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The effects of extender type, freezing and thawing rates on fertility of the cryopreserved semen of the Caspian brown trout (*Salmo trutta caspius*)

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Abstract: Cryopreservation of semen from the Caspian brown trout (*Salmo trutta caspius)* and effects of extender type, freezing and thawing rates on fertilization ability were studied. After assessment of semen quality, one part of semen was diluted with three parts of different extender and decanted into 0.5ml straws. Freezing was carried out at two freezing levels, 1.5cm and 2cm above surface of liquid nitrogen. The semen was thawed at 5°C for 90s, 15°C for 45s or 25°C for 30s in water baths and used for fertilization. Using the extender : 0.3 M glucose +10% methanol +10% egg yolk, and 0.6 M sucrose 10% DMSO + 10% egg yolk, yielded the highest post-thaw fertilization rates, with 67.05%±8.76 and 59.78%±5.08 eyeing rates, respectively. No significant differences were found in the fertilization rates with two freezing levels ($P>0.05$), however eyeing and hatching rates were higher for 2cm above the surface of the liquid nitrogen than for 1.5cm. Thawing of cryopreserved semen was best using the 25°C water bath for 30s and significant differences were seen in the eyeing rates between 25°C and 5°C or 15°C ($P<0.05$). Significant interactions ($P<0.05$) were found between effects of extender type and thawing rates and extender type and freezing rates.

Keywords: Cryopreservation, Extender, Freezing, Thawing, Fertility, *Salmo trutta caspius*

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Introduction

Cryopreservation is considered as a method in an effective strategy to save endangered species by facilitating the storage of their gametes in a gene bank (Gausen, 1993). Cryopreservation offers several benefits. Stocks can be protected from being totally eliminated due to sudden disease outbreak, natural utilization in hatcheries production and laboratory experiments can be ensured. Stocks can be maintained more economically and experimental materials for advanced studies, such as gene transfer, can be made more accessible (Chao & Liao, 2001).

The Caspian brown trout (*Salmo trutta caspius*) is distributed in southern basin of the Caspian Sea and natural stocks of this fish are critically endangered (Kiabi *et al.*, 1999). Cultivated stocks have been reared for enhancement and protection of wild populations.

Sperm cryopreservation in salmonids has been intensively investigated (Alderson & Macneil, 1984; Baynes & Scott, 1987; Holtz, 1993; Piironen, 1993; Lahnsteiner *et al.*, 1995, 1997; Babiak *et al.*, 2001; Cabrita *et al.*, 2001). Different extenders ranging from complex physiological saline solutions that mimic seminal plasma (Buyukhatipoglu & Holtz, 1978; Stoss & Holtz, 1981; Babiak *et al.*, 1995; Lahnsteiner *et al.*, 1995) to simple sugar solutions (0.3 M glucose , 0.6 M sucrose: Stoss & Holtz, 1983a; McNiven *et al.*, 1993; Piironen, 1993; Babiak *et al.*, 2001; Cabrita *et al.*, 2001) have been used. Mostly dimethylsulphoxide (DMSO) is used as the permeating cryoprotective agent for cryopreservation of salmonid fishes sperm, but other cryoprotectants like dimethyl acetamide (DMA, McNiven *et al.*, 1993) methanol (Lahnsteiner *et al.*, 1997) and DMSO+glycerol mixture (Lahnsteiner *et al.*, 1995) were also efficient. Usually, the semen diluted with extender, are frozen in pellets on dry ice (Holtz, 1993, Piironen, 1993, Babiak *et al.*, 2001) or in straws with different freezing levels in the vapor of liquid nitrogen (Lahnsteiner, 2000, Cabrita *et al.*, 2001) and after storage in liquid nitrogen for short or long periods of time are thawed and used for fertilization. Water bath with 4°C (Richardson *et al.*, 2000), 5°C (Wheeler & Thorgaard, 1991), 15-25°C (Lahnsteiner *et al.*, 1995), 25°C (Cabrita *et al.*, 2001) and 37°C (Stoss & Holtz, 1983b) successfully used for thawing of cryopreserved semen of salmonids.

Despite the progress with respect to milt cryopreservation during recent decades, the results generally are highly variable and adaptation of specific techniques for each species is necessary. Although a simultaneous analysis of the effects of more than one factor help us to avoid applying semen with different qualities in a successive experiments for a separate assessment of them, few studies have investigated the cumulative effects of procedural factors on cryopreservation success in fish semen (Baynes & Scott, 1987; Babiak *et al.*, 2001).

Therefore, the main purpose of this study was to investigate the effects of different extenders, freezing and thawing conditions and their interactions on the fertility of cryopreserved semen and development of a cryopreservation protocol for semen of this commercially valuable species.

Materials and methods

Mature wild Caspian brown trout (*Salmo trutta caspius)* were obtained from the Bahonar Salmonids Breeding and Cultivation Center (Kelardasht, Iran) at the beginning of spawning season. The fish were anesthetized (MS222, 100ppm) and their abdomens were dried before stripping. Semen was collected into 10ml syringe to prevent contamination with feces, urine, blood or water and poured into separate sterilized plastic petridishes and stored on crushed ice (0-2°C) until beginning of experiments. Sperm motility was estimated using a microscope (×400) after 1000-fold dilution and expressed as a percentage of motile spermatozoa. An activating solution, 125mM NaCl, 20Mm Tris-HCl, 30mM Glycine, pH 9 (Billard, 1992), was used for activation and estimation of motility. Samples below 70% motility were omitted and the remaining were pooled in equal volume and used for experiments. Sperm concentration was determined under a microscope (×400) using Thoma haemocytometer.

In this study, 30 experimental variants were tested to determine the effects of extenders composition, freezing conditions and thawing rates on the efficiency of the Caspian brown trout cryopreserved semen. The variants composed of 5 extenders (Table 1), 2 freezing rates and 3 thawing rates. For freezing, semen of 6 males were pooled (average semen concentrations was $7.5\times 10^{9}ml^{-1}$) and diluted

with 4°C cold different extenders in a ratio of 1:3 (semen: extenders), filled into 0.5ml straws, then frozen with vapor of liquid nitrogen at 1.5cm (freezing rate ~ 35°C min^{-1}) and 2cm (freezing rate ~ 30°C min^{-1}) above surface of liquid nitrogen (N_2). After 10 min, straws were placed in liquid nitrogen and stored for 4 weeks. After this period, straws were immersed in water bath with different thawing rates: 5°C for 90s, 15°C for 45s and 25°C for 30s. Then straws were taken immediately from water bath and thawed semen was poured onto the eggs.

Pooled eggs from 4 females were used for fertilizations assays. The ovarian fluid poured off before the fertilization. Batches of eggs (10g eggs for each replicate: 3 replicates per each treatment) were placed in plastic bowls and fertilization solution 125mM NaCl, 20Mm Tris-HCl, 30mM Glycine, pH = 9 (Billard, 1992), added in a ratio of 1:2 (fertilization solution: eggs, Lahnsteiner *et al.*, 1995). Semen was thawed and mixed immediately with eggs (one straw for each replicate, spz/egg ratio was approximately 6.2×10^6 spz per egg), and the mixture of eggs and thawed sperm stirred gently for 20-30s. After 2-3 min about 30 ml hatchery water (8-9°C) was added and then eggs were rinsed several times and left for 45-60 min under droplets of hatchery water until completing swelling. The eggs were transferred gently into plastic baskets numbered for each treatment and placed into incubator with cold running water. The eggs reached the eyed-egg stage after 30-35th days at 8-9°C. Embryos started to hatch on 60-65th days after fertilization. All percentage of eyeing and hatching were calculated based on the initial number of eggs. Control fertilization was performed in the same way as fertilization experiments with frozen semen. 10g eggs were transferred into the fertilization solution and fertilized with the same amount of semen as applied for the cryopreservation experiments.

Proportional data were converted by angular transformation (arc sin $\sqrt{p}$) prior to analysis by ANOVA. A three-way ANOVA with subsequent Tukey's test were used for comparison of mean values resulting from the various treatments at a significance level of $P<0.05$

Table 1: Experimental design for cryopreservation of the Caspian brown trout (*Salmo trutta caspius*) semen. Extender type, freezing level above N_2 and thawing temperature

Number of treatment	Type of extender*	Freezing levels (cm)	Thawing temperature (°c)	Number of treatment	Type of extender	Freezing levels (cm)	Thawing temperature (°c)
1	A	2	5	16	C	1.5	15
2	A	1.5	5	17	D	2	15
3	B	2	5	18	D	1.5	15
4	B	1.5	5	19	E	2	15
5	C	2	5	20	E	1.5	15
6	C	1.5	5	21	A	2	25
7	D	2	5	22	A	1.5	25
8	D	1.5	5	23	B	2	25
9	E	2	5	24	B	1.5	25
10	E	1.5	5	25	C	2	25
11	A	2	15	26	C	1.5	25
12	A	1.5	15	27	D	2	25
13	B	2	15	28	D	1.5	25
14	B	1.5	15	29	E	2	25
15	C	2	15	30	E	1.5	25

*: In this study five extenders were tested which listed from A to E.

A: 0.3M glucose, 10% DMSO, 10% egg yolk.

B: 0.3M glucose, 10% methanol, 10% egg yolk.

C: 0.6M sucrose, 10% DMSO, 10% egg yolk.

D: 600mg NaCl, 315mg KCl, 20mg $MgSO_4$. $7H_2O$, 15mg $CaCl_2$. $2H_2O$, 470mg Hepes, 100ml distilled water, 10% methanol, 1.5g/100ml BSA, 0.5g/100ml sucrose, 7ml/100ml egg yolk (Lahnsteiner *et al.*, 2000).

E: 7.5g/L NaCl, 2g/L $NaHCO_3$, 0.53g/L Na_2Hpo_4, 0.23g/L $MgSO_4$. $7H_2O$, 0.38g/L KCl, 0.46g/L $CaCl_2$. $2H_2O$, 1g/L glucose, 5g/L Glycine, 20% egg yolk, 10% DMSO (Stein & Bayrle, 1978).

Results

Fertilization ability of cryopreserved and control semen is shown in Table 2. The percentage of embryos reached the eyeing and hatching stages was recorded as an index of fertilization ability. Fertilization of eggs with semen frozen in 0.3 M glucose + 10% methanol + 10% egg yolk (extender B) at two freezing levels 1.5 and 2cm above LN_2 and then thawed at 25°C water bath for 30s (treatments no. 24, 23) resulted in 67.1% and 66.6% eyeing rates and 64.1% and 66.2% hatching

rates, respectively. These values did not differ significantly ($P>0.05$) from control values obtained after fertilization of eggs with fresh semen (72.5% eyeing and 69.8% hatching rates).The decrease in survival from the eyed egg stage to hatched stage was insignificant (t-test, $P>0.05$).

Three-way ANOVA showed that two of the three main effects, the extenders type (E), thawing rates (T), first-order interactions between extenders type and freezing rates (E+F) and extenders type and thawing temperature (E+T) significantly ($P<0.05$) affected fertilization rates. As shown in Figure 1a, the extenders B and C had the highest eyeing and hatching rates and significantly ($P<0.05$) differed from other extenders (A, D, E), but no significant differences ($P>0.05$) were found among the extenders A, D, E, fertilization rates in extender B was higher than the extender C, but the differences were not significant ($P>0.05$). The analysis of effect of freezing levels on fertilization rates showed no significant differences ($P>0.05$) between two freezing levels, however the values obtained with semen that froze at 2cm above N_2 were higher than at 1.5cm (Fig. 1b).

Thawing of cryopreserved semen at 25°C water bath for 30s led to higher fertilization rates than at 5°C, 15°C (Fig. 1c) and significant differences ($P<0.05$) were found among thawing temperature at 25°C with the others, but no significant differences ($P>0.05$) between 5°C and 15°C were observed.

The first-order interactions between extenders type and freezing rates (E+F) and extenders type and thawing temperature (E+T) revealed significant differences ($P<0.05$) with fertilization rates (Fig. 2a). In the tested extenders, no significant differences ($P>0.05$) were seen between two freezing levels. The highest fertilization rates were obtained with extenders B and C at both freezing levels (Fig. 2a).

Interaction between extenders type and thawing rates demonstrated that combination of two thawing rates, 5°C for 90s and 15°C for 45s with all extenders resulted in significantly lower fertilization rates ($P<0.05$) than control values, but semen frozen in extenders B and C and followed by thawing at 25°C water bath for 30s did not significantly differ ($P>0.05$) from fertilization rates of control. The

highest fertilization rates obtained with combination of extender B and 25°C water bath for 30s (Fig. 2b).

Although the first-interaction between freezing levels and thawing temperatures (F+T) was not significant (P>0.05), fertilization rates at freezing level of 2cm above N_2 were more than 1.5cm in all three thawing temperatures. The combination of freezing level of 2cm and thawing temperature of 25°C showed the highest fertilization values (Fig. 2c).

Table 2: Survival (mean±SE) of Caspian brown trout eggs inseminated with cryopreserved semen to eyed-egg stage and hatching stage. Experimental treatments were described in Table 1. Within columns, values marked with a similar range of superscripts did not differ significantly from each other at P<0.05.

N.	Eyeing Rates (%)	Hatching Rates (%)	N.	Eyeing rates (%)	Hatching rates (%)
1	$10.9\pm4.9^{e-h}$	10.8±4.8	17	$21.7\pm12.4^{e-h}$	21.7±12.4
2	$7.2\pm2.8^{f-h}$	7.2±2.8	18	$16.8\pm7.7^{e-h}$	16.8±7.7
3	$39.8\pm7.7^{a-f}$	39.8±7.7	19	$11.7\pm2^{e-h}$	11.7±2
4	$29.5\pm4.3^{b-g}$	29.2±4.2	20	$4.5\pm2^{g,h}$	4.5±2
5	$25.3\pm5.2^{b-h}$	24.3±4.3	21	$32.3\pm3.1^{a-g}$	32.3±3.1
6	$16.7\pm6.3^{e-h}$	16.3±5.9	22	$20.3\pm10.1^{e-h}$	20.3±10.1
7	$10.5\pm7.6^{f-h}$	10.5±7.6	23	$66.6\pm2.2^{a-c}$	66.2±1.8
8	$13.6\pm5.4^{e-h}$	12.5±4.4	24	$67.1\pm8.8^{a,b}$	64.1±7.9
9	$10.1\pm1.3^{e-h}$	10.1±1.3	25	$59.8\pm5.1^{a-d}$	59.8±5.1
10	1.5 ± 0.8^{h}	1.5±0.8	26	$28.9\pm7.3^{b-g}$	28.9±7.3
11	$10.3\pm2.5^{e-h}$	10.3±2.5	27	$16.7\pm3.5^{e-h}$	16.7±3.5
12	$8.2\pm3.4^{f-h}$	7.7±2.9	28	$25.4\pm9.7^{c-h}$	25.2±9.4
13	$25.3\pm5.8^{c-h}$	24.8±5.4	29	$25.2\pm9.2^{c-h}$	25.2±9.2
14	$25.6\pm3.9^{b-h}$	25.6±3.9	30	$6.6\pm1.7^{f-h}$	6.6±1.7
15	$45.8\pm9.4^{a-e}$	44.9±8.6	Cont	72.5 ± 2.7^{a}	69.8±1.9
16	$20\pm7.3^{d-h}$	20±7.3	-	-	-

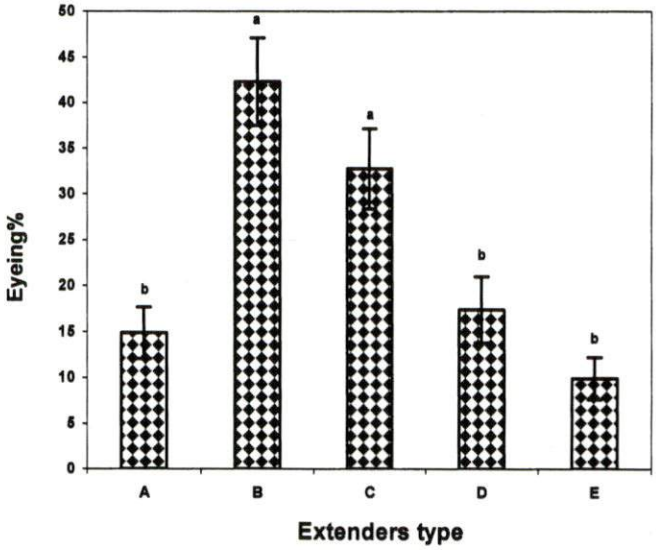

Figure 1a: A separate effect of extenders type on fertilization success of eggs inseminated with cryopreserved semen of the Caspian brown trout. Within charts, values marked with the same letters do not differ from each other (P> 0.05).

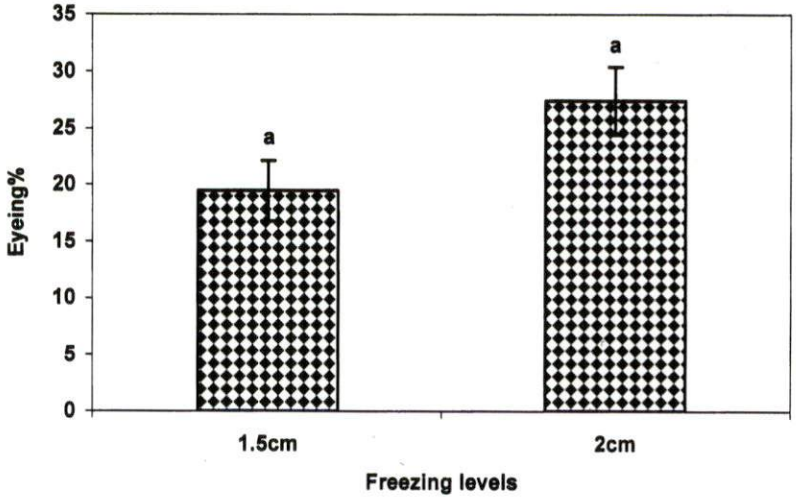

Figure 1b: A separate effect of freezing levels on fertilization success of eggs inseminated with cryopreserved semen of the Caspian brown trout. Within charts, values marked with the same letters do not differ from each other (P> 0.05).

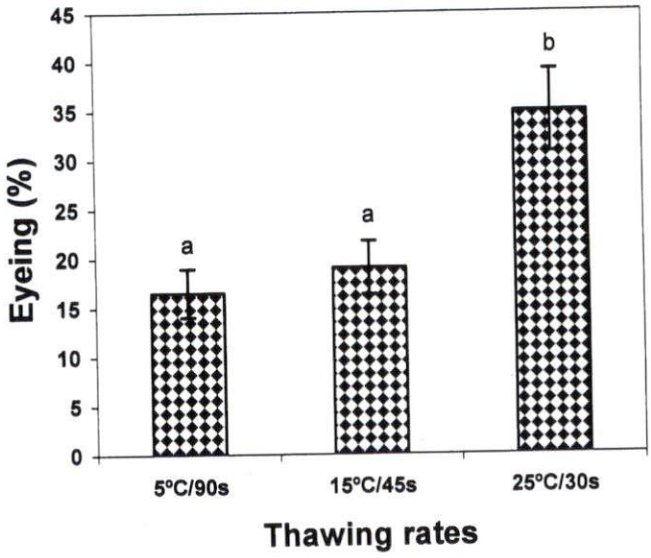

Figure1c: A separate effect of thawing temperatures on fertilization success of eggs inseminated with cryopreserved semen of the Caspian brown trout. Within charts, values marked with the same letters do not differ from each other (P>0.05).

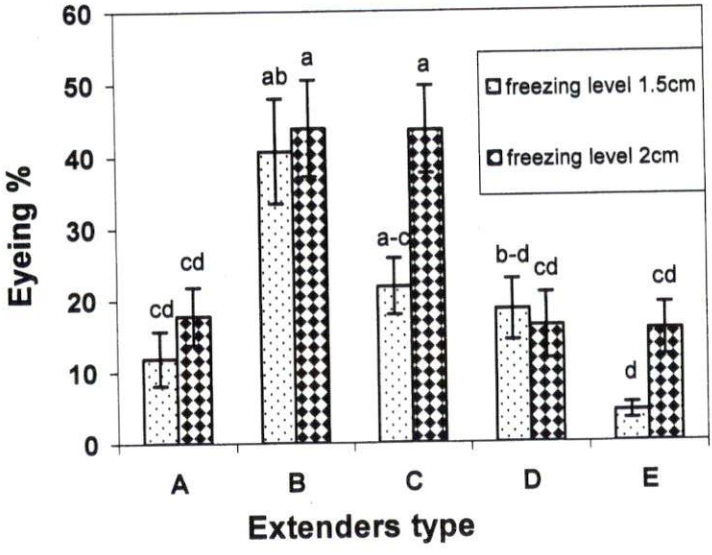

Figure 2a: First-order interaction between extenders type and freezing levels on fertilization success of eggs inseminated with cryopreserved semen of the Caspian brown trout. Within charts, values marked with the same letters do not differ from each other (P> 0.05).

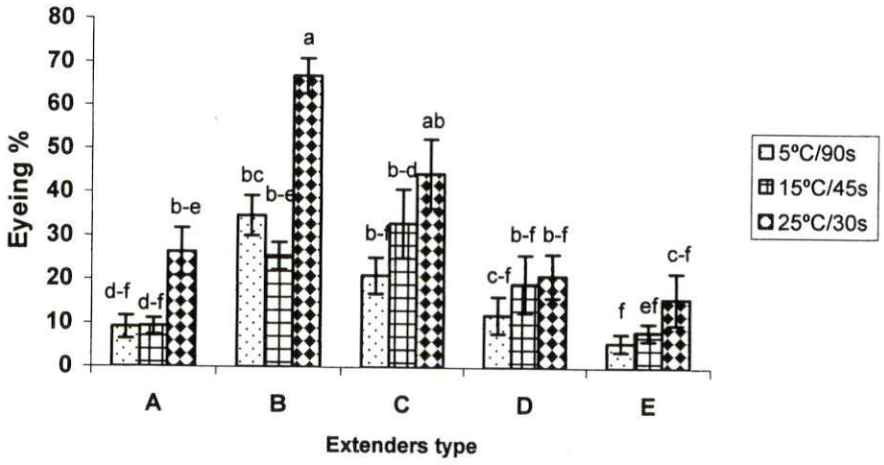

Figure 2b: First-order interaction between extenders type and thawing temperature on fertilization success of eggs inseminated with cryopreserved semen of the Caspian brown trout within charts, values marked with the same letters do not differ from each other (P> 0.05).

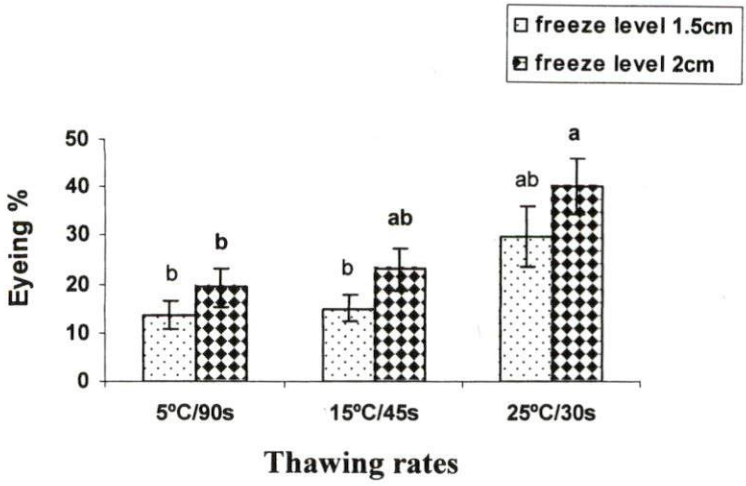

Figure 2c: First-order interaction between thawing temperature and freezing levels on fertilization success of eggs inseminated with cryopreserved semen of the Caspian brown trout Within charts, values marked with the same letters do not differ from each other (P>0.05).

Discussion

One of the difficulties faced in semen cryopreservation is that species differences require considerable adaptation of methods if they are to be successfully applied to different fish (Lahnsteiner *et al.*, 1996). Due to the scarcity of wild brood stock of the Caspian brown trout, replication of the cryopreservation tests were limited. This limit plus limited published works in this area caused the development of a new extender difficult, as a large number of potential cryoprotectans could be used in different concentrations and combinations that may interact with each other, which require different freezing and thawing rates. Therefore, in this experiment the best tested extenders in previous studies were used and main effects and interactions among tested factors were studied. Examination of influence of tested factors revealed that main effects of extenders type, thawing rates, first-order interactions between extenders type and freezing rates and extenders type and thawing rates significantly affected fertilization rates. On the basis of our results, extenders B and C indicated higher fertilization rates than the other extenders. The best fertilization rate obtained with extender B with 67.1% eyeing rate (92.5% to control). These results can be explained by the presence of 10% methanol as a cryoprotectant in this extender. Methanol has higher permeability than DMSO and Glycerol (Thomas *et al.*, 2003), by permeating into cell, causing reduced ice crystal formation. Lahnsteiner *et al.*, (1996) used 10% methanol, 10% DMSO, 10% DMA, 5% glycerol and mixture of 5% DMSO and 1% glycerol for semen cryopreservation of the grayling (*Thymallus thymallus*) and the Danube salmon (*Hucho hucho*), which methanol showed the highest fertilization rates in relation to control 95.3% and 91.1% for grayling and Danube salmon, respectively. Similar results were obtained with cryopreserved semen of *Oncorhynchus masou formosanus* in 0.3 M glucose, 10% methanol (73.9% to control) by Gwo *et al.*, (1999). The present study also confirms the usefulness of methanol as a cryoprotectant for the Caspian brown trout semen. Research by Babiak *et al.*, (2001) demonstrated that utilization of extender B for cryopreservation of rainbow

trout semen resulted in 56.3% eyeing rate in relation to control, which was lower than the results of present study, probably due to different conditions affecting these two experiments such as freezing and thawing rates, and etc. The extender C showed 59.8% (82.9% to control) eyeing rate. Conget *et al.*, (1996) reported 47-85% fertilization rates with cryopreserved semen of rainbow trout in extender containing 0.6 M sucrose and 10% DMSO. Also, Babiak *et al.*, (2001) obtained 75.19% eyeing rate with frozen semen of rainbow trout, similar to results of present study. Extender A successfully used by Alderson and Macneil (1984) (90% fertilization rates), Piironen, 1993 (31-85% fertilization rates), Gwo *et al.*, 1999 (87.5% fertilization rates), as well as extender D by Lahnsteiner *et al.*, (1995, 1996, 1997 and 2002) (85-93.8% fertilization rates), but poor fertilization rates was found in present study (44.5% and 35.1% for A and D extenders, respectively). It is probable that some different fertilization results in the mentioned studies and the present one are due to biological variation of the material.

In this study two freezing levels was applied. Although the difference was not significant, semen that was frozen at 2cm N_2 had higher fertilization rates than the 1.5cm level. Other authors used these freezing levels for salmonid semen cryopreservation. Cabrita *et al.*, (2001) reported that freezing rates lower than 30°C min^{-1} (2cm above N_2) were inefficient because the cooling rate from 4°C to -20°C was very slow (8.6°C min^{-1}) and freezing of sperm occurred slowly, exposing the cells to solvent effects for a long time before freezing. They obtained 84% fertilization rate after freezing of semen at 2cm above N_2, which is comparable with our results. Research by Conget et al., (1996) demonstrated that rainbow trout semen cryopreserved at freezing rates of 1°C min^{-1} or 10°C min^{-1} from -5°C to 80°C resulted in lower viability than 30°C min^{-1}. Also freezing at 1.5cm above N_2 was successfully used by Lahnsteiner *et al.*, (1995, 1996, and 2002). Freezing rates of 30-160°C min^{-1} suggested by Alderson and Macneil, (1984) for salmonid semen cryopreservation. Likely, at freezing rates more than 30°C min^{-1}, one part of the freezable water leaves the cell and the remaining water forms small ice crystal

which are tolerable if thawing is fast enough to avoid recrystalization (Thomas *et al.*, 2003). Since the two freezing rates tested in this study were higher than 30°C min^{-1}, only an insignificant difference was seen between them.

Thawing rate is critical in preserving viability of the spermatozoa. There is a lack of available data as regards the thawing conditions. Generally, thawing rates should be high to avoid recrystalization (Lahnsteiner, 2000). Research by Lahnsteiner *et al.*, (1995, 1996) on salmonids semen cryopreservation demonstrated that thawing was the most sensitive parameter during cryopreservation of semen and slight deviations from optimal conditions reduced the fertilization success significantly. They obtained the highest fertilization rates using semen thawed at 25°C water bath for 30s and change of the thawing period for only 5s or the thawing temperatures for 5°C led to reduction of post thaw fertilization ability of semen. Thawing at 25°C water bath for 30s has also been suggested for semen of rainbow trout (Cabrita *et al.*, 2001). It appears that either recrystalization or ice crystal formation during thawing was reduced or avoided by this thawing procedure or enzymatic activities were best reactivated (Lahnsteiner, 2000). Other thawing temperatures tested here (5°C, 15°C) had low fertilization rates, probably due to slow thawing rates and formation of large ice crystals or imperfect melting.

We may not be able to get comprehensive information on the cumulative effects of various factors on semen cryopreservation success, if these factors are considered separately; therefore, analysis of interactions is necessary. Other than the studies of Baynes and Scott, (1987) and Babiak *et al.*, (2001), there was no reliable statistical analysis of the cumulative effects of the procedural factors on cryopreservation success in fish semen until now. In this study, all possible interactions were considered. Cumulative effect of extenders type and thawing temperatures on the fertility of cryopreserved semen showed unexpected results. Thawing temperatures strongly affected usefulness of extenders. For example, semen frozen in extender B, the best extender for semen cryopreservation of this fish, resulted in the highest fertilization rates at 25°C water bath for 30s and did not

differ significantly with control, but when water bath with 5°C for 90s and 15°C for 45s was used, fertilization ability significantly decreased. These conditions were seen in other extenders. It should be noted that some interactions have been reported among extenders type, cryoprotectant, equilibration time, straw sizes, etc (Babiak *et al.*, 2001, Cabrita *et al.*, 2001). Therefore, in order to develop a suitable protocol for semen cryopreservation of the Caspian brown trout, further studies about different cryoprotectant, equilibration time, large straws, etc, will be needed to increase the efficiency of this cryopreservation method for applying in hatchery of this endangered fish.

In conclusion, cryopreserved semen of the Caspian brown trout in extenders B (0.3 M glucose, 10% methanol and 10% egg yolk) and C (0.6 M sucrose, 10% DMSO and 10% egg yolk) after fertilization resulted in 67.1% and 59.8% eyeing rates, respectively. Freezing level of 2cm above N_2 and water bath with 25°C for 30s were suitable freezing and thawing conditions for this species. Significant interaction and cumulative effect of extenders type and thawing temperature on fertilization success were revealed. This protocol is easy to use in hatchery conditions and requires no special technology.

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References

Alderson, R. and Macneil, A.J. , 1984. Preliminary investigations of cryopreservation of milt of Atlantic salmon (*Salmo salar*) and its application to commercial farming. Aquaculture. **43:**351-354.

Babiak, I.; Glogowski, J.; Luczynski, M.J.; Kucharczyk, D. and Luczynski, M. , 1995. Cryopresevation of the milt of the northern pike. Journal of Fish Biology. **46:**819-828.

Babiak, I.; Glogowski, J.; Goryczko, K.; Dobosz, S.; Kuzminski, H.; Strzezek, J. and Deminowicz, W. , 2001. Effect of extender composition and equilibration time on fertilization ability and enzymatic activity of rainbow trout cryopreserved spermatozoa. Theriogenology. **56:**177-192.

Baynes, S.M. and Scott, A.P. , 1987. Cryopresevation of rainbow trout spermatozoa: the influence of sperm quality, egg quality and extender composition on post-thaw fertility. Aquaculture. **66:**53-67.

Billard, R. , 1992. Reproduction in rainbow trout: Sex differentiation, dynamics of gametogenesis, biology and preservation of gametes. Aquaculture. **100:**263-298.

Buyukhatipoglu, S. and Holtz, W. , 1978. Preservation of trout sperm in liquid or frozen state. Aquaculture. **14:**49-56.

Cabrita, E.; Robles, V.; Alvarez, R. and Herraez, M.P. , 2001. Cryopreservation of rainbow trout sperm in large volume straws: application to large scale fertilization. Aquaculture. pp.301-314.

Chao, N.H. and Liao, I.C. , 2001. Cryopreservation of finfish and shellfish gametes and embryos. Aquaculture. **197:**161-189.

Conget, P.; Fernandez, M.; Herrera, G. and Minguell, J.J. , 1996. Cryopreservation of rainbow trout *(Oncorhynchus mykiss)* spermatozoa using programmable freezing. Aquaculture. **143:**319-329.

Gausen, D. , 1993. The Norwegian gene bank programme for Atlantic salmon (*Salmo salar*). *In*: J.C. Cloud and G.H. Thorgaad (eds.), Genetic conservation of salmonid fishes. Plenum. New York, USA. pp.181-187.

Gwo, J.C.; Ohta, H.; Okuzawa, K. and Wu, H.C. , 1999. Cryopreservation of sperm from the endangered Formosan landlocked salmon (*Oncorhynchus masou formosanus*). Theriogenology. **51:**569-582.

Holtz, W. , 1993. Cryopreservation of rainbow trout *(Oncorhynchus mykiss)* sperm: practical recommendations. Aquaculture. **110:**97-100.

Kiabi, B.H.; Abdoli, A. and Naderi, M. , 1999. Status of fish fauna in the south Caspian basin of Iran. Zoology in the Middle East. **18:**57-65.

Lahnsteiner, F.; Weismann, T. and Patzner, R.A. , 1995. A uniform method for cryopreservation of semen of the salmonid fishes *Oncorhynchus mykiss* (Walbaum), *Salmo trutta f. fario* L., *Salmo trutta f. lacustris* L., *Coregonus* sp. Aquaculture Research. **26:**801-807.

Lahnsteiner, F.; Weismann, T. and Patzner, R.A. , 1996. Cryopreservation of semen of the grayling (*Thymallus thymallus*) and the Danube salmon (*Hucho hucho*). Aquaculture. **144:**265-274.

Lahnsteiner, F.; Weismann, T. and Patzner, R.A., 1997. Methanol as cryoprotectant and the suitability of 1.2ml and 5ml straws for cryopreservation of semen from salmonid fishes. Aquaculture Research. **28:**471-479.

Lahnsteiner, F. , 2000. Semen cryopreservation in the salmonidae and in the northern pike. Aquaculture Research. **31:**245-258.

Lahnsteiner, F.; Mansour, N. and Weismann, T. , 2002. A new technique for insemination of large egg batches with cryopreserved semen in the rainbow trout. Aquaculture. **209:**359-367.

McNiven, M.A.; Gallant, R.K. and Richardson, G.F. , 1993. Dimethyl-acetamide as a cryoprotectant for rainbow trout spermatozoa. Theriogenology. **40:**943- 948.

Piironen, J. , 1993. Cryopreservation of sperm from brown trout (*Salmo trutta m. lacustris* L.) and Arctic charr (*Salvelinus alpinus* L.). Aquaculture. **116:**275-285.

Richardson, G.F.; Miller, T.L. and McNiven, M.A. , 2000. Cryopreservation of Arctic charr, (*Salvelinus alpinus* L.), semen in various extenders and in three sizes of straw. Aquaculture Research. **31:**307-315.

Stein, H. and Bayrle, H. , 1978. Cryopreservation of sperm of some freshwater teleosts. Annales de Biologie Animale Biochimie and Biophysique. **18:**1073-1076.

Stoss, J. and Holtz, W. , 1981. Cryopreservation of rainbow trout (*Salmo gairdneri*) sperm: effect of pH and presence of buffer in the diluent. Aquaculture. **25:**217-222.

Stoss, J. and Holtz, W., 1983a. Cryopreservation of rainbow trout (*Salmo gairdneri*) sperm: effect of proteins in the diluent sperm from different males and interval between sperm collection and freezing. Aquaculture. **31:**275-282.

Stoss, J. and Holtz, W. , 1983b. Successful storage of chilled rainbow trout (*Salmo gairdneri*) spermatozoa for up to 34 days. Aquaculture. **31**:269-274.

Thomas, P.C.; Rath, S.C. and Mohapatra, K.D. , 2003. Breeding and seed production of finfish and shellfish. Daya Publishing House. pp.334-354.

Wheeler, P.A. and Thorgaard, G.H. , 1991. Cryopreservation of rainbow trout semen in large straws. Aquaculture. **93**:95-100.