by the basic extender and CPA type[23]. Two types of basic extenders have generally been used for the cryopreservation of trout spermatozoa: seminal plasma-mimicking media and simple carbohydrate-based solutions[24,25].

Dimethylsulfoxide (DMSO) is usually applied as the penetrating cryoprotectant (P-CPA) in trout semen; however, other penetrating cryoprotectants (P-CPAs), such as dimethylacetamide (DMA), ethylene glycol, methanol (MeOH), glycerol and DMSO – glycerol mixtures, are also reported to provide efficient results[13,15].

Listed under the scientific name of *Salmo cettii*[26,27], the Mediterranean brown trout (*Salmo macrostigma*) inhabiting the Molise rivers is considered "critically endangered" by the Italian Red List of the International Union for Conservation of Nature and Natural Resources (IUCN). Finding an efficient freezing protocol for the semen of the Mediterranean brown trout (*Salmo macrostigma*) of Molise will allow the creation of a sperm cryobank. The sperm cryobank of autochthonous Mediterranean trout populations with high genetic variability represents an action within our financed "Life" project focusing on the recovery and conservation of this native trout in Molise rivers.

Therefore, this study was designed to improve the semen freezing procedure for native Molise trouts for the creation of a sperm cryobank by 1) investigating the effects of two basic extenders combined with three P-CPAs on *in vitro* post-thaw semen quality and 2) assessing the *in vivo* yields of the most effective P-CPA identified during phase 1) for each extender.

Methods

Chemicals. The LIVE/DEAD Sperm Viability Kit was purchased from Molecular Probes, Inc. (Eugene, OR, USA) and all other chemicals used in this study were purchased from Sigma, Chemical Co. (Milan, Italy).

Animal and gamete collection. The experiments were carried out during the spawning season (January-February) in the Bojano spring of the Biferno River (Molise, Italy). This sampling location was selected because it is a highly attractive spawning site used by the native trout population after upstream migration.

A total of 67 autochthonous individuals of *Salmo cettii* were captured by electro-fishing and identified as 60 males and 7 females, based on their phenotypic characteristics[28–30] and aged as 2+ to 5+ years old. The average total lengths of the fishes were 33.8 ± 5.4 cm for males and 36.5 ± 7.7 cm for females.

Preliminary results of genetic analysis (data not published yet) show that specimens captured in this location have low levels of introgression (approximately 0.15%) among allochthonous brown trouts.

Sperm samples were collected by gentle abdominal massage, and abdomens and urogenital papilla were dried before stripping, with special care to avoid contamination of semen with urine, mucus and blood cells. Each male was stripped once only and the total amount of expressible milt was collected individually in graduated plastic tubes.

The semen was transported from the river to the laboratory in a portable refrigerator at 4 °C. Only spermatozoa showing a motility (subjectively evaluated as described in the sperm function section) rate higher than 75% were used. Ejaculates of different males were pooled (4/5 ejaculates/pool). In total, 12 pools were created and stored in a portable fridge (4 °C) before cryopreservation.

Egg collection from the 7 mature females was also performed through gentle abdominal massage and eggs were carefully selected based on their well-rounded shape and transparency.

The experiments were carried out in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments. The Bioethics Committee of University of Molise (UNIMOL) approved all procedures performed in this study (protocol n. 450 - UNMLCLE).

This study is part of a Nat.Sal.Mo LIFE project (NAT/IT/000547) financed by the European community. In addition our Life project received "a positive opinion" from the Ministry of the Environment and the Protection of The territory and the Sea. Sampling and handling of fish followed the practices reported in the Ministerial Protocol (ISPRA) in terms of animal welfare.

All experiments were conducted with the appropriate permits of the competent authorities (Molise Region, protocol number 6192, 13/12/2017) according to the current regulations on the protection of the species,

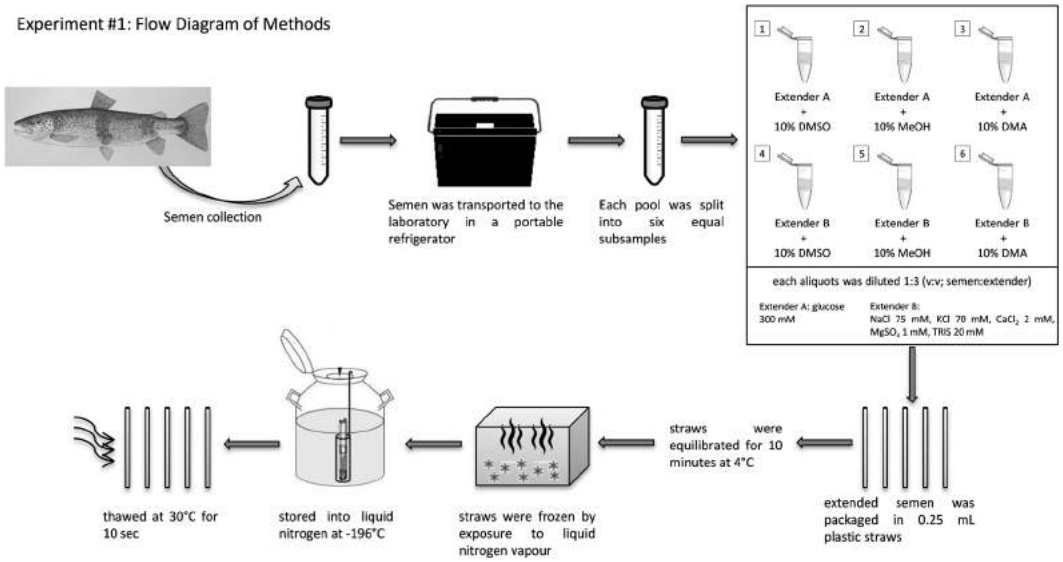

Figure 1. Flow diagram of Experiment #1 demonstrating the cryopreservation protocol used for the brown trout sperm cells as described in Experiment 1.

bio-security, protocols of sampling of fresh water and animal welfare. Gametes were transported in compliance with current national regulations (Legislative Decree 148/2008, D.L 3/08/2011).

Experiment 1. Effects of different extenders and cryoprotectants on post-thaw semen quality. The experiment was designed in a 2 × 3 factorial arrangement, in which one of the factors was the extender and the other factor was the cryoprotectant.

Extender preparation. Two basic extenders were employed: extender A containing 300 mM glucose[31] and extender B with 75 mM NaCl, 70 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 20 mM TRIS[32]. Each freezing extender contained a basic extender (A or B), +10% DMSO, DMA or MeOH as the P-CPA and 10% of egg yolk as the non-penetrating cryoprotectant (NP-CPA). We utilized the egg yolk because its combined use with P-CPA, for brown trout semen cryopreservation has been reported to be successful[20,25,33]. In total, 6 different freezing extenders (2 basic extenders × 3 penetrating cryoprotectants) were obtained.

Sperm cryopreservation. An aliquot taken from each pool was instantly used to assess fresh semen quality as described below. Each pool was split into six equal aliquots (0.4 mL), and each of them was diluted 1:3 (v:v; semen:extender) with the 6 different freezing extenders.

The extended semen was packaged in 0.25 mL plastic straws (IMV Technologies, L'Agile, France), which were later sealed with polyvinyl alcohol (PVA). In total 360 straws (5 straw for each treatment × 6 treatments × 12 pools) were used. The straws were then equilibrated for 10 minutes at 4 °C (equilibration time), and frozen by exposure to liquid nitrogen vapor at 5 cm above the liquid nitrogen surface for a period of 10 minutes. This method of exposure to liquid nitrogen vapour was previously shown to be appropriate[22]. After cryopreservation, the straws were plunged into liquid nitrogen for storage at −196 °C. Semen samples were later thawed in a water bath at 30 °C for 10 seconds (Fig. 1).

Sperm function. The following sperm quality parameters were evaluated in both fresh and thawed semen: sperm motility (%), spermatozoa movement duration (s), viability (%) and DNA integrity (%). Sperm concentration in fresh semen was also measured by a Neubauer chamber. The semen was diluted 1/1000 (v:v) with 3% NaCl (w:v) and sperm counts were performed in duplicate at a magnification of 400× and expressed as $\times 10^9$/mL.

The sperm motility of fresh semen was subjectively evaluated by placing an aliquot of semen (1 μL) on a glass microscope slide with 10 μL of an activation solution (0.3% NaCl). Observation was carried out at room temperature (15–20 °C) and sperm motility was expressed as the percentage of motile spermatozoa observed under 40× magnification. Sperm were defined as motile if they showed forward movements, whereas simply vibrating sperm were deemed immobile.

The motility parameters of cryopreserved semen were examined using a computer-aided sperm analysis system coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) employing the Sperm Class Analyzer (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain) with the Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Sperm were activated at a dilution of 1:10 with 1% $NaHCO_3$.

The following sperm motility parameters were evaluated: motile spermatozoa [MOT, (%)], curvilinear velocity [VCL, (μm/s)], straight-line velocity [VSL, (μm/s)], average path velocity [VAP, (μm/s)], amplitude of lateral head displacement [ALH, (μm)], beat cross frequency [BCF, (Hz)], linearity [LIN, (%)], straightness [STR, (%)] and wobble [WOB, (%)].

The duration of sperm movement (DSM) was measured using a sensitive chronometer as soon as the activation solution was added.

The integrity of plasma membrane was assessed using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Inc.) containing the fluorescent stains SYBR-14 and propidium iodide (PI). This procedure was performed on 1 μL of fresh or thawed semen, which were added to 40 μL of an immobilizing medium (80 mM NaCl, 40 mM KCl, 0.1 Mm $CaCl_2$, 30 mM Tris-HCl, pH 9.2) (v/v). A total of 2.5 μL of SYBR-14 working solution (50-fold dilution in distilled water of the stock solution-10-fold dilution in DMSO of the SYBR-14 commercial solution) were added to the cell suspension. After 10 minutes of incubation at room temperature in the dark, 3 μL of working PI solution (PI solution diluted 1:100 in phosphate buffered saline (PBS) diluent were added to the cell suspension. The spermatozoa were incubated for an additional 10 minutes under the same conditions. Ten microliters of this suspension was then placed on microscope slides, covered with coverslips and examined at a magnification ×1000 using a ×100 oil immersion objective under epifluorescence illumination. For each sample, approximately 200 spermatozoa were examined in duplicate aliquots. SYBR-14 is a membrane-permeant DNA stain, which only stains live spermatozoa producing a green fluorescence of the nuclei, while propidium iodide stains the nuclei of the membrane-damaged cells in red. Thus, spermatozoa showing green fluorescence are scored as alive and those showing red fluorescence as dead. The percentage of viable spermatozoa was calculated as the number of green cells ×100 divided by the total number of sperm counted.

Sperm DNA integrity was evaluated using acridine orange (AO) following the method described by Gandini *et al.*[34]. We adapted this test following the procedure used for rabbit semen in our previous paper[35,36]. AO is a cationic fluorescent cytochemical that stains cell nuclei, specifically DNA. Acridine orange fluoresces green when incorporated into native DNA (double-stranded and normal) as a monomer, and orange-red when it binds to denatured (single-stranded) DNA as an aggregate.

This test was performed on 1 μL of fresh or thawed semen, which was added to 40 μL of immobilizing medium (80 mM NaCl, 40 mM KCl, 0.1 mM $CaCl_2$, 30 mM Tris-HCl, pH 9.2) (v/v). Then, 10 μL of this solution was smeared onto a microscope slide, air-dried and fixed overnight in a 3:1 methanol:glacial acetic acid solution, and air-dried once more. The slides were washed with distilled water and the smears were stained with an AO solution (0.2 mg/mL in water) for 5 minutes in the dark at room temperature. Each smear was then washed with distilled water, mounted with a coverslip and examined using a fluorescence microscope with a 490 nm excitation light and a 530 nm barrier filter. Nuclei in at least 200 spermatozoa per slide were examined and scored as fluorescing green or yellow-orange-red (intact DNA or damaged DNA respectively) and the percentage of normal and abnormal chromatin condensation was also calculated.

Experiment 2. *In vivo* reproductive capacity of semen cryopreserved using the most effective P-CPA identified for each extender in Experiment 1. Based on the results obtained in (1), we compared *in vivo* semen cryopreserved using the most effective P-CPA identified (DMSO) for each extender (A and B) with fresh semen in an artificial fertilization trial. Fertilization was performed using 28 dry plastic dishes, and three treatment groups were created: (1) 4 dishes fertilized with fresh semen (control group); (2) 12 dishes fertilized with cryopreserved semen using extender A containing DMSO (group A); and (3) 12 dishes fertilized with semen frozen using extender B combined with DMSO (group B) (Fig. 2). Eggs were collected from seven females and mixed together. A total of 90 ± 16 eggs were placed on each dish, and 5 ml of D532 (20 mM Tris, 30 mM glycine, 125 mM NaCl, pH 9.0)[37] as a fertilization solution was subsequently added to the eggs in each of 28 dishes. The sperm was immediately added and the gametes were gently mixed for 10 seconds. For the control group, excess fresh semen was used at the beginning and end of the fertilization trials to test egg quality, while for the frozen treatment groups A and B, 0.25 mL (one straw containing approximately 0.6×10^9 spermatozoa) of thawed semen (30 °C for 10 seconds) was used for each dish. Then, approximately 20 mL of hatchery water was added to the control and frozen treatment groups. After 2 minutes, the eggs were rinsed with hatchery water and transferred to perforated baskets (diameter 6 cm), incubated in a longitudinal hatchery tank with running water at temperature of 9 °C. Unfertilized and dead eggs were continuously counted and removed. After 25–30 days, the eggs had reached the eyed-egg stage, and 45–50 days after fertilization, the embryos started to hatch. The fertilization success rate was established by calculating the percentage of embryos at both the eyed- and hatching-larvae stages. We calculated the percentage of eyed embryos and hatching larvae using the initial number of eggs calculated as the number of eyed eggs or hatchings larvae × initial egg number^{-1} × 100.

Statistical analysis. To compare the different treatments, we used a randomized block design in a 2 × 3 factorial arrangement (2 extenders × 3 P-CPAs), with 12 replicates per treatment. A generalized linear model (GLM) procedure was used to determine the fixed effects of the extender, the type of P-CPA and the effects of their interaction on the sperm quality variables. Sperm variables (motility percentage and duration, viability and DNA integrity) and fertilization and hatching measured among the different treatments were compared by ANOVA (analysis of variance) followed by Scheffe's comparison test. Significance was set at $P < 0.05$. All statistical tests were performed using the software package SPSS (SPSS 15.0 for Windows, 2006; SPSS, Chicago, Ill).

Ethics approval and consent to participate. The experiments were carried out in accordance with the guidelines of the current European Directive (2010/63/EU) on the care and protection of animals used for scientific purposes.

Results

Effects of different extenders and P-CPAs on post-thaw semen quality. Spermatozoa motility (%) and their duration (seconds) in fresh semen were 81.2 ± 5.7 and 46.3 ± 6.1, sperm viability and DNA integrity (%) were 83.8 ± 3.3 and 97.6 ± 1.2 respectively, and the average sperm concentration was $10.6 \pm 1.4 \times 10^9$ sperm/mL.

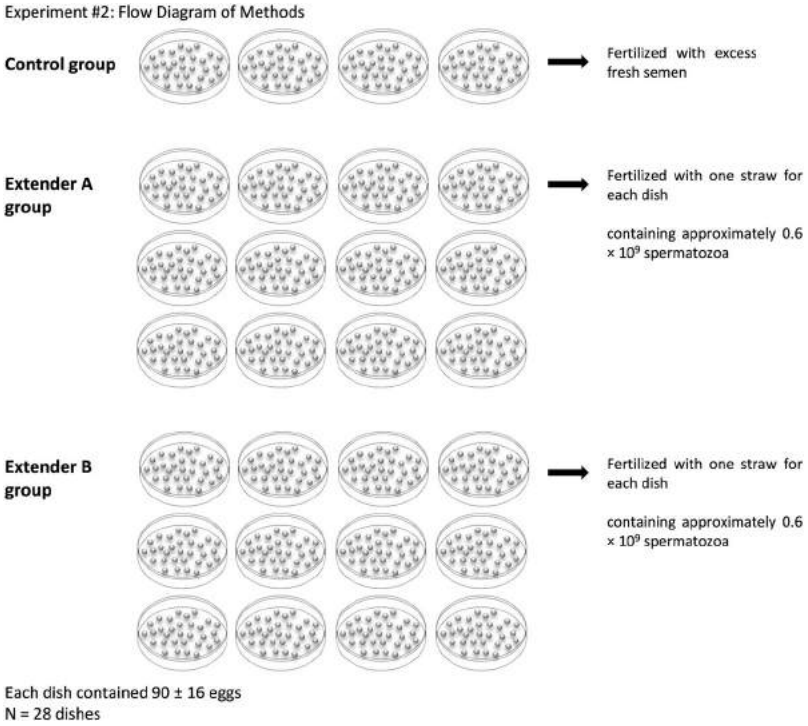

Figure 2. Flow diagram of the *in vivo* experiment (Experiment 2) to assess the reproductive capacity of cryopreserved semen.

Semen treatment		Sperm variable	
Extender	P-CPA	Viability (%)	DNA integrity (%)
A	DMSO	36.3 ± 1.1^{a}	97.3 ± 1.2^{a}
A	MeOH	22.3 ± 3.1^{b}	86.9 ± 0.4^{c}
A	DMA	22.1 ± 1.6^{b}	91.5 ± 0.8^{b}
B	DMSO	29.9 ± 1.9^{a}	97.1 ± 0.3^{a}
B	MeOH	13.3 ± 1.9^{c}	83.5 ± 0.9^{d}
B	DMA	17.9 ± 3.2^{bc}	89.9 ± 0.5^{b}
Extender effect		$P < 0.001$	$P < 0.001$
P-CPA effect		$P < 0.000$	$P < 0.000$
Extender × P-CPA effect		$P < 0.543$	$P < 0.082$

Table 1. Sperm viability and DNA integrity (mean ± SE) recorded for semen of trout from the Biferno River frozen with different extenders and P-CPAs (N = 12). [a–d]Different superscript letters within the same column indicate a significant difference ($P < 0.05$). Extender: A (glucose 300 mM); B (NaCl 75 mM, KCl 70 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1 mM, TRIS 20 mM) P-CPA: penetrating cryoprotectant; DMA: dimethylacetamide; DMSO: dimethylsulfoxide; MeOH: methanol

The fixed effects of the extender and type of P-CPA combination on sperm viability and DNA integrity are shown in Table 1. The data obtained indicate a significant effect of the extender and the P-CPA ($P < 0.05$) on both of these parameters, while no significant interaction effect was observed between the extender and the P-CPA.

Higher values of viability and DNA integrity were recorded in semen frozen in the presence of DMSO both in extender A and extender B ($P < 0.05$). Lower viability and DNA integrity values were found for extender B containing MeOH.

The motility parameters recorded in frozen/thawed semen are provided in Table 2. These data indicate a significant effect of the extender used ($P < 0.05$) on total motility, VCL, VSL, VAP, LIN and DSM. The type of P-CPA significantly affected total motility, VSL, VAP, LIN, STR, WOB and DSM, while the interaction of the extender*P-CPA affected the total motility, VCL, VSL, VAP, ALH and BCF ($P < 0.05$).

The best post-thaw total motility, VCL, VSL, VAP and DSM were recorded for semen cryopreserved with extender A/DMSO ($P < 0.05$).

For both extenders the lower values of total motility, VCL, VSL, LIN, STR and WOB were recorded in semen frozen/thawed in the presence of DMA.

Semen treatment		Sperm motility parameters									
Extender	P-CPA	Total Motility (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)	DSM (sec)
A	DMSO	42.6 ± 3.5^{a}	22.8 ± 0.5^{a}	10.2 ± 0.3^{a}	16.1 ± 0.4^{a}	44.7 ± 0.9^{a}	62.9 ± 0.6^{a}	70.9 ± 1.1^{a}	3.5 ± 0.1^{a}	2.3 ± 0.3^{a}	40.7 ± 2.1^{a}
A	MeOH	24.6 ± 2.9^{b}	19.2 ± 0.6^{b}	7.8 ± 4.5^{b}	13.1 ± 0.6^{b}	40.3 ± 1.6^{ab}	59.2 ± 1.1^{ab}	67.8 ± 1.5^{ab}	2.6 ± 0.4^{ab}	2.2 ± 0.5^{a}	29.3 ± 1.2^{b}
A	DMA	14.3 ± 2.4^{c}	18.9 ± 0.8^{b}	7.1 ± 0.6^{bc}	12.2 ± 0.9^{b}	36.9 ± 2.4^{bc}	58.1 ± 1.2^{b}	63.5 ± 3.6^{bc}	1.9 ± 0.6^{bc}	0.9 ± 0.3^{bc}	24.8 ± 1.7^{bc}
B	DMSO	20.9 ± 2.1^{bc}	19.7 ± 0.6^{b}	7.2 ± 0.3^{bc}	12.6 ± 0.5^{b}	42.9 ± 3.4^{a}	61.7 ± 2.9^{a}	67.2 ± 3.6^{ab}	0.9 ± 0.5^{c}	0.5 ± 0.2^{c}	30.6 ± 0.7^{b}
B	MeOH	18.4 ± 1.3^{bc}	16.5 ± 0.9^{c}	7.0 ± 0.5^{bc}	11.2 ± 0.9^{b}	36.6 ± 0.9^{bc}	56.9 ± 0.9^{b}	64.2 ± 1.3^{bc}	2.5 ± 0.5^{ab}	1.4 ± 0.5^{bc}	20.7 ± 2.2^{c}
B	DMA	17.5 ± 1.5^{bc}	19.5 ± 0.5^{b}	6.4 ± 0.3^{c}	11.6 ± 0.4^{b}	32.7 ± 1.4^{c}	54.7 ± 1.2^{b}	59.6 ± 1.7^{c}	2.8 ± 0.4^{ab}	1.7 ± 0.4^{ab}	21.4 ± 2.7^{c}
Extender effect		$P < 0.000$	$P < 0.002$	$P < 0.000$	$P < 0.000$	$P < 0.047$	$P < 0.196$	$P < 0.061$	$P < 0.088$	$P < 0.069$	$P < 0.000$
P-CPA effect		$P < 0.000$	$P < 0.817$	$P < 0.000$	$P < 0.029$	$P < 0.000$	$P < 0.000$	$P < 0.009$	$P < 0.749$	$P < 0.399$	$P < 0.000$
Extender × P-CPA effect		$P < 0.000$	$P < 0.000$	$P < 0.006$	$P < 0.001$	$P < 0.841$	$P < 0.386$	$P < 0.999$	$P < 0.001$	$P < 0.004$	$P < 0.196$

Table 2. CASA parameters and duration of sperm movement (mean ± SE) recorded for semen of trout of the Biferno River frozen in the presence of different extenders and P-CPAs (N = 12). [a–c]Different superscript letters within the same column indicate a significant difference ($P < 0.05$). Extender: A (glucose 300 mM); B (NaCl 75 mM, KCl 70 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1 mM, TRIS 20 mM). P-CPA: cryoprotectant; DMA: dimethylacetamide; DMSO: dimethylsulfoxide; MeOH: methanol. Total motility: the percentage of motile spermatozoa; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: linearity (VSL/VCL × 100); STR: straightness (VSL/VAP × 100), WOB: wobble (VAP/VCL × 100) and DSM: duration of sperm movement.

	Semen treatment		
		Frozen	
	Fresh	Extender A	Extender B
Eyed eggs (%)	83.7 ± 1.2^{a}	36.5 ± 5.5^{b}	27.8 ± 4.2^{b}
Hatching rate (%)	75.5 ± 1.6^{a}	32.5 ± 4.9^{b}	23.1 ± 3.5^{b}

Table 3. Fertilization ability of fresh semen or semen frozen in the presence of extender A or B combined with DMSO. Values with different superscript letters within treatments of the same row are significantly different ($P < 0.05$). Extender: A (glucose 300 mM); B (NaCl 75 mM, KCl 70 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1 mM, TRIS 20 mM); DMSO: dimethylsulfoxide.

Based on these findings, extenders A and B combined with DMSO were used in the *in vivo* artificial fertilization trial as the most effective treatments.

Fertilization ability of cryopreserved semen. The percentage of eyed eggs and hatching rates recorded for cryopreserved and fresh semen are provided in Table 3.

The percentage of eyed and hatched eggs was significantly higher in fresh semen compared to frozen semen. No significant differences emerged when we compared the frozen semen using extender A or B, although, the higher percentages of eyed eggs and hatching rates were recorded in eggs fertilized with extender A.

Discussion

Obtaining effective semen cryopreservation protocols is an important goal because sperm cryopreservation has several advantages for biodiversity conservation, such as minimizing inbreeding and reducing domestication selection. Fish semen cryopreservation is currently the only technology available to develop *ex situ* conservation programmes because oocyte and embryos cryopreservation remain unsatisfactory. Importantly, sperm cryopreservation techniques have been developed for a wide variety of endangered salmonids[2] but seldom used for *S. macrostigma*.

We recently studied the optimal freezing rate for the semen cryopreservation procedure for the Mediterranean brown trout of the Biferno River[22]. The freezing rate is only one of the steps of the semen cryopreservation procedure, and further improvements of other steps of the freezing protocol are necessary. This is even more important considering that sperm cryotolerance could vary among native trout populations.

This study sought to identify the most effective basic extender and P-CPA as well as the best combination between the type of extender and P-CPA for semen cryopreservation in wild specimens of the Mediterranean brown trout (*Salmo cettii*) population of the Molise river (Italy). In this regard, some researchers have indeed shown that the effectiveness of sperm cryopreservation may also depend on the interaction between the type of extender and cryoprotectant employed[25,38].

Effects of different extenders and P-CPAs on *in vitro* post-thaw semen quality. Our *in vitro* results clearly revealed a significant effect of the extender and P-CPA on the quality of the cryopreserved semen. In fact, extender A combined with DMSO produced an overall high post-thaw semen quality compared with all

the other combinations. Likewise, extender B combined with DMSO showed higher post-thawing quality compared with MeOH and DMA.

As reported in the literature, the choice of a suitable extender and P-CPA is key to successful cryopreservation of salmonid sperm[23].

Here, the choice of the extenders was addressed by previous research that reported a better post thawing *in vitro* semen quality with a mineral-based extender[32] and glucose-based extender[31]. Our results also revealed that the glucose-based extender (extender A) provided better *in vitro* conditions than the mineral-based extender (extender B) to preserve sperm integrity (viability and DNA) and function (motility) during the freezing/thawing procedure. This is consistent with the finding of Bozkurt and Yavas[23] reporting that carbohydrate-based extenders are preferred with respect to other extenders. These authors established that the glucose-based extender provided higher post-thaw motility and duration with respect to the mineral extender (Lahnsteiner extender). Specifically, glucose-based extenders have been used for the cryopreservation of rainbow trout[31] and brown trout sperm[23,25] with satisfactory results. Hence, the success of extender A could be explained by the ability of glucose to protect the sperm from osmolality damage as reported by Leung and Jamieson[39]; Maisse[40] also showed that the efficacy of sugars as extenders can be explained by their role as external CPA and membrane stabilizers. In this regard, various authors have recently shown the positive effects of glucose and trehalose as external CPAs in semen cryopreservation protocols of rainbow and brown trouts[33,41–43].

Another interesting point emerging from this research is that DMSO provided better post-thaw sperm quality than DMA and MeOH for both tested extenders. The CPA molecules used here are classified as permeable CPAs, and their mechanism of action is the same; therefore, the reason why DMSO provided better results is the object of our speculation. P-CPAs are membrane-permeable solutes that act intra- and extracellularly, causing the dehydration of spermatozoa because of an osmotically driven flow of water, which varies according to CPA composition[44–46]. Spermatozoa are normally equilibrated in a P-CPA, preventing cells from undergoing intracellular ice-crystal formation, which is mainly responsible for cell damage affecting the plasma membrane, mitochondria and chromatin structure[47].

Penetrating CPAs also cause membrane lipid and protein reorganization. This improves membrane fluidity, causing greater dehydration at lower temperatures, and thus an increased ability to survive cryopreservation[48]. In light of these considerations, DMSO was associated with less physical-functional injuries to the sperm and was better than DMA and MeOH at preserving the post-thaw semen quality of *Salmo cettii* under our experimental conditions. This leads us to hypothesize that although the P-CPA molecules act in the same way, they have different chemical-physical properties, specifically in terms of molecular weight (DMSO 78.13, DMA 87.12 and methanol 32.04 gmol-1) and chemical functional groups (DMA-amide groups, DMSO-hydrophilic sulfoxide group and MeOH-alcoholic group). These properties are likely to confer upon the compounds a different degree of permeability in a given phospholipid bilayer and lesser or greater cellular toxicity. In turn, this might lead to variations in the relative cryoprotection efficiency of these CPAs for *S. cettii* sperm. Our results depict a clear scenario in which, because of its lower molecular weight compared to other cryoprotectants (DMSO and DMA), MeOH is highly permeable to cell membranes but, on the other hand, is also more toxic[49], while DMSO is more permeable to sperm membranes than DMA and less toxic than the other compounds. In this regard, this notion is also substantiated by Noble[49], who reported DMSO to be the most widely used P-CPA because it showed the right compromise between its membrane permeability and toxicity.

A variety of P-CPAs, such as DMSO, DMA, MeOH, glycerol and ethylene glycol, have been tested for the cryopreservation of brown trout[11,13,14,23,25,50] and rainbow trout semen[10,15,41,51]. In general, DMSO and MeOH at different concentrations were mainly used as P-CPAs in freezing protocols for trout semen, whereas little is known about the usage of DMA. In particular, Ciereszko *et al.*[41,52] and Dietrich *et al.*[53] reported that MeOH is a suitable P-CPA for sperm cryopreservation of rainbow trout, while DMSO is widely used for brown trout[23]. However, in some papers, MeOH has also been successfully used in brown trout cryopreservation[13,20].

Fertilization capacity of cryopreserved semen. Interestingly, our *in vivo* study reported no significant differences in the number of eyed eggs and hatching rates between the considered extenders, although these fertilization parameters were notably higher (approximately 10% more) for extender A. The fertilization ability found here was higher than that observed in our previous paper[22] due to the different sperm-to-egg ratios applied. In this regard, we used a ratio of approximately 6×10^6 sperm/egg, while in our previous research, a ratio approximately 10 times lower (0.5×10^6 sperm/egg) was applied.

Therefore, we think that the sperm-to-egg ratio used here is more appropriate for artificial fertilization in the Mediterranean brown trout of the Biferno River as a result of a higher concentration of viable and motile spermatozoa suitable for each egg. This aspect emphasizes the importance of the choice of the optimal spermatozoa/egg ratio to determine the fertilization capacity of thawed sperm, as reported by different authors[20,23,43]. In addition, the sperm-to-egg ratio used here was similar to that reported in brown trout by Sarvi *et al.*[50] with 6.2×10^6 sperm/egg, while 4×10^6 sperm/egg was chosen by Dziewulska and Domagała[20].

In vivo results were similar to those recorded in brown trout by Labbé and Maisse[16] and Bozkurt and Yavas[23] and were lower than those reported by other authors[13,20,50,54].

The literature reports how the variability in the biological material and the use of multiple preservation procedures have made it impossible to reproduce either the quality or the fertilizing capacity of cryopreserved semen[12,14]. In addition, susceptibility to semen cryopreservation varies among fish species[12], within subpopulations[14]. This notion is substantiated by Martínez-Páramo *et al.*[14], who observed different fertilization rates using cryopreserved semen from two brown trout subpopulations inhabiting different rivers in the same basin. This means that the sperm of different populations belonging to the same fish species have different biological characteristics and consequently dissimilar cryoresilience, so an individualized semen cryopreservation protocol is also required.

Thus, the present study showed that an extender composed of 300 mM glucose combined with 10% DMSO and 10% egg yolk resulted in remarkably high post-thaw quality *in vitro* and in a better fertilizing ability in semen of the Mediterranean brown trout of the Molise rivers. Therefore, the achievement of an effective semen cryopreservation protocol for *S. cettii* will contribute to the creation of a sperm cryobank that is an important tool for the conservation of the biodiversity of this Molise native trout.

Conclusions

Our results identified the glucose-based extender and DMSO as the best combination for an effective cryopreservation protocol for the native trout of the Molise rivers. However, further studies are needed to improve the semen freezing protocols for this trout by studying the NP-CPA, equilibration time, thawing rate and cryopreserved sperm-to-egg ratio.

Our findings are important because they will allow the creation of a sperm cryobank that is key to the conservation and restoration of the native population of the Mediterranean brown trout (*Salmo cettii*) in Molise rivers. The use of cryopreserved semen in artificial fertilization protocols represents a valuable tool to maintain genetic diversity and fitness within self-sustaining populations. Furthermore, the creation of the first sperm cryobank of pure *Salmo cettii* populations with high genetic variability will be useful not only for Molise river basins but also for other Italian basins where this species is at risk of extinction.

Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

References

1. Fickel, J., Wagener, A. & Ludwig, A. Semen cryopreservation and the conservation of endangered species. *Eur. J Wildlife Res.* **53**, 81–89 (2007).
2. Martinez-Paramo, S. *et al*. Cryobanking of aquatic species. *Aquaculture* **472**, 156–177 (2017).
3. Dietrich, A. M. & Ciereszko, A. Proteomic characterization of fresh spermatozoa and supernatant after cryopreservation in relation to freezability of carp (*Cyprinus carpio L*) semen. *PLoS One* **13**(3), e0192972 (2018).
4. Hu, E., Yang, H. & Tiersch, T. R. High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): establishment of an approach for commercial-scale processing. *Cryobiology* **62**, 74–82 (2011).
5. Robles, V., Cabrita, E., Cuñado, S. & Herráez, M. P. Sperm cryopreservation of sex-reversed rainbow trout (*Oncorhynchus mykiss*): parameters that affect its ability for freezing. *Aquaculture* **224**, 203–212 (2003).
6. Chenais, N., Depince, A., Le Bail, P. Y. & Labbe, C. Fin cell cryopreservation and fish reconstruction by nuclear transfer stand as promising technologies for preservation of finfish genetic resources. *Aquac Int.* **22**, 63–76 (2014).
7. Zhang, T. & Rawson, D. M. Permeability of dechorionated one-cell and six-somite stage zebrafish (*Brachydanio rerio*) embryos to water and methanol. *Cryobiology* **37**, 13–21 (1998).
8. Zhang, T., Liu, X.-H. & Rawson, D. M. Effects of methanol and developmental arrest on chilling injury in zebrafish (*Danio rerio*) embryos. *Theriogenology* **59**, 1545–56 (2003).
9. Robles, V. *et al*. Biology of teleost primordial germ cells (PGCs) and spermatogonia: biotechnological applications. *Aquaculture.* **472**, 4–20 (2007).
10. Bozkurt, Y., Akçay, E., Tekin, N. & Seçer, S. Effect of freezing techniques, extenders and cryoprotectants on the fertilization rate of frozen rainbow trout (*Oncorhynchus mykiss*) sperm. *Isr. J Aquacult-Bamid.* **57**, 125–130 (2005).
11. Bozkurt, Y., Yavas, I. & Karaca, F. Cryopreservation of Brown Trout (*Salmo trutta macrostigma*) and Ornamental Koi Carp (*Cyprinus carpio*) sperm. In: Katkov I, editor. Current Frontiers in Cryopreservation. Croatia: InTech. p. 29 – 304 (2012).
12. Cabrita, E. *et al*. Cryopreservation of fish sperm: applications and perspectives. *J. Appl. Ichthyol.* **26**, 623–635 (2010).
13. Nynca, J. *et al*. Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen. *Aquaculture* **433**, 62–65 (2014).
14. Martínez-Páramo, S. *et al*. Cryobanking as tool for conservation of biodiversity: effect of brown trout sperm cryopreservation on the male genetic potential. *Theriogenology* **71**, 594–604 (2009).
15. Babiak, I. *et al*. Effect of extender composition and equilibration time on fertilization ability and enzymatic activity of rainbow trout cryopreserved spermatozoa. *Theriogenology* **56**, 177–192 (2001).
16. Labbé, C. & Maisse, G. Characteristics and freezing tolerance of brown trout spermatozoa according to rearing water salinity. *Aquaculture* **201**, 287–299 (2001).
17. Müller, K. *et al*. Characterization of sperm plasma membrane properties after cholesterol modification: consequences for cryopreservation of rainbow trout spermatozoa. *Biol. Reprod.* **78**, 390–399 (2008).
18. Cabrita, E., Alvarez, R., Anel, L. & Herráez, M. P. The hypoosmotic swelling test performed with coulter counter: A method to assay functional integrity of sperm membrane in rainbow trout. *Anim. Reprod. Sci.* **55**, 279–287 (1999).
19. Lahnsteiner, F., Mansour, N. & Kunz, F. A. The effect of antioxidants on the quality of cryopreserved semen in two salmonid fish, the brook trout (*Salvelinus fontinalis*) and the rainbow trout (*Oncorhynchus mykiss*). *Theriogenology.* **76**, 882–890 (2011).
20. Dziewulska, K. & Domagała, J. Effect of pH and cation concentrations on spermatozoan motility of sea trout (*Salmo trutta m. trutta L.*). *Theriogenology* **79**, 48–58 (2013).
21. Mansour, N., Richardson, G. F. & McNiven, M. A. Effect of extender composition and freezing rate on post-thaw motility and fertility of Arctic char, *Salvelinus alpinus* (*L.*), spermatozoa. *Aquacult Res.* **37**, 862–868 (2006).
22. Iaffaldano, N. *et al*. Effective freezing rate for semen cryopreservation in endangered Mediterranean brown trout (*Salmo trutta macrostigma*) inhabiting the Biferno river (South Italy). *Zygote* **24**, 668–675 (2016a).
23. Bozkurt, Y. & Yavas, I. Preliminary study on hybridization of brown trout (*Salmo trutta macrostigma*) and rainbow trout (*Oncorhynchus mykiss*) using cryopreserved sperm. *Isr J Aquacult-Bamid Isr.* **66**, 1–6 (2014).
24. Ciereszko, A. & Dabrowski, K. Effect of a sucrose-DMSO extender supplement with pentoxifylline or blood plasma on fertilizing ability of cryopreserved rainbow trout spermatozoa. *Prog Fish-Cult.* **58**, 143–145 (1996).
25. Bozkurt, Y., Yavas, I. & Karaca, F. Effect of extender co mposition and cryoprotectants on post-thaw motility of brown trout (*salmo trutta macrostigma)* spermatozoa. In: V international conference "aquaculture & fishery" Belgrad, Serbia (2011).
26. Bianco, P. G. *et al*. Pesci d'acqua dolce. In: Rondinini, C, Battistoni, A, Peronace, V, Teofili, C, editors. Lista Rossa IUCN dei Vertebrati Italiani. Comitato Italiano IUCN e Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Roma; p. 54 (2013).
27. Iaffaldano, N. *et al*. Semen quality of threatened native population of Mediterranean brown trout (*Salmo cettii*, Rafinesque 1810) in the Biferno river (Molise region - South Italy). *Turk. J Fish. Aquat. Sc.* **16**, 259–266 (2016b).
28. Gibertoni, P. P., Jelli, F. & Bracchi, P. Allevamento, riproduzione e reintroduzione in ambiente naturale di trote fario di "ceppo mediterraneo", *Salmo (trutta) trutta*, *L.* Annali della Facoltà di Medicina Veterinaria, XVIII, Università di Parma; P. 1–20 (1998).

29. Jelli, F. & Gibertoni, P. P. Recupero e reintroduzione di ceppi autoctoni di trota fario, *Salmo (trutta) trutta* L., nel bacino del fiume Secchia. Atti del Convegno: Recupero e reintroduzione dei ceppi autoctoni di trota fario, *Salmo (trutta) trutta* L., di "ceppo mediterraneo" in ambienti appenninici tipici. Esperienze a confronto. Reggio Emilia (1999).
30. Penserini, M. *et al*. Fenotipi della trota mediterranea: metodologia di indagine molecolare combinata e selezione morfologica per l'identificazione degli esemplari autoctoni. *J Freshwat. Biol.* **34**, 69–75 (2006).
31. Tekin, N., Seçer, S., Akçay, E. & Bozkurt, Y. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) semen. *Isr J Aquacult-Bamid.* **55**, 208–212 (2003).
32. Lahnsteiner, F., Berger, B. & Weismann, T. Effects of media fertilization technique extender straw volume and sperm to egg ratio on hatchability of cyprinid embryos using cryopreserved semen. *Theriogenology* **60**, 829–841 (2003).
33. Domagala, J., Dziewulska, K. & Czerniawski, R. Survival rate and growth in the wild of sea trout (*Salmo trutta L.*) fry obtained using frozen semen. *Arch. Pol. Fish.* **22**, 265–70 (2014).
34. Gandini, L. *et al*. Cryopreservation and sperm DNA integrity. *Cell Tissue Bank.* **7**, 91–98 (2006).
35. Iaffaldano, N., Di Iorio, M. & Rosato, M. P. The cryoprotectant used, its concentration and the equilibrium time are critical for the successful cryopreservation of rabbit sperm: Dimethylacetamide versus dimethylsulfoxide. *Theriogenology.* **78**, 1381–1389 (2012).
36. Iaffaldano, N., Di Iorio, M., Rosato, M. P. & Manchisi, A. Cryopreservation of rabbit semen using non-permeablecryoprotectants: Effectiveness of different concentrations oflow-density lipoproteins (LDL) from egg yolk versus egg yolkor sucrose. *Anim. Reprod. Sci.* **151**, 220–228 (2014).
37. Billard, R. Reproduction in rainbow trout: sex differentiation, dynamics of gametogenesis, biology and preservation of gametes. *Aquaculture* **100**, 263–298 (1992).
38. Tekin, N., Secer, S., Akcay, E., Bozkurt, Y. & Kayam, S. Effects of glycerol additions on post-thaw fertility of frozen rainbow trout sperm, with an emphasis on interaction between extender and cryoprotectant. *J. Appl. Ichthyol.* **23**, 60–63 (2007).
39. Leung, L. K. P. & Jamieson, B. G. M. Live preservation of fish gametes. In: Jamieson BGM, editor. Fish Evolution and Systematics: Evidence from Spermatozoa. Cambridge University Pres; p. 245–295 (1991).
40. Maisse, G. Cryopreservation of fish semen: a review. Proceedings of the Refrigeration Science and Technology Conference, Refrigeration and Aquaculture. Institut International du Froid, Paris, France; p. 443–467 (1996).
41. Ciereszko, A., Dietrich, G. J., Nynca, J., Dobosz, S. & Zalewski, T. Cryopreservation of rainbow trout semen using a glucose–methanol extender. *Aquaculture* **420**, 275–281 (2014).
42. Judycka, S. *et al*. The effect of supplementation of a trehalose-based extender with KCl on rainbow trout (*Oncorhynchus mykiss*) sperm freezability and post-thaw motility. *Aquaculture* **465**, 303–310 (2016).
43. Judycka, S. *et al*. Optimal sperm concentration in straws and final glucose concentration in extender are crucial for improving the cryopreservation protocol of salmonid spermatozoa. *Aquaculture* **486**, 90–97 (2018).
44. Curry, M. R. Cryopreservation of semen from domestic livestock. *Rev. Reprod.* **5**, 46–52 (2000).
45. Purdy, P. H. A review on goat sperm cryopreservation. *Small Rumin. Res.* **6**, 215–225 (2006).
46. Elliott, G. D., Wang, S. & Fuller, B. J. Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. *Cryobiology* **76**, 74–91 (2017).
47. Sieme, H., Oldenhof, H. & Wolkers, W. F. Mode of action of cryoprotectants for sperm preservation. *Anim. Reprod. Sci.* **169**, 2–5 (2016).
48. Holt, W. V. Basic aspects of frozen storage of semen. *Anim. Reprod. Sci.* **62**, 3–22 (2000).
49. Noble, D. Cryopreservation of marine fish gametes for mariculture applications. Recent advance in mariculture genetics and biotechnology. 4th to 24th November 2003.
50. Sarvi, K. *et al*. Nikṣirat H, Amiri BM, Mirtorabi SM, Rafiee GR, Bakhtiyari M. Cryopreservation of semen from the endangered Caspian brown trout (*Salmo trutta caspius*). *Aquaculture* **256**, 564–569 (2006).
51. McNiven, M. A., Gallant, R. K. & Richardson, G. F. Dimethyl-acetamide as a cryoprotectant for rainbow trout spermatozoa. *Theriogenology* **40**, 943–948 (1993).
52. Ciereszko, A., Dietrich, G. J., Nynca, J., Krom, J. & Dobosz, S. Semen from sex-reversed rainbow trout of spring strain can be successfully cryopreserved and used for fertilization of elevated number of eggs. *Aquaculture* **448**, 564–568 (2015).
53. Dietrich, G. J., Nynca, J., Dobosz, S., Zalewski, T. & Ciereszko, A. Application of glucose-methanol extender to cryopreservation of semen of sex-reversed females rainbow trout results in high post-thaw sperm motility and fertilizing ability. *Aquaculture* **434**, 27–32 (2014).
54. Golshahi, K., Shabani, N., Aramli, M. S. & Noori, E. Motility and fertilizing ability of cryopreserved Caspian brown trout (*Salmo trutta caspius*) sperm: Effect of post-thaw storage time and different sperm-to-egg ratios. *Cryobiology* **71**, 360–363 (2015).

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Author Contributions

N.I., M.D.I., S.E., A.R. and S.C. conceived and designed the experiments. S.E., G.R., M.M., M.D.I. and P.P.G. carried out the evaluation of semen quality *in vitro* and *in vivo*. N.I., S.E. and M.D.I. performed the statistical analysis of the data. M.D.I., N.I., S.E. and S.C. wrote the manuscript. A.R., S.C. and P.P.G. contributed to revisiting and reviewing the manuscript. M.D.I., S.E., N.I. and G.R. prepared Figures 1 and 2. All authors read and approved the final manuscript.

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Optimization of Sperm Cryopreservation Protocol for Mediterranean Brown Trout: A Comparative Study of Non-Permeating Cryoprotectants and Thawing Rates In Vitro and In Vivo

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Simple Summary: Cryobanking is an important tool to preserve the genetic resources of fish species. Semen cryopreservation has been extensively used in conservation programs for endangered species. Here, we aimed to find an effective cryopreservation protocol for the autochthonous Mediterranean brown trout inhabiting the Biferno river (south Italy), in order to create a sperm cryobank. Low-density lipoproteins and sucrose were tested as non-permeating cryoprotectants (NP-CPAs) to replace the egg yolk. Moreover, the thawing rate (10 °C for 30 s vs. 30 °C for 10 s) was also studied. From results obtained in vitro and in vivo, egg yolk emerged as the best NP-CPA and the lower thawing rate recorded better post-thaw semen quality in vitro and higher fertilization and hatching rates in vivo. These findings are important because they will contribute to the creation of a sperm cryobank for Molise's native trout, which is a milestone of our European project (Life Nat.Sal.Mo).

Abstract: The aim of our study was to test the effects of different non-permeating cryoprotectants (NP-CPAs), namely low-density lipoproteins (LDLs), sucrose, and egg yolk, and thawing rates on the post-thaw semen quality and fertilizing ability of the native Mediterranean brown trout. Pooled semen samples were diluted 1:3 (v:v) with 2.5%, 5%, 10%, or 15% LDL; 0.05, 0.1, or 0.3 M sucrose; or 10% egg yolk. At the moment of analysis, semen was thawed at 30 °C/10 s or 10 °C/30 s. The post-thaw semen quality was evaluated, considering motility, the duration of motility, viability, and DNA integrity. Significantly higher values of motility and viability were obtained using egg yolk/10 °C for 30 s, across all treatments. However, LDL and sucrose concentrations affected sperm cryosurvival, showing the highest post-thaw sperm quality at 5% LDL and 0.1 M sucrose. Based on the in vitro data, egg yolk, 5% LDL, and 0.1 M sucrose thawed at 10 °C or 30 °C were tested for the in vivo trial. The highest fertilization and hatching rates were recorded using egg yolk/10 °C ($p < 0.05$). According to these in vitro and in vivo results, egg yolk emerged as the most suitable NP-CPA and 10 °C/30 s as the best thawing rate for the cryopreservation of this trout sperm, under our experimental conditions.

Keywords: *Salmo cettii*; sperm freezing; egg yolk; conservation biology; sperm cryobank; fertilization rate

1. Introduction

Sperm cryopreservation is considered a valuable tool for preserving the genetic material of endangered fish species by storage of their gametes in a cryobank [1–3]. The semen cryobanks provide the opportunity to preserve representative samples and further reconstruct the original strain, population, or diversity [2,4]. In this regard, we attempted to find an effective semen cryopreservation protocol for the Mediterranean brown trout (*Salmo cettii*), a native species that inhabits the rivers of Molise [5]. Currently, this species is listed in the Italian IUNC Red List as critically endangered [6], due to river pollution, uncontrolled fishing, and hybridization following the introduction of non-native strains that have drastically reduced the number of the native species in recent centuries [7].

In our previous works [5,8], the effects of different freezing rates, types of basic extenders, and penetrating cryoprotectants (P-CPAs) were studied in vitro and in vivo. For our native trout, an adequate amount of satisfactory results had been obtained when the semen was frozen in the presence of a glucose-DMSO extender at 5 cm above the liquid nitrogen surface, for 10 min. However, further factors still need to be tweaked to improve the fertilizing ability of cryopreserved semen, in order to achieve satisfactory in vivo results that are closer to those of fresh semen.

In this regard, the optimization of temperature and thawing rate are very important tools to improve the cryopreservation protocol, because thawing rate is a critical factor in preserving the survival of the spermatozoa [9]. Nevertheless, there are few available data present in the literature regarding the thawing conditions for brown trout semen cryopreservation.

Another important factor is finding the best combination between non-permeating cryoprotectant (NP-CPA) and P-CPA in the freezing extender, which can result in a decrease in sperm damages caused by intra and extracellular ice formation, and also improve the egg fertilization rate [10,11]. Egg yolk has been used as an NP-CPA in our previous studies [5,8]. However, in recent years, there have been increasing demands to replace whole egg yolks in semen extenders for two main reasons; one being the presence of substances in the egg yolk that could inhibit the sperm respiration [12–14]; the second being the sanitary and practical disadvantages that could occur from its use [15]. In this regard, both low-density lipoproteins (LDLs) extracted from egg yolk and sucrose as alternative molecules have been considered in this paper. Several studies about the use of LDL [12,16] and sucrose [17,18] in the semen freezing protocol in mammals, in order to improve the post-thaw semen quality, have been published.

On the contrary, there are only a few reports in which the effects of an isolated fraction of LDL [19,20] and sucrose [21,22] were tested as NP-CPAs on the post-thaw sperm quality of rainbow trout. Therefore, it is completely unknown whether replacing egg yolk with LDL or sucrose in a glucose-DMSO extender can improve the freezing protocol of Mediterranean brown trout.

In light of all these considerations, in order to optimize the cryopreservation protocol of that Mediterranean brown trout that we have tested, for the first time, the effects of: (1) LDL and sucrose as NP-CPAs at different concentrations as alternatives to the egg yolk, and (2) two different thawing temperatures on motility, viability, DNA integrity, and the fertilizing capacity of Mediterranean brown trout spermatozoa (*Salmo Cettii*). Obtaining an effective semen freezing protocol represents an important milestone within our financed "LIFE" project, aiming to establish the first sperm cryobank needed for the conservation and restock of the native population of Mediterranean brown trout in the Molise River.

2. Materials and Methods

2.1. Chemicals

A LIVE/DEAD Sperm Viability Kit was obtained from Molecular Probes, Inc. (Eugene, OR, USA), and all other chemicals used in this study were purchased from Sigma, Chemical Co. (Milan, Italy).

2.2. Animals

Specimens of *S. cettii* were caught from the Biferno river in the Molise region, during spawning season (January–February 2017), by electro-fishing. Forty-seven native Mediterranean brown trout fish were identified according to their phenotypic features [23–25]. These individuals (40 males and 7 females) were aged at 2+ to 5+ years.

2.3. Semen and Egg Collection

Trout semen and eggs were gathered during spawning season. To collect the sperm, the abdomens and urogenital papilla of the fish were carefully dried before stripping to avoid contamination with urine, mucus, and blood cells. The semen of 40 males was obtained through a gentle abdominal massage. Each male was stripped only once, and the total amount of expressible milt was collected individually.

Following sperm collection in the river, the tubes containing sperm were transferred to the laboratory in a portable refrigerator at 4 °C. Only spermatozoa that showed a motility rate higher than 75% were used for experimentation.

Eggs were collected as follows: 7 mature females were wiped dry, stripped by gentle abdominal massage, and the eggs from each female were collected in a dry metal bowl. Eggs were checked visually to ensure that the those used in the fertilization experiments were well-rounded and transparent.

2.4. Experiment 1. Effects of Different Non-Permeable Cryoprotectants and Two Thawing Rates on Post-Thaw Semen Quality In Vitro

2.4.1. Extender Preparation

A basic freezing extender prepared at our laboratory was used. This extender was composed of 0.3 M glucose containing 10% DMSO (v:v) as a P-CPA. A quantity of 2.5%, 5%, 10%, or 15% (w:v) of LDL was added to this extender, obtained using the protocol described by Moussa et al. [12], and 0.05, 0.1, or 0.3 M of sucrose, or 10% whole egg yolk as NP-CPAs. In total, 8 different freezing extenders were obtained.

2.4.2. Sperm Cryopreservation Protocol

In total, 5 pools were prepared and kept at 4 °C before cryopreservation. For each semen pool (about 4 mL), five ejaculates were mixed in equal ratios to exclude interactions due to individual differences in semen quality.

A semen aliquot taken from each pool was promptly used to assess fresh semen quality as described below. Each pool was split into eight subsamples (0.4 mL), and each of them was diluted 1:3 (v:v; semen:extender), with a different freezing extender for each one.

The diluted semen was loaded in 0.25 mL plastic straws, which were sealed with polyvinyl alcohol (PVA). In total, 240 straws were used (6 straws for each treatment × 8 treatments × 5 replicates). Subsequently, the straws were equilibrated for 10 min at 5 °C (equilibration phase), and frozen by exposure to liquid nitrogen vapor at 5 cm above the liquid nitrogen level for a period of 10 min. These heights in relation to liquid nitrogen vapor resulted in being the most appropriate in our previous paper [5]. At the end of the cryopreservation process, the straws were submerged into liquid nitrogen at −196 °C, where they were stored until analysis.

Finally, before the analysis, the straws were thawed by immersion in a water bath at two different thawing rates, namely, 30 °C for 10 s, as already used in our previous works [5,8] and 10 °C for 30 s according to Bozkurt et al. [3]. This last rate was chosen in order to evaluate the effect of natural river water temperature, during the time of spawning season, on the post-thaw sperm quality of Mediterranean brown trout. For each thawing condition, 80 straws were thawed (2 straws for each thawing rate × 8 treatments × 5 replicates) and the analysis, as described below, was carried out on each straw.

2.4.3. Sperm Quality

The sperm quality parameters evaluated in both fresh and thawed semen were sperm motility (%), spermatozoa movement duration (s), viability (%), and DNA integrity (%). Moreover, the fresh semen concentration was also measured. For frozen semen, the analyses were carried out in duplicate, thawing 2 straws for each condition.

Sperm concentration was measured by using a Neubauer chamber. The semen was extended 1/1000 (v:v) with 3% NaCl (w:v), and sperm counts were carried out in duplicate, at a magnification of 400× and expressed as $\times 10^9$/mL. Sperm motility was subjectively evaluated as reported in our previous paper [5,7]. Briefly, 1 µL of semen was placed on a glass microscope slide, and 10 µL of 0.3% NaCl or 1% $NaHCO_3$ as an activation solution was added for fresh semen and frozen semen, respectively. The duration of sperm movement was evaluated using a chronometer.

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Inc.), which contained the fluorescent stains SYBR-14 and propidium iodide (PI), following the same procedure described in our previous works [5,7].

Sperm DNA integrity was assessed using acridine orange (AO) as described by Gandini et al. [26]. We adapted this test following the procedure used for rabbit semen [18,27]. Specifically, 1 µL of fresh or thawed trout semen was extended with 40 µL of immobilizing medium (80 mM NaCl, 40 mM KCl, 0.1 mM $CaCl_2$, 30 mM Tris-HCl, pH 9.2) (v/v). Then, 10 µL was smeared onto a microscope slide and fixed for at least 12 h in a 3:1 methanol:glacial acetic acid solution. Smears were then stained with an AO solution (0.2 mg/mL in water) in the dark at room temperature for 5 min; subsequently, 200 spermatozoa per slide were counted and scored as possessing green or yellow-orange-red fluorescence (intact DNA or damaged DNA, respectively), and the percentage of DNA integrity was calculated.

2.5. Experiment 2. In Vivo Reproductive Capacity of Cryopreserved Semen

Based on the results obtained in experiment 1, we compared in vivo semen samples cryopreserved using the three NP-CPAs at the concentrations that gave the best results in vitro, and thawed at both thawing rates, with fresh semen in an artificial fertilization trial.

Fertilization was performed using 34 dry plastic dishes. We had one control group, with 4 dishes fertilized using fresh semen (control group), and three treatment groups: (1) 10 dishes fertilized with cryopreserved semen using 10% egg yolk; (2) 10 dishes fertilized with semen frozen with 5% LDL; (3) 10 dishes fertilized with cryopreserved semen using 0.1 M sucrose. For each treatment group, the semen was thawed at 30 °C for 10 s and 10 °C for 30 s. By doing this, we obtained six different treatments (3 NP-CPAs × 2 thawing rates).

Eggs obtained from seven females were mixed together. An amount of 100 ± 11 eggs was placed in each dish. Next, 5 mL of D532 (20 mM Tris, 30 mM glycine, 125 mM NaCl, pH 9.0; [28]), which served as a fertilization solution, was added to the eggs. The sperm was immediately added, and the gametes were gently mixed for 10 s. Excess fresh semen was used at the beginning and the end of the fertilization trials to test the quality of the eggs.

For each treatment group, 0.25 mL (one straw containing approximately 540×10^6 sperm) of thawed semen was used for each dish. Then, about 20 mL of hatchery water was added. After 2 min, the eggs were rinsed with hatchery water and incubated in incubators at water temperature (9 °C). Unfertilized and dead eggs were counted and removed at each day of incubation. After 25–30 days, the eggs had reached the eyed-egg stage. Embryos started to hatch 45–50 days after fertilization.

The fertilization success was established by calculating the percentage of embryos at the eyed stage and hatching larvae. We calculated the percentage of eyed embryos and hatching larvae using the initial number of eggs and calculated as the number of eyed eggs or hatchings × initial egg number^{-1} × 100.

2.6. Statistical Analysis

To compare the different treatments, we used a generalized linear model (GLM) procedure to determine the fixed effects of NP-CPA concentration, thawing rate, and their interaction on the sperm quality variables in vitro; this procedure was used to assess the fixed effects of NP-CPA, thawing rate, and their interaction on fertilization and hatching rates.

Sperm variables (motility percentage, the duration of sperm movement, sperm viability, and DNA integrity) and fertilization and hatching were measured across the different treatments and were compared by analysis of variance (ANOVA) followed by Duncan's comparison test. Significance was set at $p < 0.05$. All statistical tests were conducted using the software package SPSS (SPSS 15.0 for Windows, 2006; SPSS, Chicago, IL, USA).

3. Results

3.1. Effects of Different Kinds of NP-CPAs and Thawing Rates on Post-Thaw Semen Quality

Spermatozoa motility (%) and its duration (s) in fresh semen was 78.50 ± 1.69 and 48.10 ± 2.04, while sperm viability and DNA integrity (%) was 82.75 ± 1.38 and 98.28 ± 0.87, respectively. The average sperm concentration was $8.66 \pm 1.28 \times 10^9$ sperm/mL.

The fixed effects of different NP-CPAs concentrations and thawing rates on sperm motility, the duration of sperm motility, viability, and DNA integrity are shown in Table 1. The data obtained indicated a significant effect for the concentrations of NP-CPAs and thawing rates on all parameters considered, except for on the motility duration for the thawing rate effect.

Table 1. Sperm quality variables (mean ± SE) recorded for native trout frozen with different non-permeating cryoprotectants (CPAs) (at different concentrations) and two thawing rates (N = 5).

Semen Treatment		Sperm Variables			
CPA	**Thawing Rate (°C)**	**Motility (%)**	**Motility (s)**	**Viability (%)**	**DNA Integrity (%)**
Egg yolk	30	35.00 ± 1.37 ^{b}	42.70 ± 2.61 ^{ab}	39.72 ± 1.08 ^{b}	97.70 ± 0.49 ^{a}
Egg yolk	10	51.80 ± 1.65 ^{a}	47.40 ± 3.09 ^{a}	53.58 ± 1.23 ^{a}	98.52 ± 0.40 ^{a}
LDL 2.5%	30	10.80 ± 2.03 ^{e}	21.90 ± 4.28 ^{d}	15.98 ± 0.31 ^{g}	93.18 ± 0.92 ^{d}
LDL 2.5%	10	14.70 ± 2.14 ^{de}	25.00 ± 3.55 ^{d}	16.74 ± 1.26 ^{g}	95.64 ± 0.4 ^{bc}
LDL 5%	30	19.20 ± 0.72 ^{cd}	28.50 ± 3.23 ^{cd}	22.34 ± 1.39 ^{f}	94.92 ± 0.46 ^{cd}
LDL 5%	10	22.60 ± 1.37 ^{c}	31.60 ± 3.76 ^{cd}	28.52 ± 0.85 ^{cd}	97.74 ± 0.63 ^{a}
LDL 10%	30	20.50 ± 2.39 ^{cd}	27.50 ± 2.02 ^{cd}	17.78 ± 0.60 ^{g}	94.78 ± 0.86 ^{cd}
LDL 10%	10	20.50 ± 2.89 ^{cd}	30.20 ± 3.44 ^{cd}	23.90 ± 1.04 ^{ef}	97.46 ± 0.23 ^{a}
LDL 15%	30	15.00 ± 2.24 ^{de}	25.00 ± 3.19 ^{d}	17.78 ± 0.45 ^{g}	94.94 ± 0.54 ^{cd}
LDL 15%	10	14.50 ± 1.46 ^{de}	28.10 ± 3.32 ^{cd}	21.62 ± 0.50 ^{f}	95.26 ± 0.65 ^{bc}
Sucrose 0.05 M	30	18.80 ± 1.89 ^{cd}	25.90 ± 3.17 ^{d}	24.14 ± 1.41 ^{ef}	95.36 ± 0.49 ^{bc}
Sucrose 0.05 M	10	21.90 ± 2.24 ^{c}	28.70 ± 2.21 ^{cd}	28.62 ± 0.53 ^{cd}	96.96 ± 0.47 ^{ab}
Sucrose 0.1 M	30	24.00 ± 3.32 ^{c}	36.40 ± 2.82 ^{bc}	32.08 ± 1.58 ^{c}	96.69 ± 0.42 ^{ab}
Sucrose 0.1 M	10	35.00 ± 2.50 ^{b}	42.60 ± 2.56 ^{ab}	39.02 ± 2.56 ^{b}	98.48 ± 0.96 ^{a}
Sucrose 0.3 M	30	18.00 ± 1.46 ^{cd}	22.90 ± 1.58 ^{d}	26.48 ± 1.28 ^{de}	94.44 ± 0.86 ^{cd}
Sucrose 0.3 M	10	20.00 ± 1.25 ^{cd}	23.90 ± 2.15 ^{d}	31.44 ± 1.83 ^{c}	97.38 ± 0.30 ^{a}
concentration effect		$p < 0.000$	$p < 0.000$	$p < 0.000$	$p < 0.000$
thawing rate effect		$p < 0.000$	$p < 0.052$	$p < 0.000$	$p < 0.000$
concentration × thawing rate effect		$p < 0.001$	$p < 0.763$	$p < 0.017$	$p < 0.194$

$^{a–g}$ Different superscript letters within the same column indicate a significant difference ($p < 0.05$). LDL: low density lipoproteins; CPA: cryoprotectant.

Regarding the interaction effect between the concentration and thawing rates, a significant effect was only observed with regards to motility and viability.

Significantly higher values were found for motility and viability in semen frozen in the presence of 10% egg yolk and thawed at 10 °C for 30 s, in comparison to all LDL and sucrose concentrations and thawing rates considered.

Regarding LDL, significantly higher sperm motility and DNA integrity were recorded for the semen frozen with 5% LDL and thawed at 10 °C with respect to those frozen with 2.5% and 15%, whilst the viability resulted as significant with all other LDL concentrations.

For the sucrose treatment group, significantly higher values for sperm motility and viability were found in semen frozen at a concentration of 0.1 M and at 10 °C. Moreover, this treatment also returned higher values of motility duration ($p < 0.05$) and DNA integrity compared with other sucrose concentrations. In addition, no significant differences were found for the duration of sperm movement and DNA integrity between the following treatments: 0.1 M sucrose/10 °C and 10% egg yolk/10 °C.

Based on these findings, 10% egg yolk, 0.1 M sucrose, and 5% LDL were selected as the most effective treatments, using both thawing rates (10 °C × 30 s and 30 °C × 10 s) for the in vivo artificial fertilization trial.

3.2. Fertilization Ability of Cryopreserved Semen

The percentages of fertilization and hatching rates recorded for cryopreserved and fresh semen are provided in Table 2. The percentages of fertilization rate and hatched eggs were significantly higher ($p < 0.05$) in fresh semen compared to frozen semen. The data reported in Table 2 indicate a significant effect of NP-CPA and thawing rate for both parameters considered, however no significant interaction effect was observed.

Table 2. Fertilization ability of fresh semen or frozen semen in presence of the three non-permeating cryoprotectants and two different thawing rates.

Semen Treatment	CPA	Thawing Rate (°C)	Fertilization Rate (%)	Hatching Rate (%)
Fresh	-	-	73.27 ^{a}	68.90 ^{a}
Frozen	Egg yolk 10%	10	58.62 ^{b}	54.50 ^{b}
		30	32.88 ^{cd}	29.87 ^{cd}
	LDL 5%	10	17.42 ^{e}	16.40 ^{e}
		30	9.06 ^{e}	6.89 ^{e}
	Sucrose 0.1 M	10	43.71 ^{c}	37.85 ^{c}
		30	22.87 ^{de}	20.85 ^{de}
CPA effect			$p < 0.000$	$p < 0.000$
thawing rate effect			$p < 0.000$	$p < 0.000$
CPA × thawing rate effect			$p < 0.202$	$p < 0.275$

$^{a–e}$ Different superscript letters within the same column indicate a significant difference ($p < 0.05$). LDL: low density lipoproteins; CPA: cryoprotectant.

Higher fertilization and hatching rates were recorded for the semen cryopreserved in the presence of egg yolk and thawed at 10 °C (58.62 and 54.50, respectively), with respect to sucrose and LDL at all the thawing rates tested ($p < 0.05$). On the other hand, the sucrose recorded high values for both parameters considered (43.71 and 37.85, respectively) when combined with the thawing rate at 10 °C × 30 s.

LDL, on the contrary, significantly impaired the fertilization and hatching rate compared to all other treatments, except for sucrose/30 °C.

4. Discussion

This is a comparative study that aims to evaluate the effects of different NP-CPAs and thawing rates on Mediterranean brown trout sperm characteristics, which include sperm motility parameters, viability, DNA integrity, and fertilization ability. In particular, LDL and sucrose were tested as NP-CPAs to replace the egg yolk in order to make extender preparation easier and to overcome the sanitary and practical disadvantages associated with its use. The results clearly demonstrated that the type and concentrations of NP-CPAs used affect the post-thaw quality of trout semen.

However, contrary to our expectations, the replacement of egg yolk with LDL (extracted from egg yolk) or sucrose did not improve the post-thaw in vitro quality, confirming that egg yolk is the best NP-CPA for the cryopreservation of Mediterranean brown trout semen [5,8]. On this matter,

other authors also showed that the egg yolk is a valuable component in extenders for salmonid sperm cryopreservation. The addition of egg yolk in extenders significantly increased the post-thaw sperm quality of Atlantic salmon [29], rainbow trout [19,22], and brown trout [3], compared to frozen semen without egg yolk.

In mammals, numerous authors have attributed the LDL fraction of egg yolk to an ability to protect the spermatozoa during the freeze–thaw process [12,16,30–32]. However, the mechanism in which this protection is provided to sperm remains elusive. Some authors suggest that LDL could adhere to cell membranes, protecting spermatozoa against freeze–thaw damage by stabilizing the cellular membrane [33,34]; other researchers reported that the release of phospholipids from LDL during the freezing process could substitute some of the sperm membrane's phospholipids, thus reducing the formation of ice crystals [12]. Because of this property, we have chosen to test the effect of the isolated fraction of LDL on the cryopreservation of Mediterranean brown trout spermatozoa.

In this regard, despite the fact that the replacement of egg yolk with LDL has decreased the overall post-thaw semen quality, an effect was observed in terms of its concentration. When the LDL concentration was increased from 2.5% to 10%, an improvement was noted in trout sperm cryosurvival. Conversely, the usage of 15% LDL reduced the post-thaw sperm quality. This could be due to a drop in osmotic pressure in the extender, which some authors attributed to the possible precipitation of sugars contained in the extender supplemented with high LDL concentrations [12]. However, our findings disagree with those reported by Pérez-Cerezales et al. [20]. They showed that the LDL fraction (from egg yolk) had a more cryoprotective effect compared to the overall use of egg yolk in rainbow trout spermatozoa. These conflicting results could be explained by the different experimental conditions used in the two studies. On the other hand, from results obtained here and in accordance with Babiak et al. [19], we can come to the conclusion that LDL is not the only constituent of egg yolk that can play a considerable role in sperm protection against injuries caused by the freeze–thaw process.

The choice to assess sucrose as an NP-CPA for our native trout was motivated by its beneficial effects obtained in mammal semen freezing and the fact that very little is known about sucrose in trout semen cryopreservation. In this regard, in mammals, several authors have reported sucrose as an NP-CPA agent in semen cryopreservation extenders [17,18,35–40], whilst in trout, the effects of sucrose as external cryoprotectant on the in vitro post-thaw quality have been studied in only two reports [21,22]. In fact, numerous studies have advised that sucrose can be utilized as a source of energy, as alternative sugar to glucose in the preparation of carbohydrate-based extenders, rather than being supplemented as a non-permeating agent [41–45]. However, Maisse [46] suggested that sugars play a dual role in semen extenders as energy sources and non-permeating agents. Moreover, some authors observed that disaccharides seem more effective in respect to monosaccharides when it comes to causing osmotic dehydration [47,48]. Therefore, the protective effect of sucrose as a non-permeating agent has been related to its specific osmotic effect, which induces a decrease in the intracellular freezability of water and consequently reduces the sperm injuries provoked by ice crystallization [49,50]. However, although our results showed that sucrose improves the sperm cryosurvival in comparison with LDL, no significant improvement has been observed in comparison with egg yolk when the thawing temperature of 10 °C (for 30 s) was used.

Remarkably, the results obtained in vitro were confirmed by in vivo data when cryopreserved semen, in the presence of the highest concentrations of LDL and sucrose, was compared to whole egg yolk in fertilization trials. In relation to this matter, fertilization and hatching rates were significantly higher for semen frozen in the presence of egg yolk in comparison with those recorded with 5% LDL and 0.1 M sucrose. In accordance with our findings, other authors also showed the valuable effects of egg yolk in freezing extenders in respect to LDL on fertilization rates and embryo survival of northern pike and rainbow trout [19,51].

Taking into account the overall results in vitro and in vivo obtained here, we can sustain that adding the whole egg yolk in the glucose-DMSO extender appears to be the most effective NP-CPA for cryopreserved Mediterranean brown trout spermatozoa.

Another interesting point that emerged from our study was that the thawing rate impacted significantly on post-thaw semen quality and fertilizing capacity. In this regard, it is known that the thawing rate is among the most critical factors that influence sperm frozen cryosurvival [11,52,53], other than the fact that it is also the most sensitive parameter in the cryopreservation of Salmonidae semen [54,55]. In particular, the lower thawing rate (10 °C for 30 s) recorded better post-thaw semen quality for all NP-CPAs, regardless of the concentrations used. Similarly, the thawing rate of 10 °C for 30 s showed higher fertilization and hatching rates for all the treatments tested, and in accordance with those obtained in previous studies by the same authors [5,8]. In accordance with our results, higher fertilization rates of rainbow trout semen using low thawing rates (5 °C for 90 s and 10 °C for 30 s) in comparison with high thawing rates (20 °C for 20 s and 30 °C for 15 s) were obtained by Wheeler and Thorgaard [52]. Instead, other authors found an impairment of fertilization rates when low thawing rates (5° C for 90 s vs. 15 °C for 45 s) were used [11,44]. However, the conflicting results reported in the literature may depend on the different experimental conditions adopted in the studies (extender composition, freezing rates, and different straw volume). In particular, the effect of thawing rate seems to be strongly influenced by the freezing conditions used [56]. In this regard, it is generally accepted that, whether the freezing rate is sufficiently low to induce cell dehydration, a low thawing rate is required to ensure adequate rehydration; on the contrary, high freezing rates produce induce intracellular water freezing, therefore a fast thawing rate is necessary to prevent recrystallization.

In light of this, we speculate that the freezing rate used in our study allows an intracellular water efflux and dehydration such that the lowest thawing rate is appropriate to ensure adequate restoration of the intra and extracellular equilibrium. Moreover, given that the natural reproduction of this native trout occurs on the spawning grounds at the main springs of the Volturno and Biferno rivers, whose water temperature ranges between 7 and 12 °C, this is an exceptional discovery for us, because this would facilitate the on-field artificial reproduction practices of wild breeders, directly using the spring water to thaw the straws.

5. Conclusions

In conclusion, the present study corroborated that egg yolk is the best non-permeating cryoprotectant for the cryopreservation of Mediterranean brown trout sperm, using a glucose-DMSO extender. In addition, the temperature of 10 °C improved the sperm fertilization ability, reaching fertilization and hatching rates similar to those of fresh semen. These encouraging results provide an important contribution for the creation of a sperm cryobank aiming at the restoration of Mediterranean brown trout in Molise (Italy), and it is a milestone of our European project (life Nat.Sal.Mo).

However, given the presence of important sanitary and practical disadvantages related to the use of egg yolk, hopefully with further studies the egg yolk could be replaced with another NP-CPA or an alternative freezing extender that has the same efficacy or an even better one for the sperm cryopreservation of Mediterranean brown trout will be found.

Recently, in this regard, some Polish colleagues [45,57], using a simple glucose–methanol extender to cryopreserve salmonid fish sperm (Atlantic salmon, rainbow trout, brown trout, and brook trout) obtained excellent fertilization and hatching rates. These authors sustained that their semen cryopreservation protocol seems to be "universal" for the cryopreservation of Salmonidae semen. Therefore, we believe in the possibility to test this sperm freezing protocol and compare it to ours for Molise native trout. This could be our next challenge.

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Ethics Statement: The experiments were conducted in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments. This study is part of a Nat.Sal.Mo LIFE project that received "a positive opinion" from the Ministry of the Environment and the Protection of the Territory and the Sea. The sampling and

handling of fish followed animal welfare practices as reported in the Ministerial Protocol (ISPRA). All experiments were carried out with the appropriate authorizations from the Molise Region (protocol number 6192, 13/12/2017) according to the current regulations on the protection of the species, protocols of the sampling of fresh water, animal welfare, and bio-security. Semen and eggs were transported in compliance with current national regulations (Legislative Decree 148/2008, D.L 3/08/2011).

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References

1. Akçay, E.; Bozkurt, Y.; Seçer, S.; Tekin, N. Cryopreservation of Mirror Carp Semen. *Turk. J. Vet. Anim. Sci.* **2004**, *28*, 837–843.
2. Martínez-Páramo, S.; Pérez-Cerezales, S.; Gòmez-Romano, F.; Blanco, G.; Sánchez, J.A.; Herráez, M.P. Cryobanking as tool for conservation of biodiversity: Effect of brown trout sperm cryopreservation on the male genetic potential. *Theriogenology* **2009**, *71*, 594–604. [CrossRef] [PubMed]
3. Bozkurt, Y.; Yavas, I.; Karaca, F. Cryopreservation of Brown Trout (*Salmo trutta macrostigma*) and Ornamental Koi Carp (*Cyprinus carpio*) sperm. In *Current Frontiers in Cryopreservation*; Katkov, I., Ed.; In Tech: Rijeka, Croatia, 2012; pp. 29–304.
4. Martínez-Páramo, S.; Horváth, Á.; Labbé, C.; Zhang, T.; Robles, V.; Herráez, P.; Cabrita, E. Cryobanking of aquatic species. *Aquaculture* **2017**, *472*, 156–177. [CrossRef] [PubMed]
5. Iaffaldano, N.; Di Iorio, M.; Manchisi, A.; Esposito, S.; Gibertoni, P.P. Effective freezing rate for semen cryopreservation in endangered Mediterranean brown trout (*Salmo trutta macrostigma*) inhabiting the Biferno river (South Italy). *Zygote* **2016**, *24*, 668–675. [CrossRef] [PubMed]
6. Bianco, P.G.; Caputo, V.; Ferrito, V.; Lorenzoni, M.; Nonnis Marzano, F.; Stefani, F.; Sabatini, A.; Tancioni, L. Pesci d'acqua dolce. In *Lista Rossa IUCN dei Vertebrati Italiani*; Rondinini, C., Battistoni, A., Peronace, V., Teofili, C., Eds.; Comitato Italiano IUCN e Ministero dell'Ambiente e della Tutela del Territorio e del Mare: Roma, Italy, 2013; p. 54.
7. Iaffaldano, N.; Di Iorio, M.; Manchisi, A.; Gibertoni, P.P.; Esposito, S. Semen quality of threatened native population of Mediterranean brown trout (Salmo cettii, Rafinesque 1810) in the Biferno River (Molise Region-South Italy). *Turk. J. Fish. Aquat. Sci.* **2016**, *16*, 259–266. [CrossRef]
8. Di Iorio, M.; Esposito, E.; Rusco, G.; Roncarati, A.; Miranda, M.; Gibertoni, P.P.; Cerolini, S.; Iaffaldano, I. Semen cryopreservation for the Mediterranean brown trout of the Biferno River (Molise-Italy): Comparative study on the effects of basic extenders and cryoprotectants. *Sci. Rep.* **2019**. accepted.
9. Sarvi Moghanloo, K.; Niksirat, H.; Mojazi Amiri, B.; Mirtorabi, S.M. The effects of extender type, freezing and thawing rates on fertility of the cryopreserved semen of the Caspian brown trout (Salmo trutta caspius). *Iran. J. Fish. Sci.* **2007**, *7*, 111–128. Available online: https://aquaticcommons.org/id/eprint/22309 (accessed on 30 April 2019).
10. Kopeika, J.; Kopeika, E.; Zhang, T.; Rawson, D.M. Studies on the Toxicity of Dimethyl Sulfoxide, Ethylene Glycol, Methanol and Glycerol to Loach (*Misgurnus fossilis*) Sperm and the Effect on Subsequent Embryo Development. *CryoLetters* **2003**, *24*, 365–374. Available online: https://www.ncbi.nlm.nih.gov/pubmed/14671688 (accessed on 30 April 2019).
11. Fowler, A.; Toner, M. Cryo-injury and biopreservation. *Ann. N. Y. Acad. Sci.* **2006**, *1066*, 119–135. [CrossRef] [PubMed]

12. Moussa, M.; Martinet, V.; Trimeche, A.; Tainturier, D.; Anton, M. Low density lipoproteins extracted from hen egg yolk by an easy method: Cryoprotective effect on frozen–thawed bull semen. *Theriogenology* **2002**, *57*, 1695–1706. [CrossRef]
13. Kampschmidt, R.F.; Mayer, D.T.; Herman, H.A. Lipid and Lipoprotein Constituents of Egg Yolk in the Resistance and Storage of Bull Spermatozoa. *J. Dairy Sci.* **1953**, *36*, 733–742. [CrossRef]
14. Pace, M.M.; Graham, E.F. Components in Egg Yolk which Protect Bovine Spermatozoa during Freezing. *J. Anim. Sci.* **1974**, *39*, 1144–1149. [CrossRef] [PubMed]
15. Pillet, E.; Duchamp, G.; Batellier, F.; Beaumal, V.; Anton, M.; Desherces, S.; Magistrini, M. Egg yolk plasma can replace egg yolk in stallion freezing extenders. *Theriogenology* **2011**, *75*, 105–114. [CrossRef]
16. Hu, J.H.; Li, Q.W.; Jiang, Z.L.; Li, W.Y. Effects of different extenders on DNA integrity of boar spermatozoa following freezing–thawing. *Cryobiology* **2008**, *57*, 257–262. [CrossRef]
17. Mocé, E.; Vicente, J.S. Rabbit sperm cryopreservation: A review. *Anim. Reprod. Sci.* **2009**, *110*, 1–24. [CrossRef] [PubMed]
18. Iaffaldano, N.; Di Iorio, M.; Rosato, M.P.; Manchisi, A. Cryopreservation of rabbit semen using non-permeable cryoprotectants: Effectiveness of different concentrations of low-density lipoproteins (LDL) from egg yolk versus egg yolk or sucrose. *Anim. Reprod. Sci.* **2014**, *151*, 220–228. [CrossRef] [PubMed]
19. Babiak, I.; Glogowski, J.; Goryczko, K.; Dobosz, S.; Kuzminski, H.; Strzezek, J.; Demianowicz, W. Effect of extender composition and equilibration time on fertilization ability and enzymatic activity of rainbow trout frozen spermatozoa. *Theriogenology* **2001**, *56*, 177–192. [CrossRef]
20. Pérez-Cerezales, S.; Martínez-Páramo, S.; Beirão, J.; Herráez, M. Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. *Reproduction* **2010**, *139*, 989–997. Available online: https://rep.bioscientifica.com/view/journals/rep/139/6/989.xml (accessed on 30 April 2019). [CrossRef]
21. Maisse, G. Comparaison de l'effet cryoprotecteur de différents glucides sur le sperme de truite arc-en-ciel (Oncorhynchus mykiss). *Aquat. Living Resour.* **1994**, *7*, 217–219. [CrossRef]
22. Lahnsteiner, F.; Berger, B.; Weismann, T.; Patzner, R.A. Motility of spermatozoa of *Alburnus alburnus* (*Cyprinidae*) and its relationship to seminal plasma composition and sperm metabolism. *Fish Physiol. Biochem.* **1996**, *15*, 167–179. [CrossRef]
23. Gibertoni, P.; Jelli, F.; Bracchi, P. Allevamento, riproduzione e reintroduzione in ambiente naturale di trote fario di 'ceppo mediterraneo', Salmo (trutta) trutta, L. *Annali della Facoltà di Medicina Veterinaria* **1998**, *18*, 1–20.
24. Jelli, F.; Gibertoni, P.P. Recupero e reintroduzione di ceppi autoctoni di trota fario, Salmo (trutta) trutta L., nel bacino del Fiume Secchia. In *Atti del Convegno: Recupero e Reintroduzione dei Ceppi Autoctoni di Trota Fario, Salmo (Trutta) Trutta L., di "Ceppo Mediterraneo" in Ambienti Appenninici Tipici*; Esperienze Gestionali a Confronto: Reggio Emilia, Italy, 1999; Volume 2128.
25. Penserini, M.; Nonnis Marzano, F.; Gandolfi, G.; Maldini, M.; Marconato, E.; Gibertoni, P.P. Fenotipi della trota mediterranea: Metodologia di indagine molecolare combinata e selezione morfologica per l'identificazione degli esemplari autoctoni. *J. Freshwat. Biol.* **2006**, *34*, 69.
26. Gandini, L.; Lombardo, F.; Lenzi, A.; Spano, M.; Dondero, F. Cryopreservation and sperm DNA integrity. *Cell Tissue Bank.* **2006**, *7*, 91–98. [CrossRef] [PubMed]
27. Iaffaldano, N.; Di Iorio, M.; Rosato, M.P. The cryoprotectant used, its concentration and the equilibrium time are critical for the successful cryopreservation of rabbit sperm: Dimethylacetamide versus dimethylsulfoxide. *Theriogenology* **2012**, *78*, 1381–1389. [CrossRef]
28. Billard, R. Reproduction in rainbow trout: Sex differentiation, dynamics of gametogenesis, biology and preservation of gametes. *Aquaculture* **1992**, *100*, 263–298. [CrossRef]
29. Alderson, R.; Macneil, A.J. Preliminary investigations of cryopreservation of milt of atlantic salmon (salmo salar) and its application to commercial farming. *Aquaculture* **1984**, *43*, 351–354. [CrossRef]
30. Amirat-Briand, L.; Tainturier, D.; Jeanneau, L.; Thorin, C.; Gèrard, O.; Courtens, J.L.; Anton, M. Bull semen in vitro fertility after cryopreservation using egg yolk LDL: A comparison with Optidyl®, a commercial egg yolk extender. *Theriogenology* **2004**, *61*, 895–907. [CrossRef]
31. Bencharif, D.; Amirat, L.; Anton, M.; Schmitt, E.; Desherces, S.; Delhomme, G.; Tainturier, D. The advantages of LDL (low density lipoproteins) in the cryopreservation of canine semen. *Theriogenology* **2008**, *70*, 1478–1488. [CrossRef]

32. Moreno, D.; Bencharif, D.; Amirat-Briand, L.; Neira, A.; Destrumelle, S.; Tainturier, D. Preliminary results: The advantages of low-density lipoproteins for the cryopreservation of equine semen. *J. Equine Vet. Sci.* **2013**, *33*, 1068–1075. [CrossRef]
33. Foulkes, J.A.; Stewart, D.L. Fertility of dairy cattle after artificial insemination with semen frozen in a lipoprotein diluent. *Reproduction* **1977**, *51*, 175–177. Available online: https://rep.bioscientifica.com/view/journals/rep/51/1/jrf_51_1_032 (accessed on 30 April 2019). [CrossRef]
34. Graham, J.K.; Foote, R.H. Effect of several lipids fatty acyl chain length and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. *Cryobiology* **1987**, *24*, 42–52. [CrossRef]
35. De Leeuw, F.E.; De Leeuw, A.M.; Den Daas, J.H.G.; Colenbrander, B.; Verkleij, A.J. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology* **1993**, *30*, 32–44. [CrossRef] [PubMed]
36. Molinia, F.C.; Evans, G.; Casares, P.Q.; Maxwell, W.M.C. Effect of monosaccharides and disaccharides in Tris-based diluents on motility, acrosome integrity and fertility of pellet frozen ram spermatozoa. *Anim. Reprod. Sci.* **1994**, *36*, 113–122. [CrossRef]
37. Woelders, H.; Matthijs, A.; Engel, B. Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology* **1997**, *35*, 93–105. [CrossRef] [PubMed]
38. Yildiz, C.; Kaya, A.; Aksoy, M.; Tekeli, T. Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. *Theriogenology* **2000**, *54*, 579–585. [CrossRef]
39. Farshad, A.; Akhondzadeh, S. Effects of sucrose and glycerol during the freezing step of cryopreservation on the viability of goat spermatozoa. *Asian Austral. J. Anim.* **2008**, *21*, 1721–1727. [CrossRef]
40. Khalili, B.; Farshad, A.; Zamiri, M.J.; Rashidi, A.; Fazeli, P. Effects of Sucrose and Trehalose on the Freezability of Markhoz Goat Spermatozoa. *Asian Austral. J. Anim.* **2009**, *22*, 1614–1619. [CrossRef]
41. Ciereszko, A.; Dabrowski, K. Effect of a sucrose-DMSO extender supplement with pentoxifylline or blood plasma on fertilizing ability of cryopreserved rainbow trout spermatozoa. *Prog. Fish-Cult.* **1996**, *58*, 143–145. [CrossRef]
42. Lahnsteiner, F.; Weismann, T.; Patzner, R.A. Aging processes of rainbow trout semen during storage. *Prog. Fish Cult.* **1997**, *59*, 272–279. [CrossRef]
43. Glogowski, J.; Kwasnik, M.; Piros, B.; Dabrowski, K.; Goryczko, K.; Dobosz, S.; Ciereszko, A. Characterization of rainbow trout milt collected with a catheter: Semen parameters and cryopreservation success. *Aquac. Res.* **2000**, *31*, 289–296. [CrossRef]
44. Sarvi, K.; Niksirat, H.; Amiri, B.M.; Mirtorabi, S.M.; Rafiee, G.R.; Bakhtiyari, M. Cryopreservation of semen from the endangered Caspian brown trout (*Salmo trutta caspius*). *Aquaculture* **2006**, *256*, 564–569. [CrossRef]
45. Nynca, J.; Judycka, S.; Liszewska, E.; Dobosz, S.; Grudniewska, J.; Arai, K.; Ciereszko, A. Utility of different sugar extenders for cryopreservation and post-thaw storage of sperm from Salmonidae species. *Aquaculture* **2006**, *464*, 340–348. [CrossRef]
46. Maisse, G. Cryopreservation of fish semen: A review. In *Proceedings of the Refrigeration Science and Technology Conference, Refrigeration and Aquaculture*; Institut International du Froid: Paris, France, 1996; pp. 443–467.
47. Pursel, V.G.; Johnson, L.A.; Rampacek, G.B. Acrosome morphology of boar spermatozoa incubated before cold shock. *J. Anim. Sci.* **1972**, *34*, 278–283. [CrossRef] [PubMed]
48. Platov, E.M. Cryopreservation of ram spermatozoa. In *Cryoconservation of Spermatozoa of Farm Animals*; Agropromizda: Leningrad, Russia, 1988; pp. 161–195.
49. Aisen, E.G.; Medina, V.H.; Venturino, A. Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. *Theriogenology* **2002**, *57*, 1801–1808. [CrossRef]
50. Purdy, P.H. A review on goat sperm cryopreservation. *Small Rumin. Res.* **2006**, *6*, 215–225. [CrossRef]
51. Babiak, I.; Glogowski, J.; Luczynski, M.J.; Luczynski, M.; Demianowicz, W. The effect of egg yolk, low density lipoproteins, methylxanthines and fertilization diluent on cryopreservation efficiency of northern pike (Esox lucius) spermatozoa. *Theriogenology* **1999**, *52*, 473–479. [CrossRef]
52. Wheeler, P.A.; Thorgard, G.H. Cryopreservation of rainbow trout semen in large straws. *Aquaculture* **1991**, *93*, 95–100. [CrossRef]
53. Morris, G.J. *Cryopreservation: An Introduction to Cryopreservation in Culture Collections*; Institute of Terrestrial Ecology: Cambridge, UK, 1981.

54. Lahnsteiner, F.; Weismann, T.; Patzner, R.A. Composition of the ovarian fluid in 4 salmonid species: Oncorhynchus mykiss, Salmo trutta f lacustris, Salvelinus alpinus and Hucho hucho. *Reprod. Nutr. Dev.* **1995**, *35*, 465–474. Available online: https://www.ncbi.nlm.nih.gov/pubmed/8526977 (accessed on 30 April 2019). [CrossRef]
55. Lahnsteiner, F.; Berger, B.; Weismann, T.; Patzner, R.A. Determination of semen quality of the rainbow trout, Oncorhynchus mykiss, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. *Aquaculture* **1998**, *163*, 163–181. [CrossRef]
56. Barbas, J.P.; Mascarenhas, R.D. Cryopreservation of domestic animal sperm cells. *Cell Tissue Bank.* **2009**, *10*, 49–62. [CrossRef]
57. Judycka, S.; Nynca, J.; Liszewska, E.; Dobosz, S.; Grudniewska, J.; Ciereszko, A. Optimal sperm concentration in straws and final glucose concentration in extender are crucial for improving the cryopreservation protocol of salmonid spermatozoa. *Aquaculture* **2018**, *486*, 90–97. [CrossRef]

ECOLOGY AND BIODIVERSITY OF MEDITERRANEAN TROUT IN THE MAIN BASINS OF MOLISE (ITALY)

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Keywords: Native trout, genetic variability, introgressive hybridization, PCR-RFLP, biodiversity preservation, evolutionary biology.

Abstract

The Mediterranean Basin is a global biodiversity hotspot, bearing a high number of endemic species of freshwater fishes. The introduction of alien invasive species for fishing activities implementation has compromised, during years, the genetic integrity of Mediterranean trout (*Salmo cettii*, syn. *Salmo macrostigma*), which may be headed towards extinction, being also more vulnerable to climate change compared to allochthonous species. Given the considerable economic significance for fisheries and aquaculture of this species, a deep knowledge on trout populations structures and a proper method for spawners' selection is necessary, to preserve native fauna and adopt a correct ecosystem approach in fisheries management.

Here we present the results from the genetic characterization of 300 trout specimens, descriptive of the human-mediated hybridization process established between the native trout of Molise region (Central-Southern Italy) and the inter-fertile brown trout of Atlantic origin.

Specimens were collected by electrofishing from different sampling stations of Biferno and Volturno rivers. Gene variability from LDH-C1* allozyme and mitochondrial 16S mtDNA data was evaluated by PCR-RFLP, analyzing on 2.5% agarose gel the fragments' differences obtained after specific enzyme digestions. The use of two markers combination (nuclear and mitochondrial) allows to categorize the analyzed genotypes into six introgression classes (I-VI), establishing the level of hybridization, which goes from null level to maximum level.

Obtained results show a different genetic introgression in the two rivers and a wide variability within each of them. More specifically, the Volturno exhibits a greater percentage of pure native trouts, while the Biferno has a higher percentage of Atlantic individuals, both pure and Atlantic fishes with a Mediterranean matrilineal line. Thus, the degree of heterozygous specimens in the Biferno is greater than Volturno.

These information suggest that invasive Atlantic trout is seriously threatening the autochthonous populations, especially in Biferno river, further investigations are required, to know also the influence of morphological and environmental parameters on the introgression phenomenon of Mediterranean trout.

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THE SAFEGUARD AND CONSERVATION OF *S. MACROSTIGMA* THROUGH THE FIRST EUROPEAN SEMEN CRYOBANK

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Keywords: *Salmo macrostigma*, sperm freezing, conservation biology, sperm cryobank.

Abstract

The Native Mediterranean brown trout (*S. macrostigma*) population inhabiting the Molise rivers (South of Italy), is declining as a result of river pollution, poorly regulated fishing activities and the introduction of allochthonous strains for recreational purposes, causing genetic introgression. The conservation status of *S. macrostigma* in the Mediterranean biogeographical area, according to the Italian report is considered as "critically endangered" by IUCN. The overall status at EU level, taking into account the Mediterranean area, is "bad" (U2) and "in decline". The status of Italian populations significantly contributes to the overall EU status because the Mediterranean trout populations largely represent the majority of the European population of this species.

Semen Cryobanking is a valuable tool in preserving the genetic resources of endangered fish species and it plays an important role in biodiversity conservation and restocking programmes. The semen cryobanks provide the opportunity to preserve representative samples and further reconstruct the original strain, population, or diversity. Finding an efficient freezing protocol for the Molise authotrout will allow the creation of a sperm cryobank. The sperm cryobank of autochthonous Mediterranean trout populations with high genetic variability represents an action within our financed "LIFE" project focusing on the recovery and conservation of this native trout in Molise rivers. Many efforts have been made by our research group in order to find an effective semen protocol and promising results have been obtained so far, we aim to obtain a freezing procedure that reports values of post-thaw quality and fertilization similar to fresh semen (Iaffaldano et al., 2016; Di Iorio et al., 2019; Rusco et al., 2019).

The aim of the present work was to test the effectiveness of semen cryopreservation protocol developed recently by Polish researchers for other salmonids (Ciereszko et al., 2017; Judycka et al., 2018) and adptading it for the authoctonus trout from Molise. The effects of two different thawing rates on sperm motility, spermatozoa movement duration (SMD) and sperm viability (SYBR-PI) were evaluated. Semen of five individual males was diluted to a final extender concentration of 0.15 M glucose and 7.5% methanol) and loaded into 0.25 mL plastic straws, obtaining a final sperm concentration of 3.0×10^9 sperm/mL. Subsequently, the straws were placed on a 3 cm high frame and equilibrated for 15 min on ice. After equilibration, the straws were frozen by exposure to liquid nitrogen vapor at 3 cm above liquid nitrogen level for 5 min and were then placed in liquid nitrogen. The straws were then thawed by immersion in a water bath at 40°C for 5 sec or at 10°C for 30 sec. Sperm motility (CASA system), SMD and sperm viability (SYBR-PI) were then assessed.

As expected, the motility, SMD and viability were significantly higher in fresh semen compared to the frozen semen, althought a significant effect of thawing rate was observed for the motility with a higher value at the thawing rate of 40°C. According to the results obtained, the thawing rate at 40°C for 5 sec emerged as more appropriate. However, this cryopreservation protocol seems to be more effective compared to other cryopreservation protocol developed previosuly in our laboratory. Further studies are necessary to confirm this encouraging results *in vivo*. Our findings provide a valid contribution for the creation of the first eureopean sperm cryobank aiming at the genetic restoration of Mediterranean brown trout (*S. macrostigma*) in Molise rivers.

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THE SCHEMES OF FERTILIZATION IN COMBINATION WITH FROZEN SEMEN AS A STRATEGY TO INCREASE THE GENETIC VARIABILITY OF NATIVE MEDITERRANEAN BROWN *(SALMO MACROSTIGMA)* IN MOLISE REGION (SOUTH ITALY)

(ITALY)

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Keywords: native Mediterranean brown, genetic variability, biodiversity preservation, frozen semen

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Abstract

The native trout population originally inhabiting the Biferno and Volturno rivers basin (correspondents respectively to the Adriatic and Tyrrhenian drainages) in the Molise region (South Italy) is the Mediterranean brown trout species. It is listed by the annex II of Habitat Directive, and reported as critically threatened and endangered under the taxonomical term *Salmo cettii* by the International Union for Conservation of Nature (IUCN).

The distribution range of native Mediterranean brown trout has suffered a gradual reduction over the last few centuries. This could be largely attributed to the detrimental effects of anthropogenic disruptions, such as dam building, river straightening and local pollution. Moreover, the hybridization introduction of domesticated Atlantic strains of brown trout for recreational purposes has caused genetic introgression of autochthonous population. Hybridization by non-native genes is considered to be one of the most serious threats to the long-term conservation of diversity within the species.

The native Mediterranean brown trout population inhabiting the Molise rivers basin is characterized by migratory life-history, is threatened by genetic introgression and the loss of suitable reproductive sites as a result of high density antropic actions at spawning grounds.

After distinctive migratory patterns, the native breeders reach the few suitable and available spawning sites resulting in high densities caused by the loss of other optimal reproductive sites due to pollution, water captations and anthropogenic barriers. Thus, the high breeder densities in the spawning grounds cause a strong male competition for females which strongly influences the individual and population fitness.

In this regard, thank to the project "LIFE" Nat.Sal.mo, recently funded by the EU, aiming to the recovery and the conservation of native Mediterranean trout (*Salmo macrostigma*) in the Molise river basins, has many specific objectives one of which is to produce eggs from wild breeders by artificial reproduction ensuring the genetic variability of the offspring. Supporting breeding in the management of salmonids is discussed at a global level, where it is not strictly necessary due to the need to select native breeders and decrease the degree of population introgression. The main problem derives from the possibility to produce thousands of eggs from a few breeders, causing loss of genetic variability.

Moreover, if fry are released, there is a risk of domestication. The restoration of genetic integrity of native Mediterranean brown trout during the LIFE Nat.Sal.Mo Project will occur using a strategy of fertilization schemes that uses frozen semen in artificial reproduction, increasing the number of genetically typed males always available (frozen semen) and crossing the individual males into a scheme designed to increase the breeding pairs. Eggs will be released in nature by artificial nesting, directly on the river grounds, increasing thus the genetic variability of the offspring and avoiding domestication.

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