fishes

MDPI

Article

Cryobank of Mediterranean Brown Trout Semen: Evaluation of the Use of Frozen Semen up to Six Hours Post-Collection

Giusy Rusco [1], Michele Di Iorio [1], Roberta Iampietro [1], Alessandra Roncarati [2], Stefano Esposito [3] and Nicolaia Iaffaldano [1,*]

[1] Department of Agricultural, Environmental and Food Sciences, University of Molise, Via De Sanctis snc, 86100 Campobasso, Italy; giusy.rusco@unimol.it (G.R.); michele.diiorio@unimol.it (M.D.I.); robertaiampietro@hotmail.it (R.I.)
[2] School of Biosciences and Veterinary Medicine, University of Camerino, Viale Circonvallazione 93-95, 62024 Matelica, Italy; alessandra.roncarati@unicam.it
[3] Mediterranean Trout Research Group "MTRG", 42037 Collagna, Italy; dott.stefanoesposito@gmail.com
* Correspondence: nicolaia@unimol.it; Tel.: +39-0874-404697; Fax: +39-0874-404855

Abstract: The aim of this study was to evaluate the effects of different cold-storage time intervals between collection and semen-freezing on both fresh and cryopreserved semen motility parameters and the post-thaw fertilizing ability of Mediterranean brown trout semen. The ejaculates were split into six aliquots and stored on ice from 1 to 6 h, until freezing. Fresh and post-thaw sperm motility was evaluated by a Computer-Assisted Sperm Analysis system, whilst the fertilizing ability was assessed by in vivo trials. In fresh semen, at 3 h of storage, a significant decrease of total motility, linear movement (STR, LIN) and beat cross frequency (BCF) was recorded, whilst the amplitude of lateral displacement of the spermatozoon head (ALH) underwent a significant increase. In frozen semen, no significant difference was observed for all the motility parameters evaluated, except for the total motility between 1 and 6 h of storage and the duration of sperm movement between 1 and 5 h. Cold-storage time did not significantly affect the percentage of live embryos following the use of frozen semen. In conclusion, our results showed that, if necessary, the Mediterranean brown trout semen can be frozen even until 6 h post-collection without losing its fertilizing ability.

Keywords: Mediterranean brown trout; cold-storage time; semen cryopreservation; safeguarding biodiversity

Citation: Rusco, G.; Di Iorio, M.; Iampietro, R.; Roncarati, A.; Esposito, S.; Iaffaldano, N. Cryobank of Mediterranean Brown Trout Semen: Evaluation of the Use of Frozen Semen up to Six Hours Post-Collection. *Fishes* **2021**, *6*, 26. https://doi.org/10.3390/fishes6030026

Academic Editor: Maria Angeles Esteban

Received: 1 July 2021
Accepted: 26 July 2021
Published: 2 August 2021

1. Introduction

Over the last two decades, because of population reduction and extinction in many European countries, native salmonid species have been the focus of some important conservation projects [1–5]. The Mediterranean brown trout is one of the freshwater fish species complexes at a greater risk of extinction in the Mediterranean area; it is also listed on the Italian IUCN Red List as "critically endangered" [6] under the taxon *S. cettii*. The introduction of alien invasive species, such as the Atlantic strain, and their interaction with the native fauna represents a major threat to the survival of this species, as the traits of their native gene pool are altered [7–13].

In this context, the EU has recently funded the "LIFE" Nat.Sal.Mo project, which aims to ensure the recovery of native genetic variability and the conservation of the Mediterranean brown trout (*S. macrostigma* = *S. cettii*) inhabiting the Biferno and Volturno river basins (Molise region—Southern Italy).

The restoration of the Mediterranean brown trout's genetic integrity is among the main objectives of the project. It is being realised through the use of artificial reproduction, using frozen semen from pure wild breeders in combination with appropriate fertilization schemes, as a strategy to maximize the genetic variability within the offspring and ensure the maintenance of the fitness within self-sustaining populations. In this regard, the

creation of the first European semen cryobank plays a key role for conserving extant genomic diversity of *S. cettii* to be used for artificial breeding activities. In order to obtain an effective freezing protocol aimed at the implementation of the first European sperm cryobank for the native Mediterranean brown trout, a series of systematic studies were performed [14–17].

Among the factors that affect the success of the cryopreservation procedure, good initial quality of semen is an indispensable prerequisite. The latter depends on the selected donor, a correct semen collection method, the appropriate conditions for transportation and the semen storage times before freezing [18]. Concerning this last factor, the time that elapses between collection and processing of semen results as an important variable in our project, since the sampling sites in the project area are not easily accessible and are distant, not only from each other but also from the laboratory. Due to this logistic problem, we have estimated that the time range that elapses between collection and semen-freezing could vary from a minimum of 1 h to a maximum of 6 h, depending on the sampling site and the time taken to capture the broodstock. As a result of these large time intervals, we questioned if the longer intervals of time prior to freezing could cause a loss in the quality of fresh semen and, consequently, adversely affect the freezability of the spermatozoa.

In light of these considerations, six possible scenarios have been simulated to evaluate the effect of different cold-storage time intervals (from 1 h to 6 h) between collection and semen-freezing on both fresh and cryopreserved semen motility parameters and post-thaw fertilizing ability of Mediterranean brown trout semen.

2. Results

2.1. Effect of Storage Time Post-Semen Collection on Fresh Sperm Motility Parameters

Some motility parameters of the fresh semen were significantly affected ($p < 0.05$) by the cold-storage time following sperm collection (Figure 1). Total sperm motility was not affected until 2 h, after this time-frame a significant decrease was recorded (Figure 1A). No significant differences for kinetic parameters (VCL, VAP and VSL), during storage were recorded (Figure 1B–D). The lowest percentage of ALH was registered at 2 h of storage, this resulted as significant in respect to the values recorded at 3, 4, 5 and 6 h (Figure 1G).

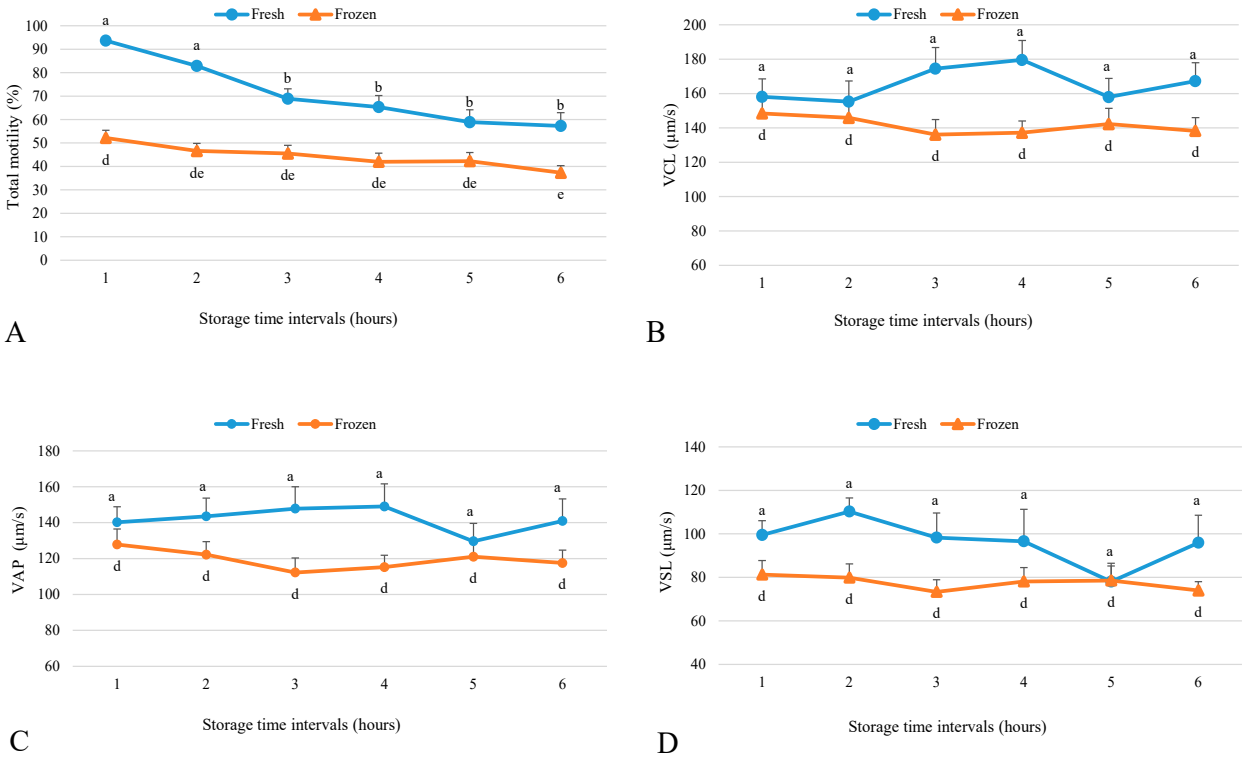

Figure 1. *Cont.*

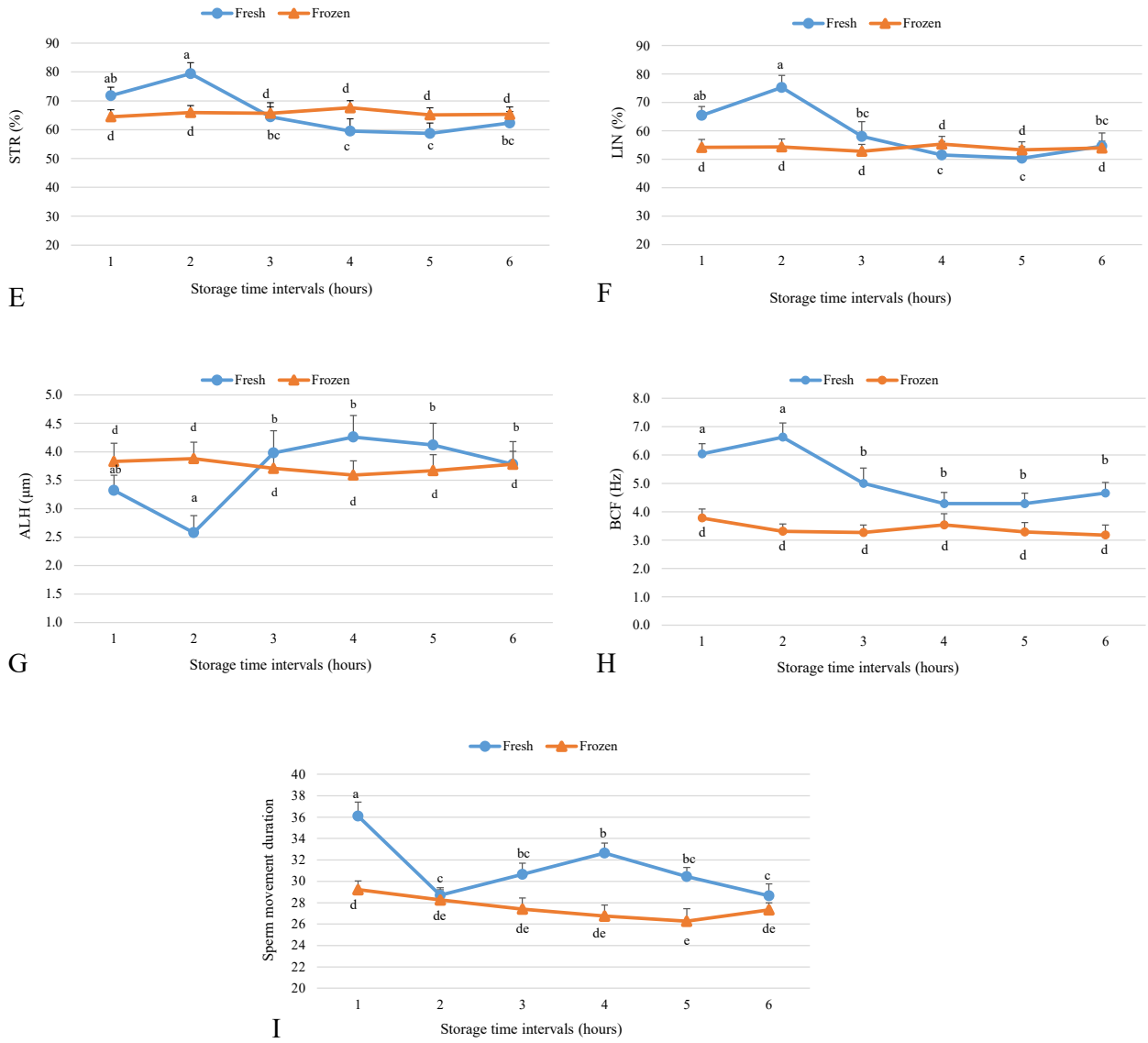

Figure 1. Effect of storage time post-collection on fresh and frozen sperm motility parameters (**A**–**I**). Total motility: the percentage of motile spermatozoa; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity (VSL/VCL × 100); STR: straightness (VSL/VAP × 100); BCF: beat cross frequency; ALH: amplitude of lateral displacement of the spermatozoon head. [a,b,c] Different superscript letters within the time intervals of fresh semen are statistically different ($p < 0.05$). [d,e] Different superscript letters within the time intervals of frozen semen are statistically different ($p < 0.05$).

The highest values of STR, LIN and BCF were found at 2 h, these values were significantly different in comparison to those recorded at 3, 4, 5 and 6 h.

The duration of sperm movement was significantly higher at 1 h compared to the other storage times and it reached the lowest value at 2 and 6 h. At 4 h of storage, an increase in duration of sperm movement was observed; this was a significant result in respect to those found at 2 h and 6 h of storage (Figure 1I).

2.2. Effect of Storage Time Post-Collection on Frozen Sperm Motility Parameters

The cold-storage time after sperm collection significantly decreased the total post-thaw motility between 1 and 6 h of storage (Figure 1A) and the duration of sperm movement between 1 and 5 h (Figure 1I), whilst no significant differences were observed for all the other parameters tested.

2.3. Effect of Storage Time Post-Collection on Post-Thaw Fertilization Rate

The percentage of eyed embryos obtained using fresh semen was of 79.4% ± 5.0%, confirming the good quality of the eggs used for the fertilization trial.

Cold-storage time did not significantly affect the percentage of eyed embryos following the use of frozen semen (Table 1). In fact, the average fertilization percentages ranged from 65% to 55% without significant differences among the storage times post-collection tested.

Table 1. Fertilization rate recorded in frozen semen after different storage time intervals.

Storage Time	Fertilization Rate (%)	
	Means ± SE	Min—Max
1 h	65.2 ± 6.3 [a]	43.0—87.5
3 h	60.4 ± 6.9 [a]	40.8—79.3
6 h	54.8 ± 3.5 [a]	41.3—64.4

[a] Different superscript letters within the same column indicate a significant difference ($p < 0.05$).

3. Discussion

In this study, we evaluated the effect of different cold-storage time intervals (from 1 h to 6 h) that elapsed between collection and semen-freezing on both fresh and cryopreserved semen motility parameters and post-thaw fertilizing ability of Mediterranean brown trout semen. The rationale of this research was to understand if *Salmo cettii* sperm could be kept on ice up to a maximum of six hours storage time without losing its suitability for freezing. The results showed that the cold-storage interval significantly influenced some motility parameters of fresh semen, but surprisingly no significant effects were observed on all post-thaw sperm motility parameters tested (except for total motility recorded at 6 h of storage) and on fertilization rates.

In accordance with the results found on rainbow trout by Lahnsteiner [19], in fresh semen, no significant difference of sperm motility parameters was recorded up until 2 h of storage on ice. After the second hour, the total sperm motility, linear movement parameters (STR, LIN) and BCF showed a progressive decrease ($p < 0.05$), whilst ALH values underwent a significant increase. Thus, our results showed that as the amount of storage time was increased, the trajectory of sperm movement tended to become increasingly circular and less progressive. Cremades et al. [20], showed that changes in boar sperm movement patterns are the results of physiological events in spermatozoon. Similar to what has been observed in mammalian sperm, the movement patterns that occur in fresh trout sperm, starting from the second hour onwards equate to those of mammalian spermatozoa in a hyperactive state. Indeed, the movement pattern of hyperactive sperm is generally characterized by low STR and LIN values, high amplitude and asymmetrical flagellar beating, representing an increased torsional force [20–26]. As a result, hyperactive sperm tend to swim in vigorous circles [27].

In mammals, hyperactivation usually occurs during sperm capacitation, which allows spermatozoa to penetrate the pellucida zone and fertilize the oocyte. Therefore, it is considered a critical event in the success of fertilization [28,29]. Moreover, sperm in a state of hyperactivation were observed in mammals during the cryopreservation process. This phenomenon is called "cryocapacitation" and it is triggered during the cooling process when the temperature is in the vicinity of 5 °C. This is due to the enhancement of cold shock [30,31], which provokes a pathologic influx of Ca^{2+} in sperm and the hyperactivation of its motility [20,32–34], thereby decreasing the life span of sperm [20,32]. Unlike mammalian spermatozoa, there is no evidence of the presence of hyperactivation and cryocapacitation phenomena in fish sperm, including in salmonids, as they do not possess acrosome. However, we can speculate that changes in the sperm motility patterns observed in our study during cold storage, may be due to mechanisms that trigger the hyperactive movement, similar to those that are found in cryocapacitated mammalian sperm.

Several studies on different fish species such as rainbow trout [35–37], common carp [38], Atlantic croaker [39], and sterlet sturgeon [40] have reported that calcium chan-

nels play a key role in regulating sperm motility. Recently, some authors have identified the presence of CatSper-like protein in the spermatozoa of many fish, including rainbow trout [41,42]. In particular, CatSper is a Ca^{2+}-specific channel of mammalian spermatozoa plasma membrane, which by mediating $Ca^{2+}{}_i$ influx induces the initiation of the vigorous and hyperactive sperm motility prior to fertilization [43,44]. A recent study [42], demonstrated, for the first time, that this ionic channel plays a key role even in the sperm motility of Atlantic salmon. In the light of these considerations, we could assume that a cold-storage time longer than 2 h, prior to freezing, could cause cold shock injury in the plasma membrane of trout spermatozoa and an increase in membrane permeability, resulting in a pathologic influx of Ca^{2+} into the cell. Consistent with our results, Labbè and Maisse [45] claimed that semen from rainbow trout does not undergo any cold shock when it is stored for 1 h, at 4 °C just after stripping. Our data led us to think that possible physical, biochemical and physiological changes in trout sperm begin to be triggered from the second hour of cold storage. However, further analysis to support our hypothesis, such as the measurement of concentration of the cholesterol in sperm membrane and the intracellular Ca^{2+} in the cryopreserved semen samples, is needed

The most interesting result that emerged in our study is that in contrast to in vitro results observed in fresh semen, the cold-storage time did not significantly affect the post-thaw sperm motility parameters, with the exception of the total motility recorded at the sixth hour of storage. Thus, we can postulate that the cold-storage time eliminates the weakest sperm population leaving those that are more cryoresistant. To be specific, two distinct sperm populations could coexist: one cool-sensitive population, which 2 h after semen collection undergoes cold shock injury, thereby losing its freezability features, and a second cryoresistant population, which survives cold injuries up to 6 h and keeps the sperm motility parameters constant even after freezing. The cold-sensitive population could be the product of defective spermatogenesis, resulting in membrane weakness, defective enzymatic activity, low glycolytic activity and mitochondrial respiration, or a consequence of the stripping method, which induces the release of non-completely mature spermatozoa [46].

The post-thaw motility results were consistent with those of post-thaw fertilization obtained from in vivo trials, using semen stored on ice for 1, 3 or 6 h prior to freezing. Although a progressive decrease in fertilization rate was observed over time, no significant differences were recorded from 1 to 6 h of storage. In agreement with values of sperm total motility and the duration of movement recorded in fresh semen, the highest percentage of fertilization was achieved at 1 h of storage, of which we assume that fewer spermatozoa were affected by cryo-injuries. In this regard, it is important to stress that the number of spermatozoa affected by cryo-injuries is always related to the individual ejaculates' initial quality. Indeed, the range of the fertilization rates reported in Table 1 show a wide inter-individual variability to the response to storage time on the post-thaw fertilization rate. However, regardless of the storage time and inter-male variability, a minimum success of fertilization rate that ranged from 40–43% was always guaranteed.

4. Materials and Methods

4.1. Animal Capture and Sperm and Eggs Collection

Specimens of autochthonous *Salmo cettii* were caught in the Biferno River (Molise region, latitude: 41°28′47.8″ N and longitude: 14°28′40.9″ E) during the spawning season (January–February 2020), by electro-fishing. Twelve individuals were first identified according to their phenotypic traits [47–49] and, subsequently, were crossed with genetic data to ensure that the individuals were autochthonous. These individuals (10 males and 2 females) were aged between 2+ to 5+ years, and the average total lengths of the fish were 24.9 ± 6.1 cm for males and 29.7 ± 7.3 cm for females.

Sperm samples were collected by gentle abdominal massaging; abdomens and urogenital papilla were dried with special care before stripping, in order to avoid contamination of semen with urine, mucus and blood cells. The tubes containing sperm were transferred to

the laboratory in a cooler that contained ice, where the experimental design (see Section 2.2) took place.

Eggs were stripped by gentle abdominal massage into a dry metal bowl and were checked visually to ensure that those used in the fertilization experiments were well-rounded and transparent.

The experiments were conducted in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments. This study took place within Nat.Sal.Mo LIFE project, which 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–Dipartimento Governo del Territorio, Mobilità e Risorse Naturali cod. DP.A4.02.4N.01 (protocol number 3969, 3 August 2018), according to the current regulations on the protection of the species, biosecurity, protocols of sampling of fresh water and animal welfare.

4.2. In Vitro Experimental Design and Cryopreservation Procedure

To begin, six aliquots of equal volume from each sperm sample (n = 10 male) were split into 1.5 mL cryovials and stored on ice for 1, 2, 3, 4, 5 and 6 h respectively, until freezing (6 intervals × 10 males = 60 total aliquots). After each storage time interval, an aliquot of fresh semen was subjected to sperm motility evaluation by a Computer-Assisted Sperm Analysis (CASA) system, whilst another one was frozen using the cryopreservation procedure optimized in our previous work [17] (Figure 2). Briefly, each semen aliquot was diluted with a freezing extender to reach 0.15 M of glucose, 7.5% of methanol and sperm concentration of 3.0×10^9 sperm/mL. The diluted semen was charged into 0.25 mL plastic straws and equilibrated for 15 min on ice (at the height of 3 cm), lastly the straws were cryopreserved by exposure to liquid nitrogen (LN_2) vapor at 3 cm above the LN_2 level for 5 min and plunged into LN_2. Frozen semen samples were tested after each post-collection storage time, and were thawed at 40 °C for 5 s and immediately evaluated using the sperm motility analysis.

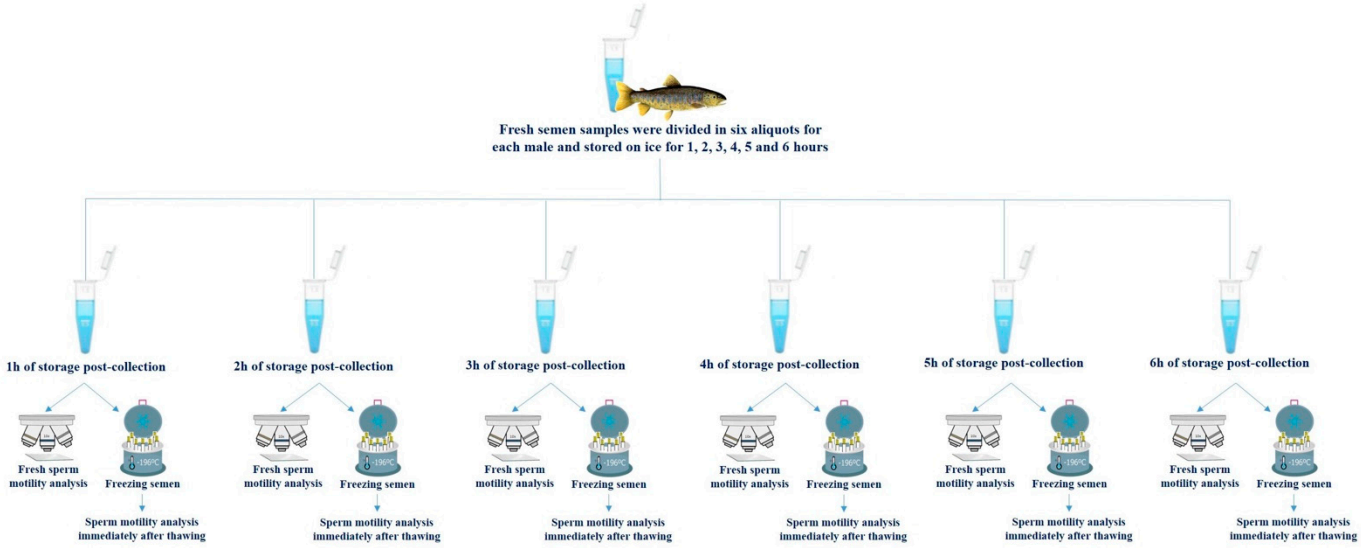

Figure 2. In Vitro experimental design.

4.3. Sperm Analysis

The fresh semen concentration was measured with a Neubauer chamber. The samples were diluted 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. The average sperm concentration was $15.3 \pm 4.9 \times 10^9$ sperm/mL.

The sperm motility parameters were determined with the use of a CASA system coupled to a phase contrast microscope (Nikon model Ci-L) using the Sperm Class Analyser (SCA) software (VET Edition, Barcelona, Spain). For the sperm motility activation, fresh and frozen spermatozoa were diluted as reported in our previous paper [16], reaching a concentration of 6.0×10^7 sperm/mL. After, an aliquot of 3 µL was loaded onto a 20 micron Leja slide (Leja Standard Count, Nieuw Vennep The Netherlands) and 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]), linearity (LIN, [%]) and straightness (STR, [%)]), beat cross frequency (BCF, [Hz]) and amplitude of lateral displacement of the spermatozoon head (ALH, [µm]). The duration of sperm movement was evaluated using a chronometer. Three replicates were performed for sperm motility evaluation of each fresh and frozen semen sample.

4.4. Fertilizing Ability Trials of Cryopreserved Semen

The fertilization trial was performed in February 2020. Pooled eggs from two females were divided into batches of 80 ± 9 eggs, using 36 glass laboratory jars: the eggs in six of the jars were fertilized using excess fresh semen at the beginning and at the end of the fertilization trial (control groups), in order to test the quality of the eggs, whilst the eggs in the remaining 30 jars were divided into three treatment groups. Each treatment group was fertilized using the semen of individual males (n = 10) frozen/thawed after a cold-storage interval of 1, 3 or 6 h, using a spermatozoa-to-egg ratio of 4.5×10^5:1. Before adding the semen, 5 mL of fertilization solution D532 (20 mM Tris, 30 mM glycine, 125 mM NaCl, pH 9.0 [50]) was added to the eggs. The sperm was gently mixed with the eggs for 10 s, and then about 20 mL of hatchery water was added. After 2 min, the eggs were washed with hatchery water and placed into an incubator with running water at about 10 °C.

Unfertilized and dead eggs were counted and removed every day during the incubation. After 25–30 days, the eggs reached the eyed-egg stage. The fertilization success was established by calculating the percentage of embryos at the eyed stage, using the initial number of eggs (number of eyed eggs × initial egg number^{-1} × 100).

4.5. Statistical Analysis

Values are expressed as mean ± standard error (SE). The statistical analysis was conducted with the software package SPSS (SPSS 15.0 for Windows, 2006; SPSS, Chicago, IL, USA). Sperm motility parameters and fertilization rates measured across the different storage time intervals were compared by one-way analysis of variance (one-way ANOVA) followed by Duncan's comparison test. The level of significance for all statistical tests was set to 5% ($p < 0.05$).

5. Conclusions

In conclusion, our results showed that although it is recommendable to freeze semen of *S. cettii* within 1–2 h of collection, good fertilization rates are achievable even 6 h after semen collection from wild breeders. This last scenario is a valid expedient for us when the sampling sites are far away from the laboratory. These results provide an important contribution to improving the sampling management in the case of our wild specimen. Finally, further studies are needed to understand the biological mechanisms involved in the trout sperm movement pattern, and if these patterns are similar to the hyperactivity triggered following the cooling steps.

Author Contributions: Conceptualization, N.I., G.R. and M.D.I.; Data curation, N.I., S.E. and M.D.I.; Formal analysis, G.R., M.D.I., R.I. and S.E.; Methodology, G.R., M.D.I. and R.I.; Writing—original draft, N.I., G.R. and M.D.I.; Writing—review and editing, A.R. and S.E. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the LIFE Nat.Sal.Mo. project (LIFE17 NAT/IT/000547).

Institutional Review Board Statement: The study was conducted in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments and approved by the Ministero dell'Ambiente e della Tutela del Territorio e del Mare (Divisione II – Biodiversità, Aree Protette, Flora e Fauna – Prot. number 17975, 11/06/2019).

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: The authors thank Amber Burchell for the English revision of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Crivelli, A.J.; Poizat, G.; Berrebi, P.; Jesensek, D.; Rubin, J.F. Conservation biology applied to fish: The example of a project for rehabilitating the Marble trout (Salmo marmoratus) in Slovenia. *Cybium* **2000**, *24*, 211–230.
2. Araguas, R.M.; Sanz, N.; Fernández, R.; Utter, F.M.; Pla, C.; García-Marín, J.-L. Genetic refuges for a self-sustained fishery: Experience in wild brown trout populations in the eastern Pyrenees. *Ecol. Freshw. Fish* **2008**, *17*, 610–616. [CrossRef]
3. Araguas, R.M.; Sanz, N.; Fernández, R.; Utter, F.M.; Pla, C.; García-Marín, J.-L. Role of Genetic Refuges in the Restoration of Native Gene Pools of Brown Trout. *Conserv. Biol.* **2009**, *23*, 871–878. [CrossRef]
4. Caudron, A.; Champigneulle, A.; Guyomard, R.; Largiadèr, C.R. Assessment of three strategies practiced by fishery managers for restoring native brown trout (Salmo trutta) populations in Northern French Alpine Streams. *Ecol. Freshw. Fish* **2011**, *20*, 478–491. [CrossRef]
5. Caputo Barucchi, V.; Carosi, A.; Giovannotti, M.; La Porta, G.; Splendiani, S.; Lorenzoni, M. Life+ Trout Project (LIFE12 NAT/IT/0000940) for the Recovery and Conservation of Mediterranean Trout (Salmo Trutta Complex) in the Central Apennines (Italy). Front. Mar. Sci. Conference Abstract. In Proceedings of the XV European Congress of Ichthyology, Porto, Portugal, 7–11 September 2015.
6. Bianco, P.G.; Caputo Barucchi, 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. Berrebi, P.; Povz, M.; Jesensek, D.; Cattaneo-Berrebi, G.; Crivelli, A.J. The genetic diversity of native, stocked and hybrid populations of marble trout in the Soča river, Slovenia. *Heredity* **2000**, *85*, 277–287. [CrossRef] [PubMed]
8. Berrebi, P.; Jesenšek, D.; Crivelli, A.J. Natural and domestic introgressions in the marble trout population of Soča River (Slovenia). *Hydrobiologia* **2017**, *785*, 277–291. [CrossRef]
9. Marzano, F.N.; Corradi, N.; Papa, R.; Tagliavini, J.; Gandolfi, G. Molecular Evidence for Introgression and Loss of Genetic Variability in Salmo (trutta) macrostigma as a Result of Massive Restocking of Apennine Populations (Northern and Central Italy). *Environ. Boil. Fishes* **2003**, *68*, 349–356. [CrossRef]
10. Caputo, V.; Giovannotti, M.; Cerioni, P.N.; Caniglia, M.L.; Splendiani, A. Genetic diversity of brown trout in central Italy. *J. Fish Biol.* **2004**, *65*, 403–418. [CrossRef]
11. Querci, G.; Pecchioli, E.; Leonzio, C.; Frati, F.; Nardi, F. Molecular characterization and hybridization in Salmo (trutta) macrostigma morphotypes from Central Italy. *Hydrobiologia* **2012**, *702*, 191–200. [CrossRef]
12. Gratton, P.; Allegrucci, G.; Sbordoni, V.; Gandolfi, A. The evolutionary jigsaw puzzle of the surviving trout (*Salmo trutta* L. complex) diversity in the Italian region. A multilocus Bayesian approach. *Mol. Phylogenet. Evol.* **2014**, *79*, 292–304. [CrossRef]
13. Splendiani, A.; Ruggeri, P.; Giovannotti, M.; Pesaresi, S.; Occhipinti, G.; Fioravanti, T.; Lorenzoni, M.; Cerioni, P.N.; Barucchi, V.C. Alien brown trout invasion of the Italian peninsula: The role of geological, climate and anthropogenic factors. *Biol. Invasions* **2016**, *18*, 2029–2044. [CrossRef]
14. 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* **2015**, *24*, 668–675. [CrossRef] [PubMed]
15. Di Iorio, M.; Esposito, S.; Rusco, G.; Roncarati, A.; Miranda, M.; Gibertoni, P.P.; Cerolini, S.; Iaffaldano, N. 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**, *9*, 1–9. [CrossRef] [PubMed]

16. Rusco, G.; Di Iorio, M.; Gibertoni, P.P.; Esposito, S.; Penserini, M.; Roncarati, A.; Cerolini, S.; Iaffaldano, N. Optimization of Sperm Cryopreservation Protocol for Mediterranean Brown Trout: A Comparative Study of Non-Permeating Cryoprotectants and Thawing Rates In Vitro and In Vivo. *Animals* **2019**, *9*, 304. [CrossRef] [PubMed]
17. Rusco, G.; Di Iorio, M.; Iampietro, R.; Esposito, S.; Gibertoni, P.P.; Penserini, M.; Roncarati, A.; Iaffaldano, N. A Simple and Efficient Semen Cryopreservation Method to Increase the Genetic Variability of Endangered Mediterranean Brown Trout Inhabiting Molise Rivers. *Animals* **2020**, *10*, 403.
18. Jawahar, K.T.P.; Betsy, J. Cryopreservation of Fish Gametes: An Overview. In *Cryopreservation of Fish Gametes*; Springer Science and Business Media LLC: Berlin/Heidelberg, Germany, 2020; pp. 151–175.
19. Lahnsteiner, F.; Weismann, T.; Patzner, R.A. Aging Processes of Rainbow Trout Semen during Storage. *Progress. Fish Cult.* **1997**, *59*, 272–279. [CrossRef]
20. Cremades, T.; Vazquez, J.M.; Martínez, E.A.; Roca, J.; Rodriguez-Martinez, H.; Abáigar, T. Kinematic Changes during the Cryopreservation of Boar Spermatozoa. *J. Androl.* **2005**, *26*, 610–618. [CrossRef]
21. Katz, D.F.; Yanagimachi, R. Movement Characteristics of Hamster and Guinea Pig Spermatozoa upon Attachment to the Zona Pellucida. *Biol. Reprod.* **1981**, *25*, 785–791. [CrossRef]
22. Suarez, S.S.; Katz, D.F.; Overstreet, J.W. Movement Characteristics and Acrosomal Status of Rabbit Spermatozoa Recovered at the Site and Time of Fertilization. *Biol. Reprod.* **1983**, *29*, 1277–1287. [CrossRef]
23. Suarez, S.S. Hamster sperm motility transformation during development of hyperactivation in vitro and epididymal maturation. *Gamete Res.* **1988**, *19*, 51–65. [CrossRef]
24. Shivaji, S.; Peedicayil, J.; Devi, L.G. Analysis of the motility parameters of in vitro hyperactivated hamster spermatozoa. *Mol. Reprod. Dev.* **1995**, *42*, 233–247. [CrossRef] [PubMed]
25. Casas, I.; Sancho, S.; Briz, M.D.; Pinart, E.; Bussalleu, E.; Yeste, M.; Bonet, S. Freezability prediction of boar ejaculates assessed by functional sperm parameters and sperm proteins. *Theriogenology* **2009**, *72*, 930–948. [CrossRef] [PubMed]
26. Curtis, M.; Kirkman-Brown, J.C.; Connolly, T.; Gaffney, E. Modelling a tethered mammalian sperm cell undergoing hyperactivation. *J. Theor. Biol.* **2012**, *309*, 1–10. [CrossRef] [PubMed]
27. Suarez, S.S.; Ho, H.C. Hyperactivation of mammalian sperm. *Cell. Mol. Boil.* **2003**, *49*, 351–356.
28. Yanagimachi, R. In Vitro Capacitation of Hamster Spermatozoa by Follicular Fluid. *Reproduction* **1969**, *18*, 275–286. [CrossRef]
29. Ho, H.-C.; Suarez, S.S. An Inositol 1,4,5-Trisphosphate Receptor-Gated Intracellular Ca2+ Store Is Involved in Regulating Sperm Hyperactivated Motility. *Biol. Reprod.* **2001**, *65*, 1606–1615. [CrossRef]
30. Watson, P. The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci.* **2000**, *60–61*, 481–492. [CrossRef]
31. Andrabi, S.M.H. Fundamental principles of cryopreservation of Bos taurus and Bos indicus bull spermatozoa (Minireview). *Int. J. Agric. Biol.* **2007**, *9*, 367–369.
32. Green, C.; Watson, P. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. *Reprodoction* **2001**, *122*, 889–898. [CrossRef]
33. Harrison, R.A.; Gadella, B.M. Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology* **2005**, *63*, 342–351. [CrossRef]
34. Saravia, F.; Hernández, M.; Wallgren, M.; Johannisson, A.; Rodriguez-Martinez, H. Controlled cooling during semen cryopreservation does not induce capacitation of spermatozoa from two portions of the boar ejaculate. *Int. J. Androl.* **2007**, *30*, 485–499. [CrossRef] [PubMed]
35. Tanimoto, S.; Morisawa, M. Roles for potassium and calcium channels in the initiation of sperm motility in rainbow trout (K^+/Ca^{2+}/channel/sperm motility/rainbow trout). *Develop. Growth Differ.* **1988**, *30*, 117–124. [CrossRef]
36. Tanimoto, S.; Nakazawa, T.; Kudo, Y.; Morisawa, M. Implication that potassium flux and increase in intracellular calcium are necessary for the initiation of sperm motility in salmonid fishes. *Mol. Reprod. Dev.* **1994**, *39*, 409–414. [CrossRef]
37. Kho, K.H.; Tanimoto, S.; Inaba, K.; Oka, Y.; Morisawa, M. Transmembrane Cell Signaling for the Initiation of Trout Sperm Motility: Roles of Ion Channels and Membrane Hyperpolarization for Cyclic AMP Synthesis. *Zool. Sci.* **2001**, *18*, 919–928. [CrossRef]
38. Krasznai, Z.; Morisawa, M.; Morisawa, S.; Krasznai, Z.T.; Trón, L.; Gáspár, R.; Márián, T. Role of ion channels and membrane potential in the initiation of carp sperm motility. *Aquat. Living Resour.* **2003**, *16*, 445–449. [CrossRef]
39. Detweiler, C.; Thomas, P. Role of ions and ion channels in the regulation of Atlantic croaker sperm motility. *J. Exp. Zool.* **1998**, *281*, 139–148. [CrossRef]
40. Bondarenko, O.; Dzyuba, B.; Rodina, M.; Cosson, J. Role of Ca^{2+} in the IVM of spermatozoa from the sterlet Acipenser ruthenus. *Reprod. Fertil. Dev.* **2017**, *29*, 1319–1328. [CrossRef]
41. Yanagimachi, R.; Harumi, T.; Matsubara, H.; Yan, W.; Yuan, S.; Hirohashi, N.; Iida, T.; Yamaha, E.; Arai, K.; Matsubara, T.; et al. Chemical and physical guidance of fish spermatozoa into the egg through the micropyle†,‡. *Biol. Reprod.* **2017**, *96*, 780–799. [CrossRef]
42. Lissabet, J.F.B.; Belén, L.H.; Lee-Estevez, M.; Risopatrón, J.; Valdebenito, I.; Figueroa, E.; Farias, J. The CatSper channel is present and plays a key role in sperm motility of the Atlantic salmon (Salmo salar). *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2020**, *241*, 110634. [CrossRef]
43. Kirichok, Y.; Navarro, B.; Clapham, D. Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. *Nat. Cell Biol.* **2006**, *439*, 737–740. [CrossRef] [PubMed]

44. Darszon, A.; Guerrero, A.; Galindo, B.E.; Nishigaki, T.; Wood, C.D. Sperm-activating peptides in the regulation of ion fluxes, signal transduction and motility. *Int. J. Dev. Biol.* **2008**, *52*, 595–606. [CrossRef] [PubMed]
45. Labbé, C.; Maisse, G. Influence of rainbow trout thermal acclimation on sperm cryopreservation: Relation to change in the lipid composition of the plasma membrane. *Aquaculture* **1996**, *145*, 281–294. [CrossRef]
46. Martínez-Pastor, F.; Fernández-Santos, M.R.; Del Olmo, E.; Domínguez-Rebolledo, A.E.; Esteso, M.C.; Montoro, V.; Garde, J.J. Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. *Reprod. Fertil. Dev.* **2008**, *20*, 547–556. [CrossRef] [PubMed]
47. Gibertoni, P.P.; Jelli, F.; Bracchi, P. Allevamento, riproduzione e reintroduzione in ambiente naturale di trote fario di "ceppo mediterraneo", *Salmo (trutta) trutta*, L. *Ann. Della Fac. Med. Vet.* **1998**, *18*, 1–20.
48. Jelli, F.; Gibertoni, P.P. *Recupero e Reintroduzione di Ceppi Autoctoni di Trota Fario, Salmo (trutta) trutta L., nel Bacino del Fiu-me Secchia. Atti del Convegno: La Trota Fario, Salmo (trutta) trutta L. di Ceppo Mediterraneo*; Esperienze Gestionali a Confronto: Reggio Emilia, Italy, 1999; pp. 21–28.
49. Penserini, M.; Nonnis Marzano, F.; Gandolfi, G.; Maldini, M. Fenotipi della trota mediterranea: Metodologia di indagine molecolare combinata e selezione morfologica per l'identificazione degli esemplari autoctoni. *J. Freshwat. Biol.* **2006**, *34*, 69–75.
50. Billard, R. Reproduction in rainbow trout: Sex differentiation, dynamics of gametogenesis, biology and preservation of gametes. *Aquaculture* **1992**, *100*, 263–298. [CrossRef]