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Cryopreservation of rainbow trout semen using a glucose-methanol extender

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ABSTRACT

The goal of this study was to improve post-thaw quality of cryopreserved rainbow trout semen. Quality was assessed by measuring sperm motility and fertilization rates at the eyed, hatching, and swim-up larvae stages. We first tested how varying glucose concentrations in a methanol extender (0.1, 0.2, and 0.3 M, $n = 9$ different males per concentration) and different semen-extender ratios (1:3, 1:1, and 3:1, $n = 3$ males and in a separate experiment 1:3 and 1:5, $n = 6$) affected post-thaw motility. Sperm motility and fertilization rates of samples with 0.18 M glucose in 9% methanol as an extender, a 1:5 sperm-to-extender ratio, and a 15 min equilibration period then were measured at sperm-to-egg ratios of 100,000, 300,000, and 600,000:1. The optimal cryopreservation conditions were found to be 0.15 M glucose in the extender, a 15 min equilibration period, and a 1:5 semen-to-extender ratio. This combination resulted in high post-thaw motility (49.9%) and hatching rates (67.1 ± 18.7, 78.6 ± 17.0, and 84.4 ± 10.0% for sperm-to-egg ratios of 100,000, 300,000 and 600,000:1, respectively). To our knowledge, this is the first report to demonstrate that the post-thaw fertilization ability of rainbow trout semen can be similar to that of fresh semen at a sperm-to-egg ratio as low as 600,000:1.

1. Introduction

Providing a sufficient number of cryopreserved spermatozoa for fertilization is a major challenge in the fish hatchery industry. The quality of cryopreserved rainbow trout sperm is low, so the number of cryopreserved spermatozoa required for successful fertilization is about 10 times higher than that for fresh semen (Billard, 1992). Low fertilization ability of cryopreserved semen is accompanied by low post-thaw motility (Lahnsteiner et al., 1996a). The low quality of cryopreserved semen negatively impacts rainbow trout breeding efforts. Therefore, improved techniques for rainbow trout sperm cryopreservation are needed.

Cryopreservation is used to preserve sperm for later use, but sperm can be damaged by the freezing and thawing process. To mitigate damage, extenders (media used to dilute sperm) containing cryoprotectants (compounds used to protect sperm from cold and heat shock and cytotoxicity) are used. Simple extenders that contain only permeable cryoprotectants and sugars as nonpermeable cryoprotectants have been used successfully to cryopreserve salmonid fish sperm. Permeable cryoprotectants include methanol, DMSO, DMA, and glycerol at concentrations of 5–25% and nonpermeable cryoprotectants, such as sucrose or glucose, at concentrations of 0.3–0.6 M (Bozkurt et al., 2005; Ekici et al., 2012; Holtz, 1993; Lahnsteiner et al., 1996b, 1997; Mansour et al., 2006; Piironen, 1993; Sarvi et al., 2006; Tekin et al., 2003). An extender consisting of 0.3 M glucose and 10% methanol has been used successfully to cryopreserve Arctic char semen (Mansour et al., 2006) and whitefish semen (Ciereszko et al., 2008; Nynca et al., 2012). Our preliminary data suggest that such a simple extender is also effective for the cryopreservation of rainbow trout spermatozoa. However, our unpublished observations showed that post-thaw motility was low (Dietrich et al., unpublished), as is typical for cryopreserved rainbow trout spermatozoa (Lahnsteiner et al., 1996a).

Cryopreservation involves several parameters that need be fine-tuned to improve post-thaw survival. These factors include cryoprotectant type and concentration (Lahnsteiner et al., 1996a; Maisse, 1994), equilibration time (Babiak et al., 2001; Lahnsteiner et al., 1996b; Perez-Cerezales et al., 2010), and number of spermatozoa (Ciereszko et al., 2013). The aim of this study was to identify the optimal conditions (i.e., glucose concentration in the extender, equilibration time, and the sperm-to-extender ratio for semen dilution) for cryopreservation of rainbow trout semen. Sperm motility and fertilization rate were used as quantitative endpoint metrics.

2. Materials and methods

2.1. Source of milt

Milt was collected during the spring spawning in April from rainbow trout males of the Rutki strain (3 years of age). The fish were anesthetized using Propiscin (1 ppm IFI, Żabieniec, Poland) prior to milt collection. Milt samples were collected by gentle abdominal massage, taking care not to contaminate them with feces, mucus, or urine. This study

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was approved by the Animal Experiments Local Committee in Olsztyn, Poland.

2.2. Cryopreservation

Cryopreservation of semen from individual males followed previously described procedures (Ciereszko et al., 2008, 2013; Nynca et al., 2012). The milt and extender were stored on ice, and milt samples were diluted with the appropriate extender at a desired ratio (from 3:1 to 1:5, depending on the experiment), mixed, and drawn into 0.25 ml plastic straws (IMV Technologies, L'Agile, France). Dilution and mixing took about 30 s. The straws then were placed on a 3 cm high Styrofoam frame floating on liquid nitrogen for 3 min before being plunged into liquid nitrogen. Straws were then thawed 1–2 h later by immersion in a 40 °C water bath for 5 s.

2.3. Effect of glucose concentration on sperm motility parameters of fresh and cryopreserved semen

Three extenders with different glucose concentrations were prepared; they consisted of 10% methanol and 0.1, 0.2, and 0.3 M glucose, respectively. We did not use a variant without glucose, because an external cryoprotectant is needed for successful cryopreservation. Cryopreservation was performed using a 1:3 semen-to-extender dilution ratio. Therefore, the final concentrations of glucose were 0.075, 0.15, and 0.25 M, respectively, and the final concentration of methanol was 7.5% for all variants. Sperm motility was measured for fresh-diluted, and frozen-thawed semen ($n = 9$ different males) using CASA (see 2.9). Mean sperm concentration and osmolality were $9.87 \pm 2.74 \times 10^9$ spermatozoa and 235 ± 63 mOsm/kg, respectively. Based on the motility results shown in Fig. 1, the extender (producing final concentrations of 0.15 M glucose and 7.5% methanol) was used in the following experiments. Analyses were run in duplicates for all of the experiments in the present study.

2.4. Effectiveness of concentrated extenders for cryopreservation of rainbow trout spermatozoa

Three extenders were prepared (Ciereszko et al., 2013): 0.2 M glucose and 10% methanol, 0.3 M glucose and 15% methanol and 0.6 M glucose and 30% methanol. These extenders were used at semen-to-extender dilution ratios of 1:3, 1:1, and 3:1, respectively. Therefore, the final concentration of glucose and methanol in all variants was the same: 0.15 M and 7.5%, respectively. The aforementioned final concentrations were used in all of the subsequent experiments. Sperm motility was measured for fresh, diluted, and frozen-thawed semen ($n = 3$,

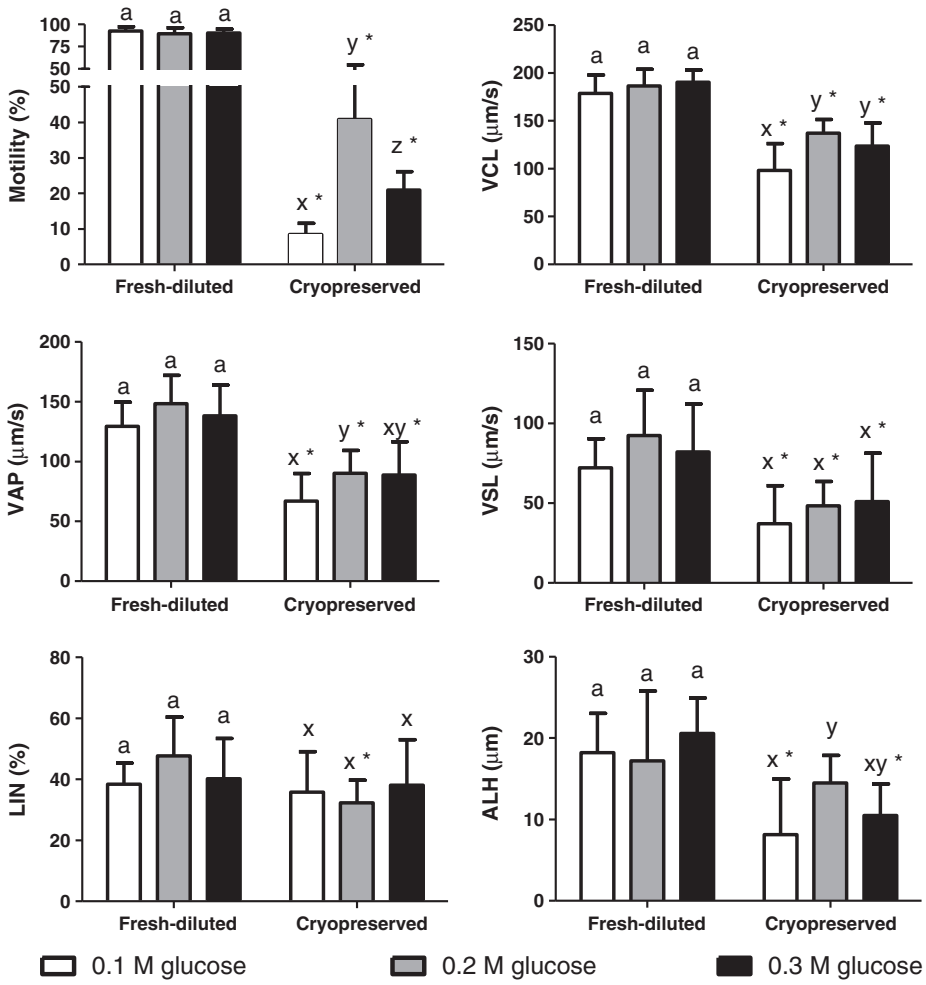

Fig. 1. Sperm motility of fresh-diluted and cryopreserved semen in relation to the concentration of glucose in the extender ($n = 9$). Results are expressed as mean ± SD. Different superscripts indicate statistical differences ($P < 0.05$) among fresh (a) and cryopreserved (x–z) semen. Asterisks indicate significant differences between the parameters for fresh and cryopreserved semen (*$P < 0.05$).

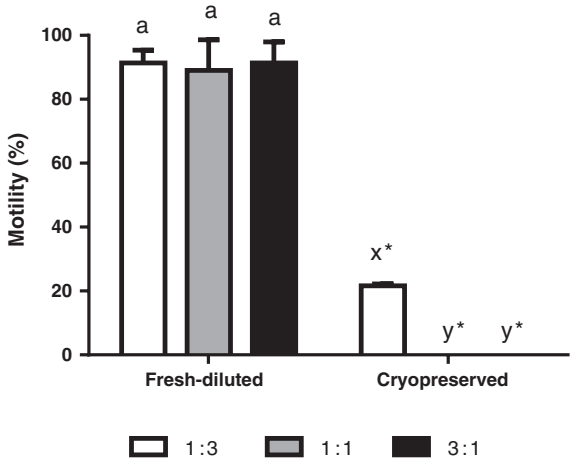

Fig. 2. Changes in the percentage of sperm motility of fresh and cryopreserved semen in relation to the semen-to-extender dilution ratios. Different superscripts indicate statistical differences ($P < 0.05$) among fresh (a) and cryopreserved (x–y) samples. Asterisks indicate significant differences between the parameters for fresh and cryopreserved semen ($^*P < 0.05$).

each of diluted-fresh and frozen-thawed samples from different males) (Fig. 2).

2.5. Effect of 15 min equilibration on sperm motility parameters of fresh semen and semen cryopreserved using 0.2 M glucose and 10% methanol at a 3:1 dilution ratio

Semen was cryopreserved with the use of an extender consisting of 0.2 M glucose and 10% methanol and a 1:3 semen-to-extender dilution ratio. Straws were either cryopreserved immediately after dilution or after 15 min of incubation on ice at 4 °C. Sperm motility was measured for fresh, diluted, and frozen-thawed semen ($n = 9$ different males), and mean sperm concentration and osmolality were $9.63 \pm 2.65 \times 10^9$ spermatozoa and 253 ± 33 mOsm/kg, respectively. Because the 15 min equilibration period has had a positive effect on sperm motility, it was used in the two subsequent experiments.

2.6. Effects of 1:3 and 1:5 sperm-to-extender dilutions on sperm motility parameters of fresh and cryopreserved semen

Two extenders were prepared: 0.2 M glucose and 10% methanol for a 3:1 dilution and 0.18 M glucose and 9% methanol for a 5:1 dilution. The final concentrations of glucose and methanol after dilution were the same for both extenders (0.15 M glucose and 7.5% methanol). Cryopreservation was performed as described above and included the 15 min equilibration period. Sperm motility was measured for fresh, diluted, and frozen-thawed semen ($n = 6$ different males). Mean sperm concentration and osmolality were $11.35 \pm 1.78 \times 10^9$ spermatozoa and 255 ± 24 mOsm/kg, respectively.

2.7. Sperm motility characteristics and fertilization rates of fresh and cryopreserved semen

Sperm samples ($n = 9$ different males) were cryopreserved using an extender consisting of 0.18 M glucose and 9% methanol at a 5:1 extender-to-semen ratio and a 15 min equilibration period. Sperm motility was measured for fresh, diluted, and frozen-thawed semen. Fertilization trials were conducted for diluted and cryopreserved semen as described below. Mean sperm concentration and osmolality of fresh undiluted semen were $10.87 \pm 2.48 \times 10^9$ spermatozoa and 251 ± 39 mOsm/kg, respectively.

2.8. Fertilization

Fertilization was performed on April 26, 2013 at 100,000, 300,000, and 600,000:1 sperm-to-egg ratios. Before fertilization, the fresh sperm sample was diluted with an extender to obtain the same sperm concentration as that of the cryopreserved semen (Cabrita et al., 2001). Eggs were collected from two females and mixed together. Next, 10 ml of D532 (20 mM Tris, 30 mM glycine, 125 mM NaCl, pH 9.0; Billard, 1992) were added to the eggs in a plastic 120 ml cup (15 g, 231 ± 10 eggs). The sperm sample was immediately added and the gametes were swirled. After 2 min, the eggs were rinsed with hatchery water, incubated for 5 min to water-harden the eggs, and then incubated in upwelling incubation trays. Mean values (± SD) of semen volumes added to the eggs were 11.6 ± 2.8, 34.8 ± 8.3, and 69.6 ± 16.5 μl at the 100,000, 300,000, and 600,000:1 sperm-to-egg ratios, respectively. Excess fresh semen (100 μl) combined from three males was used at the beginning and at the end of the fertilization trials to test the quality of the eggs. Fertilization rates were measured at the eyed (May 25), hatching (June 3), and swim-up larvae (June 18) stages.

2.9. Measurements of sperm motility, concentration, and seminal plasma osmolality

Sperm motility was measured in fresh semen within 10 min after dilution and in frozen samples immediately (within 5 s) after thawing. The motility of the spermatozoa was measured and analyzed by Computer Assisted Sperm Analysis (CASA) using the Hobson Sperm Tracker. Video recordings were made using a microscope with a 10× negative phase lens and a Sony CCD black and white video camera. Semen was diluted 1:400 with sperm activating solution (1 mm $CaCl_2$, 20 mm Tris, 30 mm glycine, 125 mm NaCl pH 9.0; Billard, 1992) supplemented with 0.2% bovine serum albumin to prevent the spermatozoa from sticking to the glass. After rapid mixing, 0.7 μl of this solution was immediately placed in a well of a 12-well multi-test glass slide (ICN Biomedicals Inc., Aurora, OH, USA) and covered with a cover slip. Sperm motility parameters were measured over a 12-second period, with a post-activation time between 5 and 17 seconds. Video recordings were analyzed using the Hobson Sperm Tracker (Hobson Vision Ltd., Baslow, UK). The program settings for the image analysis at the 10 x objective magnification were: search radius = 9.69 μm; predict = off; video = pal; aspect = 1.49; refresh time = 1 s; threshold +20/−100; filter weightings 1 = 2, 2 = 2, 3 = 2, 4 = 2; image capture rate = 50 Hz. The tracker simultaneously assessed 15 sperm motility parameters, but for simplicity only straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN = 100 × VSL/VCL), amplitude of lateral head displacement (ALH), and percentage of motile sperm were chosen for further analysis. Motility analysis was performed for fresh, diluted, and cryopreserved semen, and evaluations were made in duplicate. Sperm concentration was measured following the method described by Ciereszko and Dabrowski (1993). Osmolality of seminal plasma was measured using the KG apparatus (Minitüb Abfüll Labortechnik GmbH &Co., Tiefenbach, Germany).

2.10. Statistical analysis

All values were expressed as mean values ± SD. The percentage data were subjected to normalization by arcsine transformation. Data were tested for normal distribution and equal variances (Bartlett's test). Data then were subjected to repeated measures two-way analysis of variance (ANOVA) followed by Sidak's post hoc test using the statistical package GraphPad Prism (San Diego, CA, USA). One-way ANOVA followed by Tukey's test and the Kruskal Wallis test followed by Dunn's test were used to evaluate the effects of concentrated extenders on post-thaw sperm motility. The alpha level of significance was set at 0.05.

3. Results

3.1. Effect of glucose concentration on sperm motility parameters of fresh and cryopreserved semen

Glucose concentration in the extender did not have any effect on the fresh-diluted semen but significantly affected post-thaw sperm motility parameters (Fig. 1). At 0.2 M glucose concentration, sperm motility was highest (41%), so this concentration (final 0.15 M and 7.5% for glucose and methanol, respectively) was used in the subsequent experiments.

3.2. Effectiveness of concentrated extenders for cryopreservation of rainbow trout spermatozoa

The dilution of fresh semen (89–91%) did not change the percentage of sperm motility. However, post-thaw sperm motility only observed for the 1:3 sperm-to-extender dilution (22%); no motility was recorded for the 1:1 and 3:1 dilutions. Therefore, the 1:3 dilution was used in the subsequent experiments.

3.3. Effect of 15 min equilibration on sperm motility parameters of fresh semen and semen cryopreserved using 0.2 M glucose and 10% methanol at a 3:1 dilution ratio

Equilibration did not cause significant changes in the percentage of motility of fresh and cryopreserved spermatozoa. A decrease in VCL and an increase in LIN were found between treatments for fresh-diluted semen. VCL, VAP, VSL, and ALH were significantly higher in the cryopreserved spermatozoa after the 15 min equilibration period. Therefore, a 15 min equilibration period was used in the subsequent experiments (Fig. 3).

3.4. Effects of 1:3 and 1:5 sperm-to-extender dilution ratios on sperm motility parameters of fresh and cryopreserved semen

The sperm motility parameters of fresh-diluted semen were not affected by the dilution ratio. In contrast, the 1:5 dilution of cryopreserved semen exhibited a significantly higher percentage of sperm motility compared to the 1:3 dilution (60% vs. 30%, respectively). Other sperm motility parameters of cryopreserved semen were not affected by the dilution ratio (Fig. 4).

3.5. Sperm motility characteristics and fertilization rates of fresh and cryopreserved semen

The sperm motility parameters of fresh (data not shown) and cryopreserved semen were similar to those observed in the previous experiment. For cryopreserved semen, the percentage of sperm motility was 49.9 ± 6.8%, VCL was 132.2 ± 11.6 μm/s, VAP was 98.6 ± 10.5 μm/s, VSL was 61.7 ± 8.8 μm/s, LIN was 47.3 ± 6.7%, and ALH was 11.8 ± 2.7 μm.

Fertilization rates measured at the eyed stage of fresh-diluted semen were high (87–90%) and did not differ statistically among the different sperm-to-egg ratios (Fig. 5). The fertilization rates of eggs fertilized

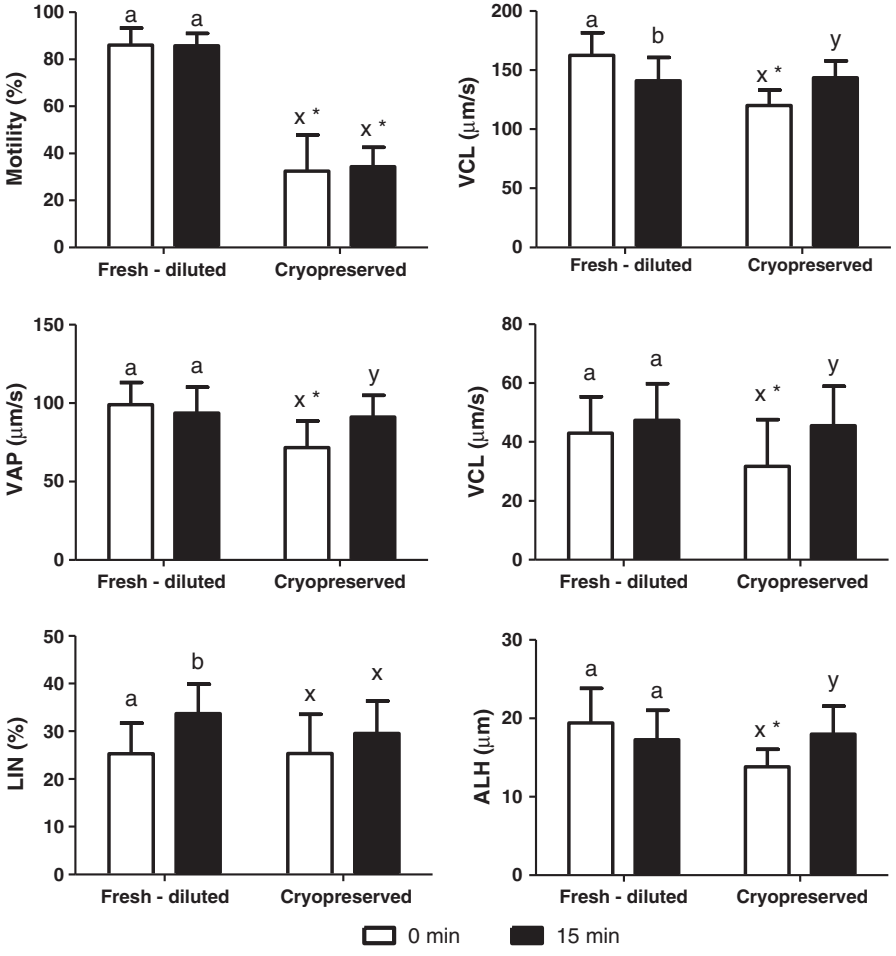

Fig. 3. Sperm motility of fresh-diluted and cryopreserved semen in the 0 and 15 min equilibration period treatments ($n = 9$). Results are expressed as mean ± SD. Different superscripts indicate statistical differences ($P < 0.05$) among fresh (a-b) and cryopreserved (x–y) semen. Asterisks indicate significant differences between the parameters for fresh and cryopreserved semen (*$P < 0.05$).

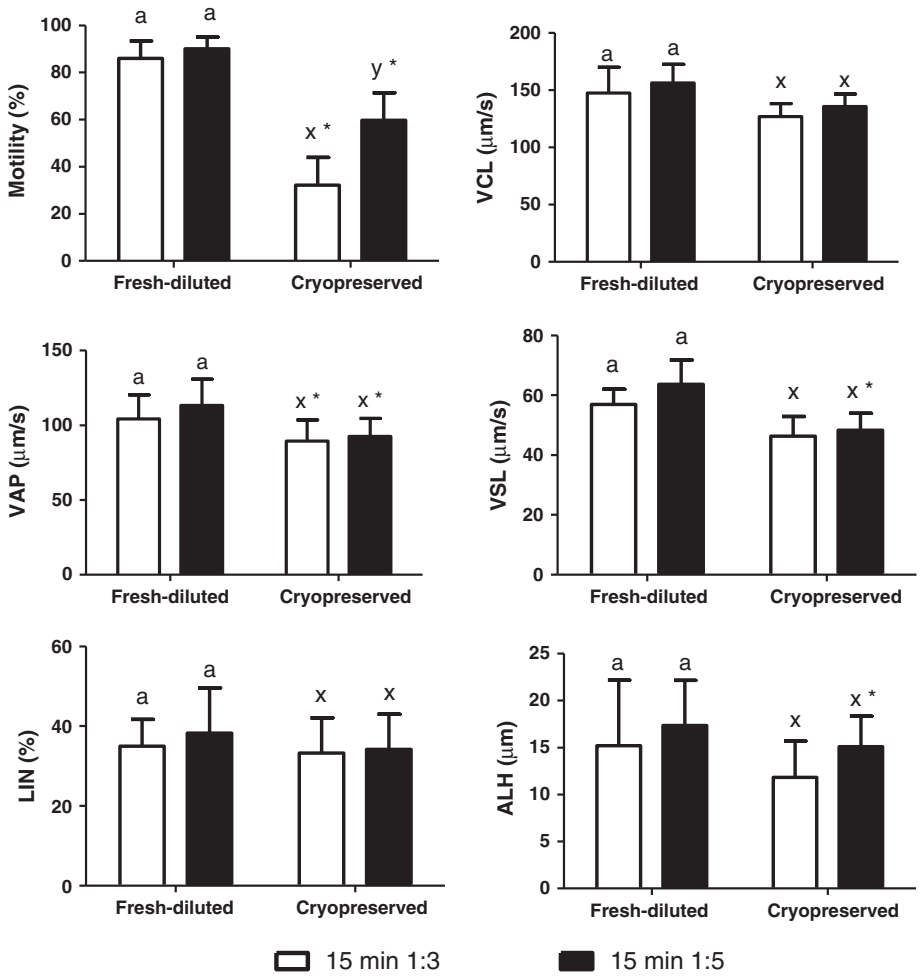

Fig. 4. Sperm motility of fresh-diluted and cryopreserved semen at 1:3 and 1:5 semen-to-extender dilution ratios ($n = 6$). Samples were equilibrated for 15 min before cryopreservation. Results are expressed as mean ± SD. Different superscripts indicate statistical differences ($P < 0.05$) between fresh (a) and cryopreserved (x–y) semen. Asterisks indicate significant differences between the parameters for fresh and cryopreserved semen (*$P < 0.05$).

with an excess of fresh semen at the beginning and end of fertilization trials were 89.0% and 90.4%, respectively. The fertilization ability of cryopreserved semen did not differ significantly from that of fresh semen at the 600,000:1 sperm-to-egg ratio. At the 300,000:1 ratio, the fertilization rate for the cryopreserved samples was significantly lower than that of fresh semen, but it still was quite high (79%). It should be stressed that fertilization rates of cryopreserved semen were lower partially due to values for one male exhibiting 29%, 37%, and 61% fertilization rates, for 100,000, 300,000 and 600,000:1 sperm-to-egg ratios, respectively.

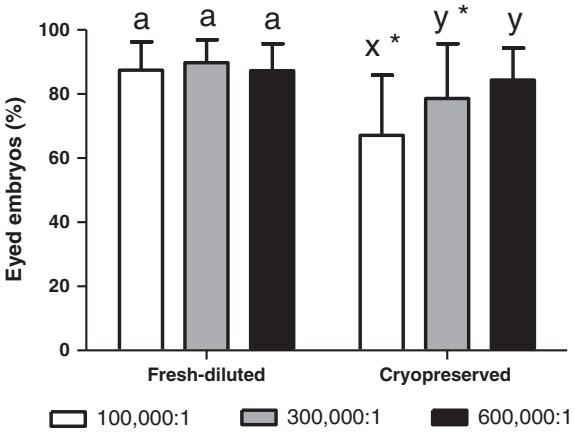

Fig. 5. The sperm fertilization ability of fresh-diluted and cryopreserved semen in relation to sperm-to-egg ratio ($n = 9$). Different superscripts indicate statistical differences ($P < 0.05$) among fresh (a) and cryopreserved (x–y) semen. Asterisks indicate significant differences between the parameters for fresh and cryopreserved semen (*$P < 0.05$).

Very little mortality was observed at the hatching and swim-up fry stages. For the 100,000, 300,000, and 600,000:1 sperm-to-egg ratios, the percentages of hatched larvae for the fresh semen treatments were 87.2 ± 8.8, 89.7 ± 7.3, and 86.9 ± 8.6%, and for the cryopreserved semen treatments they were 67.0 ± 18.6, 78.6 ± 17.0, and 84.1 ± 10.0%, respectively. For swim-up fry, the values were 86.3 ± 9.3, 88.5 ± 7.5, and 85.7 ± 9.2% for fresh semen and 66.2 ± 18.3, 77.7 ± 16.8, and 82.7 ± 9.7% for cryopreserved semen, respectively. The fertilization rates of eggs fertilized with an excess of fresh semen at the beginning and end of fertilization trials were 88.6% and 90.4%, respectively, for the hatching stage and 86.9% and 89.5%, respectively, for the swim-up larvae stage.

4. Discussion

In this study we demonstrated that glucose concentration in the extender is important for the cryopreservation of rainbow trout semen. Under our experimental conditions, the use of concentrated extenders did not increase the number of motile spermatozoa in the straw; instead, a decrease in the number of spermatozoa in the straw led to

better post-thaw motility. The conditions for the optimized cryopreservation procedure were as follows: the glucose concentration in the extender was 0.15 M final concentration, an equilibration time of 15 min was used, and the semen-to-extender ratio was 1:5. These modifications resulted in high post-thaw motility and fertilization rates.

External cryoprotectants that do not permeate sperm cells often are used to protect spermatozoa from damage during the freezing and thawing process. For fish spermatozoa, including rainbow trout, the effects of egg yolk (Babiak et al., 2001; Baynes and Scott, 1987; Lahnsteiner et al., 1996a), low density lipoproteins (Perez-Cerezales et al., 2010), soybean proteins (Cabrita et al., 2001; Stoss and Holtz, 1983), and bovine serum albumin (Stoss and Holtz, 1983) have been tested. Although these non-permeating cryoprotectants usually have a positive effect, high variability in the results often is observed, probably due to significant interactions related to their effects (Babiak et al., 2001; Tekin et al., 2007). To our knowledge, few studies of the effects of sugar concentrations on cryopreservation success have been conducted for fish. For rainbow trout, Maisse (1994) tested the usefulness of six sugars (sucrose, maltose, trehalose, galactose, fructose, and glucose) at three concentrations (125, 250, and 500 mM) and found that sucrose at 125 mM was the most beneficial. Our results demonstrated that optimal concentration of glucose plays a very important role in the effectiveness of the glucose-methanol extender. This finding suggests that rainbow trout spermatozoa are sensitive to external damage and that the cryoprotective effect of glucose is effective within a narrow range of concentrations. The mechanism for the cryoprotective action of glucose and its modulation by glucose concentration need to be elucidated in further studies.

In contrast to the processing of mammalian semen, an equilibration period usually is not used for cryopreservation of fish semen. However, Lahnsteiner et al. (1996b) reported that equilibration times up to 15 min did not affect the post-thaw fertility of salmonid semen, and even equilibration times of 30 and 45 min have been used (Bozkurt et al., 2005; Cabrita et al., 1998). Babiak et al. (2001) and Perez-Cerezales et al. (2010) used a 10 min equilibration time. The former found that equilibration led to decreased fertilization rates of semen cryopreserved with DMSO- and ethylene glycol- but not DMA-based extenders. The latter suggested that interaction between equilibration time and extender constituents may explain conflicting information about the use of equilibration in cryopreservation of fish spermatozoa. Our results showed that for the glucose-methanol extender, use of an equilibration period had beneficial effects on the sperm motility parameters of cryopreserved rainbow trout spermatozoa.

Recently, we demonstrated that the use of concentrated extenders consisting of glucose and methanol is a very simple and effective method for increasing the number of motile whitefish spermatozoa in straw samples (Ciereszko et al., 2013). Use of the 3:1 dilution ratio allowed for the cryopreservation of up to three times more spermatozoa than that of the currently recommended method (dilution 1:3). However, the results of the current study clearly demonstrated that this strategy is not effective for rainbow trout, as rainbow trout spermatozoa appear to be more sensitive than whitefish spermatozoa to cryogenic injuries. Thus, the effectiveness of concentrated extenders for the cryopreservation of fish semen is species specific. Part of the mechanism related to species-specific sensitivity may involve sperm compression effects due to limited intercellular space in the straw (Lahnsteiner, 2000; Lahnsteiner et al., 1996b).

The post-thaw motility of rainbow trout spermatozoa is usually quite low ($\leq$20%), which indicates that severe cryogenic injuries occur during the freezing and thawing process (Babiak et al., 2002; Glogowski et al., 1996; Lahnsteiner et al., 1996a). In our experiments, however, we recorded more than 40% post-thaw sperm motility (with the exception of one experiment (3.2), in which 22% was recorded). High percentage (50–60%) of post-thaw motility of rainbow trout semen was also observed by Cabrita et al. (2001, 2010), Tekin et al. (2003), and Ekici et al. (2012), but fertilization rates in these studies were lower than that of fresh semen. Our data suggest that rainbow trout spermatozoa cryopreserved using the method described in our study have high post-thaw motility, which in turn results in high fertilization ability.

To our knowledge this is the first report demonstrating that the post-thaw fertilization ability of rainbow trout semen can be similar to that of fresh semen at a sperm-to-egg ratio as low as 600,000:1, and it can still be high at a ratio of 300,000:1. In most studies, a ratio of 3,000,000:1 (Babiak et al., 2001; Billard, 1992; Bozkurt et al., 2005; Conget et al., 1996; Tekin et al., 2007) or even higher has been employed (Cabrita et al., 1998, 2001; Perez-Cerezales et al., 2010; Salte et al., 2004). The lowest semen-to-eggs ratios ($1.8–2.4 \times 10^6$ spermatozoa per egg) securing high post-thaw fertilization rates were achieved by Lahnsteiner et al. (1997). Our results demonstrate that a lower number of cryopreserved spermatozoa may be used to obtain high fertilization rates. If scaling up the method to higher straw volumes proves successful, it should be possible to introduce use of cryopreserved rainbow trout semen into hatchery practice.

We are aware that the low number of males used in our study does not allow us to make general recommendations at present, especially in view of the poor fertility data recorded for one male. It is possible that in the real population there are more males like the outlier male. Such males would originate from different genetic variations, environmental conditions, health, diet, etc. Consequently, the sperm of such males would not be suited for the cryopreservation method developed in our study and a specific protocol would need to be developed for them, including the types and concentrations of sugars in the extender (see Maisse, 1994). Further research using a larger number of males should address these issues in detail.

In conclusion, our data show that the post-thaw fertilization ability of rainbow trout semen can be similar to that of fresh semen at a sperm-to-egg ratio as low as 600,000:1. This method should be improved to allow for the higher straw volumes needed to meet requirements of hatchery operations during spawning. Glucose seems to be essential for cryopreservation, which agrees with studies documenting the importance of using external cryoprotectants to preserve rainbow trout semen. Rainbow trout sperm appears to be sensitive to cryogenic damage resulting from high concentrations of spermatozoa in the straws.

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