Could the Storage Time Post-collection Affect the Post-thaw Motility and Fertilizing Ability of Mediterranean Brown Trout Semen?

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Abstract

The aim of this study was to evaluate the effect of different cool 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 for 1 to 6 hours, until freezing. Fresh and post-thawing sperm motility were evaluated by 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 was recorded, whilst the amplitude of lateral displacement of the spermatozoon head underwent a significant increase. Velocity parameters (VCL, VAP and VSL) were not affected by the cold storage time, whilst the duration of sperm movement was significantly higher at 1h compared to the other times tested. Freezing procedure overall decreased almost all post-thaw sperm motility parameters, however no significant differences was observed over time, both in term of fast and linear movement. Cool storage time did not significantly affect the percentage of post-thaw eyed embryos.

Our results showed that Mediterranean brown trout semen can be stored on ice even up to 6 hours before freezing, without decreasing its post-thawing quality and fertilizing ability.

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 complex at a greater risk of extinction in the Mediterranean area and it is listed in 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 as a major threat to the survival of this species, by altering the traits of the native gene pool [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 realised by using the technique 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 results as a "tank" of genetic variability for *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 successfulness of cryopreservation procedure, the good initial quality of semen is an indispensable prerequisite. The latter depends on the donor selected, a correct semen collection method, the use of appropriate conditions for transport and the semen storage times before freezing [18]. Relatively to this last factor, in our project the time that elapses between collection and processing of semen resulted as variable due to the fact that the sampling sites in the project area are not easily accessible and are distant from each other as well as from the laboratory. Because of this, we have estimated that the time range that elapses between collection and semen freezing could vary from a minimum of 1h to maximum of 6h, depending on the sampling site and the time that is consumed to capture the broodstock. Given these large time intervals, we questioned if the longer intervals of time prior to freezing could cause a loss of fresh semen quality and consequently adversely affect the spermatozoa freezability.

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

2. Materials And Methods

2.1 Animal capture and sperm and eggs collection

Specimens of autochthonous *S. cettii* were caught from the Biferno river (Molise region, (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 individual Mediterranean brown trout fish were first identified according to their phenotypic traits [19–21], and were subsequently crossed with genetic data to ensure that the individuals were autochthonous. These individuals (10 males and 2 females) were aged at 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. Following samples collection, the tubes containing sperm were transferred to the Laboratory of Zoocolture at the University of Molise in a cooler that contained ice, where the experimental design (see Sect. 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 that received “a positive opinion” from the Ministry of the Environment and the Protection of the Territory and the Sea.

2.2 In vitro experimental design and cryopreservation procedure

In order to evaluate the effects of cool storage time intervals (from 1h to 6h) between collection and semen freezing on both fresh and cryopreserved semen motility parameters and post-thaw fertilizing ability, six possible scenarios were developed. In this regard, 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 hours respectively, until freezing (6 intervals × 10 males = 60 total aliquots). After each storage time interval, an aliquot of fresh semen was subjected to the sperm motility evaluation by a Computer-Assisted Sperm Analysis (CASA) system, whilst the other one was frozen using the cryopreservation procedure optimized in our previous work [17]. Briefly, each semen aliquot was diluted with freezing extender in order to reach the concentration of 0.15 M of glucose, 7.5% of methanol and a sperm concentration of 3.0×10^9 sperm/mL. The diluted semen was loaded into 0.25 mL plastic straws and equilibrated for 15 min on ice (at the height of 3 cm), lastly the straws were cryopreserved through exposure to liquid nitrogen vapor at 3 cm above the liquid nitrogen level for 5 min. They were then plunged into liquid nitrogen.

2.3 Sperm analysis

The fresh semen concentration was measured with a Neubauer chamber. In this regard, the samples were diluted 1:1,000 (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 sperm motility parameters were determined with the use of CASA system coupled to a phase contrast microscope (Nikon model Ci-L) using the Sperm Class Analyser (SCA) software (VET Edition, Barcelona, Spain). Fresh spermatozoa were activated in 0.3% NaCl at a dilution ratio of 1:400. To evaluate the post thawing semen motility the straws were thawed in a 40°C water bath [17] and then activated using a solution with 1% $NaHCO_3$ at a dilution ratio of 1:30 in order to reach a sperm concentration of 100×10^6 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.

2.4 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 cool 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 (20mM Tris, 30 mM glycine, 125 mM NaCl, pH 9.0 [22])

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).

2.5 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 analysis of variance (ANOVA) followed by Duncan's comparison test. To compare the difference between fresh and frozen semen at each storage time interval independent-samples t-tests were used. The level of significance for all statistical tests was set to 5% ($P < 0.05$).

3. Results

3.1 Effect of storage time post-semen collection on fresh sperm motility parameters

Some fresh motility semen parameters were significantly affected ($P < 0.05$) by the cool storage time following the sperm collection (Fig. 1). Total sperm motility was not affected until 2 h, after this time a significant progressive decrease was recorded (Fig. 1A). No significant differences, during storage intervals, for kinetic parameters (VCL, VAP and VSL) was recorded (Fig. 1B-D), although a slight sperm velocity increase (VAP and VCL) at 3 and 4 h of storage was observed (Fig. 1B, C). The lowest percentage of ALH was noted at 2 h of storage and no significant difference was recorded with respect to 1 h of storage, while a significant increase from 3 to 6 h in respect to the second hour was observed (Fig. 1G).

The highest values for derived parameters (STR and LIN) and BCF were recorded at 2 h, although no significant differences compared to 1 h of storage were observed (Fig. 1E-F, H). After 2 h, a significant time-dependent decrease for all these parameters was recorded. The duration of sperm movement was significantly higher at 1h compared to the other storage times, whilst at 2h of storage it underwent a significant decrease, and it reached its lowest value. After 2 h cool storage an increase that resulted as significant was observed at 4 h, and then a decrease that resulted significant was observed at 6h of storage ($P < 0.05$) (Fig. 1I).

3.2 Comparison between fresh and frozen semen quality parameters at the different intervals of storage time post-

collection

The cryopreservation procedure decreased almost all post-thaw sperm motility parameters compared to those of the fresh counterparts (Fig. 1). A significant decrease of total sperm motility of post thawed semen in respect to that of the fresh semen for all times considered was observed (Fig. 1A). For VCL and VAP a significant decrease was recorded at 3 and 4 h of storage (Fig. 1B, C), whilst for VSL the decrease was significant only at the second hour (Fig. 1D). The post-thaw ALH values at 1 and 2 h were higher compared to the fresh counterpart, for which a significant difference was registered only at the second hour (Fig. 1G). BCF values declined ($P < 0.05$) at 1–3 and 6 h of storage (Fig. 1H). The lowest ($P < 0.05$) post-thaw values of LIN were observed at 1 and 2 h and only at the second hour for STR (Fig. 1E-F). Duration of sperm movement was significantly diminished for all intervals of storage time, except for at 2 and 6 h (Fig. 1I).

3.3 Effect of storage time post-collection on frozen sperm motility parameters

Cool storage time after sperm collection did not significantly affect the sperm motility parameters of frozen/thaw semen (Fig. 1), except for the total sperm motility, where a decrease was observed with a significant difference between 1 and 6 h of storage (Fig. 1A) and for the duration of sperm movement between 1 and 5 h (Fig. 1I).

3.4 Effect of storage time post-collection on post-thaw fertilization rate

Cool storage time did not significantly affect the percentage of post-thaw eyed embryos (Table 1), although a gradual decrease in the fertilization rate was observed over time. The highest fertilization rate was recorded at 1h of storage (65.2 ± 6.3) and no significant differences were observed when compared to fresh semen (79.4 ± 5.0) and subsequent storage times (3 and 6 h).

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

Storage time	Fertilization rate (%)	
	Means ± SE	Min -Max
Fresh	79.4 ± 5.0 ^{a}	62.3–95.8
Frozen 1h	65.2 ± 6.3^{ab}	43.0–87.5
3h	60.4 ± 6.9^{b}	40.8–79.3
6h	54.8 ± 3.5^{b}	41.3–64.4
^{a,b}Different superscript letters within the same column indicate a significant difference ($p < 0.05$).		

4. Discussion

In this study we evaluated the effect of different cool storage time intervals (from 1h to 6h) 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 *S. 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 cool storage interval significantly influenced some motility parameters of fresh semen, but surprisingly no significant effects were observed on post-thaw sperm motility and fertilization rate.

In fresh semen, in accordance with the research on rainbow trout by Lahnsteiner et al. [23]no significant difference of sperm motility parameters was recorded up to 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 ($P < 0.05$), conversely the percentage of ALH underwent a significant increase. At the same time, even if not significant, visible increases for sperm velocity parameters, VCL and VAP, at 3 and 4 hours of storage were observed, while a simultaneous and progressive decrease of the VSL was recorded.

In other words, as storage time increased, the trajectory of movement became increasingly circular and less progressive. Cremades et al. [24], showed that changes in boar sperm movement patterns are the result of physiological events in spermatozoon. In agreement with what has been observed in mammalian the movement patterns of fresh trout sperm, as shown from the second storage hour onwards, equates to that of mammalian spermatozoa in the hyperactive state. Indeed, the movement pattern of hyperactive sperm is generally characterized by low VSL, STR and LIN values and an increase in ALH and VCL parameters [24–29]. The flagella of hyperactivated sperm forms deeper bends and their beating is usually asymmetric. As a result, hyperactivated sperm tends to swim vigorously in circles [30].

In mammals, hyperactivation usually occurs during sperm capacitation, allowing spermatozoa to penetrate the zona pellucida and fertilize the oocyte, therefore it is considered a critical event to the success of fertilization [31, 32]. However, also during the cryopreservation process mammalian spermatozoa in state of hyperactivation were observed. This phenomenon is called "cryocapacitation" and it triggers during the cooling process in the vicinity to 5°C due to the enhancement of cold shock [33, 34], which provokes a pathologic influx of Ca^{2+} in sperm and the hyperactivation of its motility [24, 35–37], decreasing the life span of sperm [24, 35].

Unlike mammalian spermatozoa, there is no mention in literature of hyperactivation and cryocapacitation phenomena of spermatozoa in fish species. However, we can speculate that changes in the fish sperm motility pattern before fertilization, and then probably even during the cooling process, may have something in common with the hyperactivated movement of mammalian spermatozoa which are about to fertilize eggs [38], or that occurs as a result of cryocapacitation [33, 34]. Our speculation can be supported by recent findings [39], in which the presence of cAMP-dependent protein kinase and CatSper-

like protein in the spermatozoa of many fish, including trout, were identified. 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 [40, 41].

In the light of these considerations, we assume that a cool storage time longer than 2 h, prior to freezing, could causes cold shock injury in plasma membrane of trout spermatozoa and an increase of membrane permeability, resulting in a pathologic influx of Ca^{2+} into the cell.

Consistent with our results, Labbè and Maisse [42] 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 adds further evidence that the possible physical, biochemical and physiological changes in trout sperm are triggered starting from the second hour of cool storage. These changes are reflected in turn in spermatozoa swimming patterns, which exhibit similar behaviour to that of hyperactive mammalian spermatozoa [24, 29, 43].

The most interesting result that emerged in our study is that on the contrary to *in vitro* results observed in fresh semen, the cool storage time didn't significantly affect the post-thaw sperm motility parameters. Although the cryopreservation process caused an overall decrease in the most of the sperm motility parameters at almost each time point tested compared to fresh semen, no significant differences was observed among post-thaw values recorded over time, both in term of fast and linear movement.

Thus, we can sustain that the cool storage time eliminates the weakest sperm leaving those cryoresilient. In this regard, we could hypothesize the presence of two distinct sperm populations: one cool-sensitive population which, after 2 h collection, underwent cold shock injury losing its freezability features and a second cryoresilient population, which survived cold injuries up to 6 hours and kept constant the average sperm motility parameters 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 [44].

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 are affect 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 the Table 1, show a wide inter-individual variability of 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.

Conclusion

In conclusion, our results showed that even after 6 hours of cool storage time post collection, the post thawing semen quality is preserved, and its fertilizing capacity is not compromised. These results provide an important contribution to improve the sampling management in the case of our wild specimen.

Finally, further studies are needed to find out what are the biological mechanisms involved in the trout sperm movement pattern similar to that hyperactive triggered following the cooling steps.

Declarations

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Availability of data and materials

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

Authors' contributions

NI, GR and MDI conceived and designed the experiments. GR, MDI, RI and SE carried out the evaluation of semen quality in vitro and in vivo. NI, SE and MDI performed the statistical analysis of the data. GR, MDI and NI, wrote the manuscript. AR and SE contributed in revisiting and reviewing the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

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

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Figures

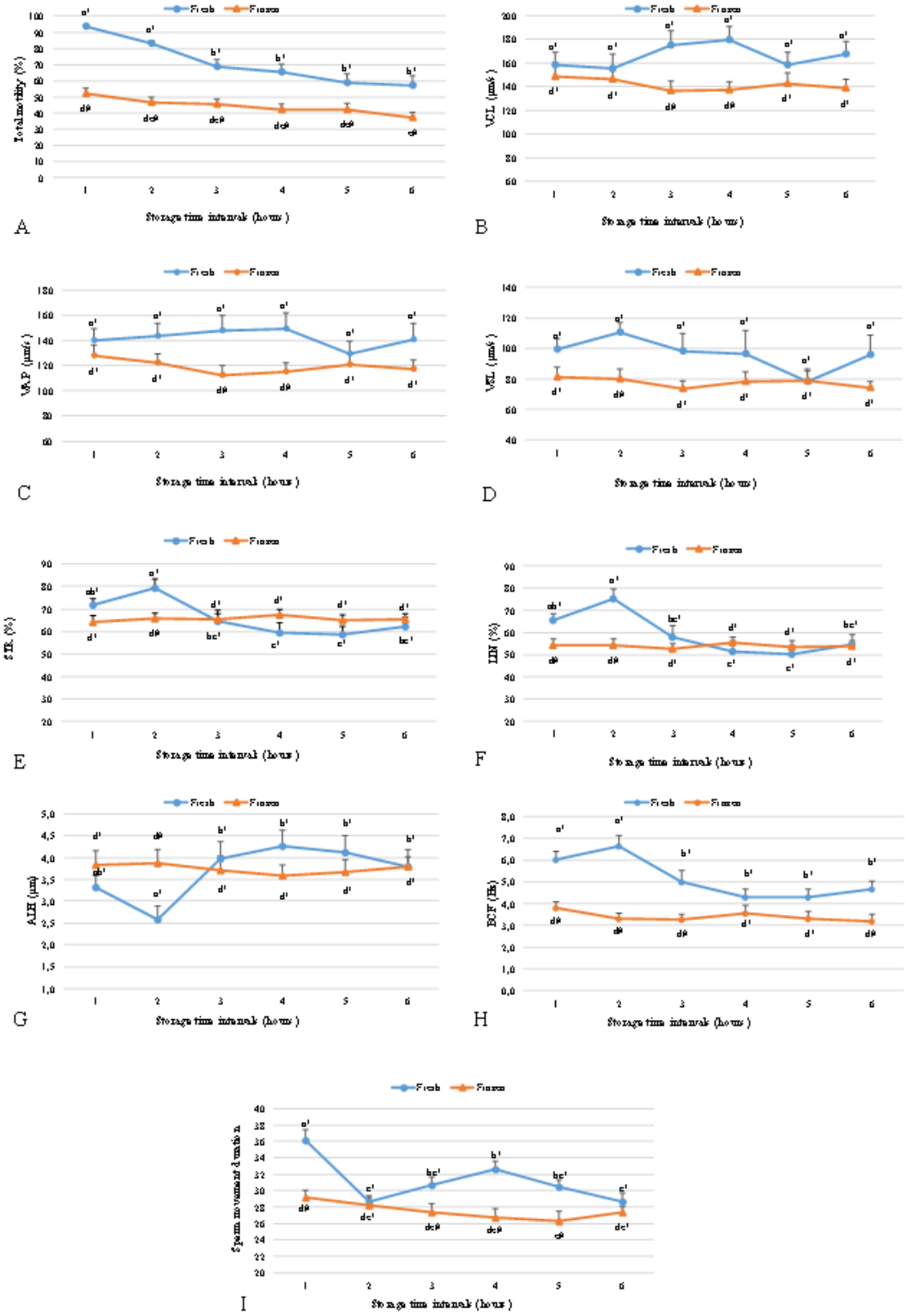

Figure 1

a,b,cDifferent superscript letters within the time intervals of fresh semen are statistically different (P<0.05) d,eDifferent superscript letters within the time intervals of frozen semen are statistically different (P<0.05) *,#Fresh and frozen groups within the same time interval are statistically different (P<0.05) according to independent sample t-test Effect of storage time post-collection on fresh and frozen sperm motility parameters. Total motility: the percentage of motile spermatozoa. Total motility: the percentage

of motile spermatozoa; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity (VSL/VCL x 100); STR: straightness (VSL/VAP x 100); BCF: beat cross frequency; ALH: amplitude of lateral displacement of the spermatozoon head.