



## Effect of freezing rate on motility, adenosine triphosphate content and fertilizability in beluga sturgeon (*Huso huso*) spermatozoa <sup>☆</sup>



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### ABSTRACT

Broodstock selection programs are currently underway for sturgeon species. To complement and further these selection programs we need to develop sperm cryopreservation procedures. In the present study, we describe the effects of freezing rate (−10 °C, −15 °C, −20 °C, −30 °C and −40 °C/min) on gamete quality characteristics (i.e., duration of motility (s), motility percentage (%), ATP content (nmol/10<sup>8</sup> cells), fertilization rate (%), and hatching rate (%)) in beluga sturgeon, *Huso huso*. After sampling, beluga sturgeon sperm were diluted in an extender composed of 23.4 mM sucrose, 0.25 mM KCl, and 30 mM Tris–HCl, pH 8.0 containing 10% methanol and subsequently frozen in a programmable freezer. Sperm frozen at −40 °C/min resulted in means for duration of motility (134 s), motility percentage (69%), ATP concentration (4.8 nmol/10<sup>8</sup> cells), fertilization rate (72%) and hatching rate (65%) that were higher ( $P < 0.05$ ) than those for slower cooling rates. Based on our results, −40 °C/min was the best freezing rate (among those tested) for cryopreservation of beluga sturgeon sperm.

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### Introduction

In aquaculture, refrigerated storage is a simple and inexpensive procedure often needed to deal with logistics of large-scale hatchery operations. Cryopreservation is a powerful tool that allows sperm to be stored indefinitely. This method has been recognized as the most appropriate way for gene banking aimed to conserve specific genetic diversity [17]. There are numerous of factors affecting cryopreservation success including origin of the broodfish, initial quality of sperm, extender, cryoprotectant, equilibration time, dilution ratio, volume of straws, freezing rate, thawing rate, time between thawing and activation as well as physiological aspects of sperm that might be species-specific [12,16,36,46]. Therefore, determining the 'optimal' procedure for sperm cryopreservation from a given species is not a simple task.

Sturgeons are considered to be "living fossils" [10]. Their evolutionary history goes back to the early Jurassic period (approximately 100–200 million years ago). Caspian Sea is the habitat for the four commercial species of sturgeon and *Huso huso* is one of them [34], where their populations are declining due to excessive fishing

for meat and caviar production, habitat destruction, and water pollution [13]. Now worldwide production of cultured sturgeon has increased from 2500 metric tons in 1999 to 25,600 metric tons in 2008, and aquaculture caviar production increased from 1.69 metric tons in 2003 to 27.32 metric tons in 2007 [25].

Although sturgeon farming has a history of more than one hundred years, the basis for intensive artificial reproduction and methods of in vitro gamete manipulation were developed only in the second half of the 20th century [22]. The importance of semen storage to efficient husbandry was emphasized by Billard [14]. In addition, cryopreservation of sperm has been well developed in many fish species including sturgeons for resource conservation and aquaculture practices, such as Atlantic sturgeon *Acipenser sturio* by Kopeika et al. [35]; Siberian sturgeon *Acipenser baerii* by Glogowski et al. [29]; Russian sturgeon *Acipenser gueldenstaedtii* by Huang et al. [32]; Persian sturgeon *Acipenser persicus* by Aramli and Nazari [4]; Sterlet *Acipenser ruthenus* by Dzyuba et al. [25]; Common carp *Cyprinus carpio* by Wamecke et al. [49]; Brown trout *Salmo trutta* by Nynca et al. [41] and Rainbow trout *Oncorhynchus mykiss* by Ciereszko et al. [18].

Some experiments have been conducted in an attempt to determine a protocol for cryopreservation and short-term storage of beluga semen [2,3]. To our knowledge there are no data on the effect of freezing rate on the sperm quality of beluga sturgeon. Hence, in the completion of previous studies, the present study

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was conducted to confirm the best freezing rate (from  $-10^{\circ}\text{C}$  to  $-40^{\circ}\text{C}/\text{min}$ ) for beluga sperm and to quantify additional information on the effects of freezing rate on fertilizing ability, motility parameters, and energetics (ATP content).

## Materials and methods

### Fish and gamete collection

Males ( $n = 12$ ; weight, 30–40 kg; total length, 1–2 m) and females ( $n = 4$ ; weight, 40–45 kg; total length, 1.5–2 m) were captured using gillnets (length 18 m, width 5.4 m, mesh size 15 cm) from the southwestern part of the Caspian Sea and transported to the Rajae Sturgeon Hatchery Center (Sari, Mazandaran, Iran) between March and April 2011. The fish were maintained in tanks with a water temperature of  $15\text{--}16^{\circ}\text{C}$ , an oxygen content of  $>5\text{ mg/L}$  and a pH of 7.6–7.9. Spermiation was stimulated by a single intramuscular injection of the luteinizing hormone-releasing hormone agonist (LHRHA<sub>2</sub>) at  $5\text{ }\mu\text{g/kg}$  body weight at 18 h before sperm collection. Semen was collected from the urogenital papilla by aspiration through a plastic catheter (5–7 mm diameter) connected to a 50 mL syringe. Special care was taken to avoid contamination with mucus, feces, or water. Females were injected with the same hormone at  $10\text{ }\mu\text{g/kg}$  body weight at 14 h before stripping. Fish were anesthetized and placed in lateral recumbency on a table. A finger was inserted into the gonopore to stretch the opening slightly. A scalpel (with a straight blade narrower than the gonopore) was inserted carefully into the gonopore opening, and a 1.5–3 cm incision was made through the ventral area of the oviductal (Mullerian duct) wall. The scalpel was withdrawn and the incision probed with one finger to ensure that the opening was not obstructed. The fish was inverted and slight pressure applied to the abdominal region by two individuals: the ova flowed through the incised opening in the oviduct and out of the gonopore [5,45]. The analysis in each trial was replicated three times.

### Cryopreservation protocol

Sperm was frozen using conventional freezing procedures: prior to freezing the samples were diluted 1:1 in an extender composed of 23.4 mM sucrose, 0.25 mM KCl, and 30 mM Tris–HCl, pH 8.0 [29] containing 10% methanol [27]. Diluted sperm were placed in 0.5 mL straws (CRYO-VET, France) and then placed into a programmable freezer (Planer Kryosave-Model KS30, Sunbury-on-Thames, Middlesex, UK) and frozen at  $-10^{\circ}\text{C}$ ,  $-15^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$ ,  $-30^{\circ}\text{C}$  or  $-40^{\circ}\text{C}/\text{min}$ . The straws were removed from the freezer and immediately placed into dewars containing liquid nitrogen for storage. The initial freezing rate used was rotated for each day of each sampling week (to account for potential differences in the time samples were frozen relative to when they were collected). Each sample was removed from storage 30 days after it was frozen, thawed for 6 s in a  $40^{\circ}\text{C}$  water bath, and re-evaluated for fertilizing ability, motility parameters and ATP.

### Evaluation of sperm motility parameters and concentration

Tris–HCl buffer (10 mM, pH 8.0) containing 0.25% pluronic (a substance that prevents spermatozoa from sticking to slides) was used as activating medium (AM). To trigger motility, the post-thaw sperm and the fresh sperm were diluted in AM with dilution rates 1:500, and 1:1000, respectively [26,27,42]. Spermatozoa motility was recorded with a dark-field microscopy (400 $\times$ , Olympus CK2, Tokyo, Japan). The percentage motility was determined arbitrarily on a 0–10 point scale, where 0 denoted 0% motility and 10 denoted 100% motility. The duration of motility was determined

by recording the time taken from activation to the complete cessation of activity by the last spermatozoa in a field. One person conducted all of the sperm motility observations to reduce the degree of variation [7]. Sperm density was estimated using a Burkner cell hemocytometer (Meopta, Czech Republic) at 200 $\times$  magnification on an Olympus BX 50 phase contrast microscope (Olympus).

### Fertilization assay

Eggs from the four females were pooled in equal parts 3 g eggs (approx. 200 eggs) and inseminated in a Petri dish with sperm stored from  $-10^{\circ}\text{C}$  to  $-40^{\circ}\text{C}/\text{min}$ . Based on the sperm concentration of the sample, the volume of sperm was adjusted to obtain a  $10^5$  sperm/egg ratio. To measure the fertilization rate, living and dead eggs were counted in each Petri dish during incubation and dead eggs were removed. Live embryos were counted after the second cleavage division at 4 h post-fertilization. Fertilization rate was expressed as the proportion of live embryos at corresponding post-fertilization times of the initial number of eggs incubated according to recommendations for sturgeon fishery practices according to Dettlaf et al. [22]. Hatching rate was determined by the proportion of yolk suck larvae from fertilized eggs.

### ATP bioluminescence assay

The ATP contents of spermatozoa were determined using the bioluminescence method described by Boryshpolets et al. [15]. Sperm samples were added to a boiling extraction medium, which contained 100 mM Tris–HCl (pH 7.75) and 4 mM EDTA. After boiling for 2 min at  $100^{\circ}\text{C}$ , samples of the sperm suspension were centrifuged at  $12,000\times g$  for 20 min. The ATP contents of the supernatants were evaluated using a Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany). The luminescence was read using a SpectraFluor Plus plate reader (Tecan Group, 1-40 Miyamachi, Japan) and the data were expressed as picomoles (pmol) of ATP per  $10^8$  sperm.

### Statistical analyses

Data are presented as mean  $\pm$  SD. All analyses were performed at a significance level of 0.05 using SPSS v 11.5 (Chicago, IL, USA). Normally distributed data were analyzed by ANOVA followed by Fisher's LSD test. For the statistical model, freezing rate was considered a fixed effect and week of semen sampling for each fish as a repeated measure. All variables were tested for rate by week interactions. A nonparametric Kruskal–Wallis test followed by the Mann–Whitney  $U$ -test with Bonferroni correction was used for comparison of motility parameters, fertilization rate and ATP content.

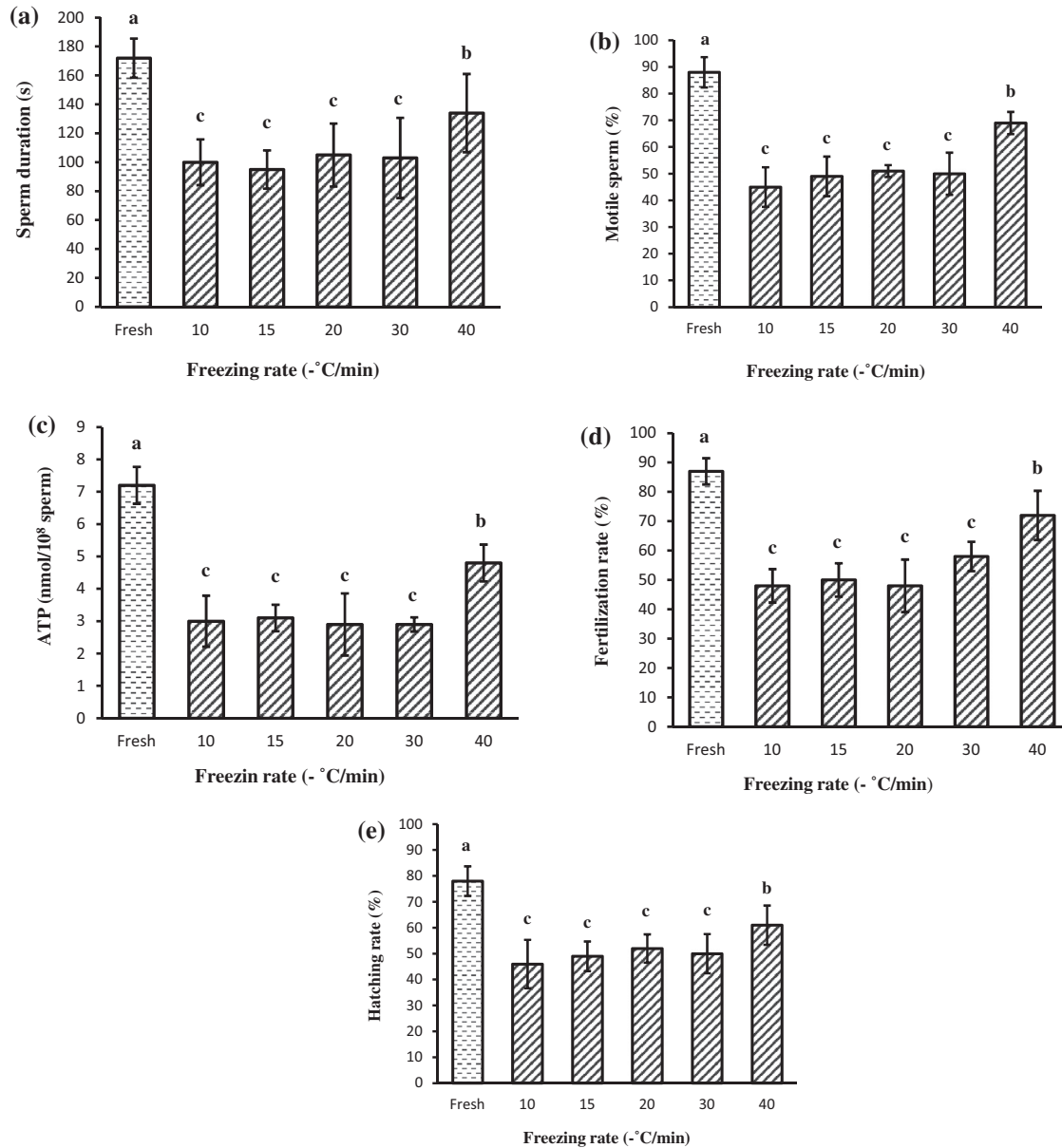
## Results

### Spermatozoa motility

For post-thaw sperm that were frozen at the  $-40^{\circ}\text{C}/\text{min}$  rate, total duration (s) and motile sperm (%) ( $134 \pm 27.01$  and  $69 \pm 4.18$  respectively), were significantly higher than sperm frozen at any of the four slower rates. No significant differences observed among the four slower rates for both motion characteristics (Fig. 1a and b). In addition, total duration was  $172 \pm 13.5$  s and percentage of motile cell was  $88 \pm 5.7$  (%) for fresh samples.

### ATP content

Fresh samples had an average ATP concentration of  $7.2 \pm 0.57\text{ nmol}/10^8$  sperm. Sperm cryopreserved using the fastest



**Fig. 1.** Mean ( $\pm$ SD) total duration of sperm movement (a), percentage of motile cell (b), ATP content (c), fertilization rate (d), and hatching rate (e) for fresh and post-thaw male beluga sturgeon sperm when cryopreserved at various freezing rates. The same letter in each chart indicates no significant difference ( $P > 0.05$ ).

freezing rate ( $-40\text{ }^{\circ}\text{C}/\text{min}$ ) had significantly higher concentrations of ATP ( $4.8 \pm 0.57\text{ nmol}/10^8\text{ sperm}$ ) than all other freezing rates (which were not significantly different from each other) (Fig. 1c).

#### Fertilization assay

Fertilization and hatching rates using frozen–thawed spermatozoa are shown in Fig. 1d and e respectively. Significant increases in both parameters were observed in the samples that were frozen at the  $-40\text{ }^{\circ}\text{C}/\text{min}$  rate. Similarity, no significant differences found among the four slower rates for both parameters. Fertilization rate was  $87 \pm 4.47\text{ }(\%)$  and hatching rate was  $78 \pm 5.7\text{ }(\%)$ .

#### Discussion

In today's farming industry, sperm cryopreservation has become routine practice. Freezing techniques of fish spermatozoa allows the preservation of a relatively large volume of diluted

ejaculate or prepared spermatozoa, with satisfactory results achieved in terms of the cells morphological and functional properties after thawing [21,24,32].

Programmable freezing units are preferred as they ensure the same freezing rates, thus providing more consistent results. The freezing conditions described here, offer consistency, precision, and accuracy. Furthermore, the whole procedure is rapid, since after obtaining the ejaculates, cells are processed, frozen and ready to be stored under liquid nitrogen in less than 15 min. Although too sophisticated to be employed at the hatchery site, this method of cryopreservation should be also useful for the establishment of spermatozoa banks to handle material that has been obtained after selective breeding programs or genetic manipulation [6,8,18]. Unfortunately, we were unable to examine faster freezing rates, because our programmable freezer was only able to create consistent, repeatable freezing rates up to  $-40\text{ }^{\circ}\text{C}/\text{min}$ .

The assessment of sperm quality using the available tools such as viability tests, ATP content, motility analysis, DNA integrity, etc. will be decisive for the selection of good sperm samples and for the

**Table 1**

Comparison of some semen characteristics in sturgeon species following freezing–thawing process.

Species	Semen type	Semen motility (%)	Fertilization rate (%)	Hatching rate (%)	References
Siberian sturgeon	Frozen–thawed	23	53		Tsvetkova et al. [48]
	Fresh	88	89		
Sterlet	Frozen–thawed	15	23		Tsvetkova et al. [48]
	Fresh	68	53		
Shortnose sturgeon	Frozen–thawed	16	39–40	31–32	Horvath et al. [30,31]
	Fresh	77	78–79	51–53	
Stellate sturgeon	Frozen–thawed	37.5	37		Alipour et al. [1]
	Fresh	74	72		
Paddlefish	Frozen–thawed	70	38–60		Linhart et al. [39]
	Fresh				
Persian sturgeon	Frozen–thawed	69**	61**	58**	Aramli and Nazari [4]
	Fresh	87	83	77	
Beluga sturgeon	Frozen–thawed	52–70**	55–67**	51–62**	Aramli et al. [2] and present study
	Fresh	88–90	87–90	78–85	

\* Mean of frozen–thawed semen samples stored from  $-10^{\circ}\text{C}$  to  $-40^{\circ}\text{C}/\text{min}$ .

\*\* Mean of frozen–thawed semen samples after 0, 30 and 60 min of storage.

standardization of the designed cryopreservation protocols [37]. For the purpose of our study, the best freezing rate was defined as the rate that produced the highest duration of motility, motility percentage, and ATP content in post-thaw beluga sturgeon sperm. In the present study, motility parameters and energetics as well as fertilization success were significantly higher in samples frozen using the  $-40^{\circ}\text{C}/\text{min}$  rate than all other rates. Similar to our study, Frankel et al. [28] tested four different cooling rates ( $-10^{\circ}\text{C}$ ,  $-15^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$ , and  $-40^{\circ}\text{C}/\text{min}$ ) using striped bass sperm and obtaining the best motility and fertility with the cooling rate of  $-40^{\circ}\text{C}/\text{min}$ . Trukshin [47] reported a deleterious effect of a  $10^{\circ}\text{C}/\text{min}$  freezing rate (0% fertilization) compared to 22% at  $4^{\circ}\text{C}/\text{min}$  in *Acipenser stellatus*. In data published by Liu et al. [40] on Chinese sturgeon *Acipenser sinensis* sperm, a freezing rate of  $-2^{\circ}\text{C}/\text{min}$  to a temperature of  $-6^{\circ}\text{C}$  produced the highest post-thaw motility. In *A. baerii*, research has shown a large inter-male variability in the changes in the ATP content of sperm during the freezing procedure [11]. Ciereszko et al. [20] have suggested the inclusion of theophylline in the extender and in the fertilization medium to increase the availability of ATP of thawed sperm.

In the present study, the motility parameters and fertilizing capacity were significantly decreased after thawing. The decrease in sperm motility was related to some loss of fertilizing capacity. However, Dettlaff et al. [22] stated that when the motility of thawed sperm was as low as 20–40%, there was almost no fertilization in some Ponto–Caspian sturgeons. This suggests that factors other than motility were involved such as alteration of the acrosome. Higher concentrations of acrosomal enzymes have been reported in frozen/thawed sperm compared to fresh sperm suggesting partial release of these enzymes from sperm cells into the seminal fluid [6,36,44]. Lahnsteiner et al. [38] also showed loss or decrease of acrosomal reaction in frozen/thawed sperm as compared to fresh sperm. These results suggest that cryopreservation affect sperm fertilizing ability in sturgeon via both decrease of sperm motility function and induction of acrosomal damage [43]. Therefore, it is prerequisite to examine cryopreservation success by performing fertilization tests. In contrast, Jahnichen et al. [33] reported a high loss of motility but not of fertilizing capacity in thawed sperm. This may be due to the use of sperm in excess, which compensate for the loss of motility.

Almost all studies on sturgeon sperm cryopreservation showed significant lower sperm motility and velocity of frozen/thawed sperm compared to that of the corresponding fresh sperm (Table 1). The observed decrease in sperm motility and velocity might be due to decrease of the percentage of sperm viability, high damage of sperm cells, or decrease of ATP content following cryopreservation

[11,30,40,48]. In addition, there is wide variation in the ability of sperm to sustain cryogenic freezing and thawing among individuals [9,23]. Such differences have been related to seminal plasma composition (e.g., antioxidants, enzymes, proteins, minerals, etc.), spermatozoa morphology, nutritional status, age, and stage of maturation (reviewed by Ciereszko [19]). Further research is required to develop statistical models that discriminate whether spermatozoa of an individual male are able to sustain cryopreservation before attempting freezing. Such predictive models would have many benefits for aquaculture production and conservation of wild stocks.

As a consequence, our data revealed that, when beluga sturgeon sperm have been cryopreserved at freezing rates of  $-10^{\circ}\text{C}$ ,  $-15^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$ ,  $-30^{\circ}\text{C}$  and  $-40^{\circ}\text{C}/\text{min}$ , the fastest rate ( $-40^{\circ}\text{C}/\text{min}$ ) yielded significantly higher post-thaw motility than the slower rates tested and when used in fertilization trials, provided a fertilization and hatching rate that was close to fresh semen. However, for future studies, it would be desirable to examine the effect of controlled, repeatable freezing rates faster than  $-40^{\circ}\text{C}/\text{min}$  and novel cryoprotectants on post-thaw quality of beluga sturgeon sperm. Furthermore, to increase efficiency of artificial reproduction using stored or cryopreserved sperm, further studies are required to optimize composition of sperm activation medium, and to determine optimal sperm/egg ratio.

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