CRYOPRESERVATION OF SPERM OF THE ADRIATIC GRAYLING (*Thymallus thymallus*) AND THE MARBLE TROUT (*Salmo marmoratus*) FROM THE SOČA RIVER IN SLOVENIA

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Abstract

A cryopreservation protocol was tested on the sperm of genetically distinct Adriatic grayling (*Thymallus thymallus)* and the Natura2000-listed marble trout (*Salmo marmoratus*) from the Soča River basin in Slovenia. Sperm was frozen in an extender composed of 200 mM glucose, 40 mM KCl, 30 mM Tris (pH 8.0) and 10% methanol as a cryoprotectant in the vapour of liquid nitrogen. Sperm dilution ratios of 1:1, 1:4 and 1:9 were tested. The cooling rate was 57 ± 1°Cmin-1 in all cases. In the grayling, the highest ratio of eyed eggs (74 ± 4 % vs. 69 ± 6 % in the control) and the highest hatch percentage (63 ± 6 % vs. 56 ± 10 % in the control) was observed with a dilution ratio of 1:1. In the marble trout, the highest percentage of eyed eggs (84 ± 4 % vs. 88 ± 3 in the control) and the highest hatch rate (70 ± 3 % vs. 76 ± 2 % in the control) was again found using the 1:1 dilution ratio of the sperm. In both species, individual sperm samples had a significant effect on the results. Sperm-to-egg ratios ranging from 2.5 to 5.7 × 10^4 spermatozoa per egg yielded satisfactory hatch rates (67 ± 7 % to 73 ± 7 %) in the grayling.

Keywords: grayling, marble trout, sperm, cryopreservation, conservation

Introduction

The Adriatic grayling indigenous to the northern part of the peri-Adriatic river system including the Soča river basin in Slovenia is a phylogenetically distinct lineage within the species *Thymallus thymallus* (Sušnik et al. 2001). Due to intense stocking of a non-native grayling lineage from the Sava River, and successive introgression between the two lineages, at present only hybrids with different amounts of the original "Adriatic" genes exist in the Soča river basin. Based on genetic analysis the Angling Club of Tolmin (http://www.flyfishing.si/) that manages the Soča river basin has, conducted extensive selection of individuals with the highest amount of "Adriatic genes" and carries out systematic spawning and stocking of these fish into the river (Sušnik et al. 2004; Jesenšek & Šumer 2004). Another species of the same river system, marble trout (*Salmo marmoratus*), has also been severely affected by the introduction of non-native brown trout (*Salmo trutta m. fario*) from the Danubian drainage and subsequent hybridization (Povž 1995).

Marble trout is a Natura 2000- as well as Habitats Directive-listed (Council Directive 92/43/EEC 1992) species. Although most rivers in the Soča river basin are inhabited by hybrids, pure marble trout populations remained in the upper sections of several streams that were inaccessible to introduced brown trout (Fumagalli et al., 2002). Broodstocks consisting of individuals of several pure populations are cultured at the facilities of the Angling Club of Tolmin as a live gene bank. Grayling are due to their sensitivity to aquaculture conditions (e.g., sensitivity to infections and diseases, nutritional deficits and territorial and aggressive behaviour) extremely difficult to rear, particularly adult (sexually mature) animals. In order to diminish these technological inconveniencies, an efficient system for long-term sperm preservation, such as cryopreservation,

should be established enabling drastic reduction of broodstock size. This system would also enable storage of sperm obtained from wild males, until it is genetically tested.

In case of the marble trout, cryopreservation of sperm from individuals of the remaining pure populations could ensure their survival. These populations that inhabit the headwaters of rivers are severely limited in numbers of fish and are continuously threatened by natural hazards such as earthquakes, landslides or floods (Povž et al., 1996).

Although the cryopreservation of salmonid sperm (particularly of the rainbow trout *Oncorhynchus mykiss*) has been studied extensively (for a review see Lahnsteiner 2000a), only a few reports have been published on the sperm of the grayling (Lahnsteiner et al. 1992, 1996a) and several issues concerning fertilizing capacity of frozen sperm, optimal sperm-to-egg ratio, lesions of preserved cells due to freezing and thawing remain to be solved. Fine structural changes in spermatozoa as a result of cryopreservation were investigated using electron microscopy systems (SEM and TEM) (Lahnsteiner et al., 1992). After thawing only 10-20% of sperm cells displayed intact morphology, although fertilization percentages of up to 80 % were observed. In the second study (Lahnsteiner et al. 1996a), a method originally developed for the rainbow trout was adapted to the grayling and the Danube salmon (*Hucho hucho*). Fertilization rates of 90 – 100 % of the control were observed using 10 % methanol as cryoprotectant, cooling at 1.5 cm above the level of liquid nitrogen and thawing at 25°C for 30 seconds. To the best of our knowledge there has been no report on the cryopreservation of marble trout sperm.

In order to develop a protocol for cryopreservation of the Adriatic grayling and the marble trout, the objective of this study was to experimentally investigate the effect of sperm dilution ratio on the fertilizing capacity of sperm after thawing. A further objective of the work was to optimize the sperm-to-egg ratio with respect to fertilization success using thawed sperm in the grayling.

Materials and methods

Gamete collection

In case of the grayling, approximately 30 spawning males and four spawning females kept in the fish farm of the Angling club of Tolmin were included in the investigations.

Before collecting sperm and eggs, all individuals were anesthetized using 2-phenoxyethanol at a dose of 0.8 ml l-1. Anesthetized fish were laid on a towel, their urogenital openings were wiped dry and sperm were stripped into 12-ml test tubes applying a silicone catheter (1 mm internal diameter, 1.5 mm external diameter) according to the protocol described by Glogowski et al. (2000). Females were inspected for the presence of ovulated eggs and eggs were stripped into a plastic bowl by gentle massage of the abdomen.

Milt was successfully collected from 12 individuals: three of them, having a sufficient milt volume (samples 10, 11, 13), were selected for individual cryopreservation, while three more samples were due to small milt volumes pooled before freezing. The remaining six samples were excluded from cryopreservation experiments due to poor motility.

Sperm motility of each specimen was estimated before cryopreservation and after thawing (post-thaw motility) under a light microscope at 100 × magnification by mixing 1 µl of sperm with 19 µl of hatchery water on a microscope slide.

Marble trout males were collected by electrofishing at three sections of the river Trebuščica. Collected fish were anesthetized on collection site using 2-phenoxyethanol at a dose of 0.8 ml l-1. Fish were laid on a towel, their urogenital pores were wiped dry and sperm was collected by aspiration through an elastic pipe into a dry test tube. At each sampling site pooled sperm was collected from 5-8 individuals, thus, they were numbered 1, 2 and 3 according to the sampling site. Sperm samples were then transported into the hatchery at 4°C and motility

estimation was conducted as described with the grayling. Eggs from females of the broodstock maintained at the fish farm were collected during a routine spawning work and also transported into the hatchery at 4°C.

Sperm cryopreservation

Conditions of freezing and thawing were identical for both species. Before freezing, sperm were mixed at a ratio of either 1:1, 1:4 or 1:9 with an extender composed of 200 mM glucose, 40 mM KCl, 30 mM Tris (pH 8.0, set with concentrated HCl) and 10% methanol adjusted to have a final concentration of 10% following dilution with sperm. All chemicals used in the experiments were purchased from Reanal Laborvegyszer Kft (Budapest, Hungary). Diluted sperm were loaded into 0.5 ml straws (Minitüb, Tiefenbach, Germany) and frozen in the vapour of liquid nitrogen in a polystyrene box. Straws were placed on a 3-cm high polystyrene frame floating on the surface of liquid nitrogen and allowed to cool for 3 minutes. After cooling straws were plunged into liquid nitrogen. Frozen sperm samples were stored in a Statebourne BIO 10 storage dewar (Statebourne Cryogenics, Washington Tyne & Wear, UK). Sperm samples were used for fertilization after two to four days of storage. Samples were thawed in a 40°C water bath for 13 seconds.

Cooling rates were measured using a K-type thermocouple inserted into a straw filled with sperm of marble trout diluted with the extender and cryoprotectant at a ratio of either 1:1, 1:4 or 1:9 as described above. The thermocouple was connected to a Digi-Sense DualLogR thermometer (Eutech Instruments, Singapore). The straw was placed onto a 3 cm high polystyrene frame which was floating on the surface of liquid nitrogen in a styrofoam box and temperature readings were logged with the interval of 2 seconds. Cooling rate measurements were conducted in three replicates. Average cooling rates were calculated using the initial temperature values and the values at 3 minutes and the time elapsed.

Effect of dilution ratio on the fertilizing capacity of cryopreserved sperm

To investigate the effect of sperm dilution ratio on the fertilizing capacity of sperm after thawing, pooled eggs from two females were used in the grayling and from three females in the marble trout. Batches of 13g (N = 837 – 1029) eggs were used for fertilization with a single straw of thawed grayling sperm, whereas, in the marble trout batches of 20g (N = 209-224) of eggs were fertilized with one straw, in triplicates for both species. Sperm samples 10, 11 and the pooled sample were used in the grayling, while in the marble trout the pooled samples collected at the three sampling sites were used. Freshly stripped sperm served as a control in both species. Contents of a straw were released onto the eggs and gametes were activated using a DIA523 fertilizing extender (in 1 liter of water: 2.42 g Tris, 3.76 g glycine, 5.5 g NaCl prepared according to the protocol of Billard (1977)). Simultaneously, post-thaw motility of sperm was observed under a light microscope at 100× magnification by diluting 1 µl of thawed sperm in 19 µl of the above mentioned fertilizing extender on a microscope slide. After incubation in the fertilizing extender for approximately 1 hour, the extender was replaced by water and eggs were distributed into experimental incubators with a water flow of 90 l min-1. Fertilization percentages were calculated at eyed stage, and hatch percentages were calculated at the stage of hatching.

Effect of sperm-to-egg ratio on the fertilizing capacity of cryopreserved sperm

To optimize the sperm-to-egg ratio using thawed sperm of the grayling, again, pooled eggs of two females were divided into the batches of 10 g (N = 622 – 743) eggs in triplicates and fertilized with the cryopreserved sperm of sample 13 diluted at a ratio of 1:4 prior to cryopreservation. Fertilization was carried out using 200, 100 or 50 µl of thawed sperm (meaning 40, 20 or 10 µl of pure sperm). Details of fertilization, incubation and determination of fertilization and

hatch percentages were the same as described in the previous experiment. Sperm concentration was determined by counting spermatozoa in a Bürker-type counting chamber at 100 × dilution (in the extender used for cryopreservation) in order to calculate the sperm-to-egg ratio. Finally, sperm-to-egg ratio was calculated by dividing the number of spermatozoa in the volume of fresh sperm used for fertilization with the number of eggs in the given batch.

Statistical analyses

Post-thaw motility, fertilization and hatch percentages were subjected to two-way analysis of variance (ANOVA) with Bonferroni's post test at $P < 0.05$ to investigate the main effects of individual sperm samples and dilution ratios. One-way ANOVA with Tukey's Multiple Comparison test at $P < 0.05$ was used to investigate the effects of the used sperm-to-egg ratios on fertilization and hatch rates. All statistical analyses were conducted using the statistical software GraphPad Prism 4.0 for Windows.

Results

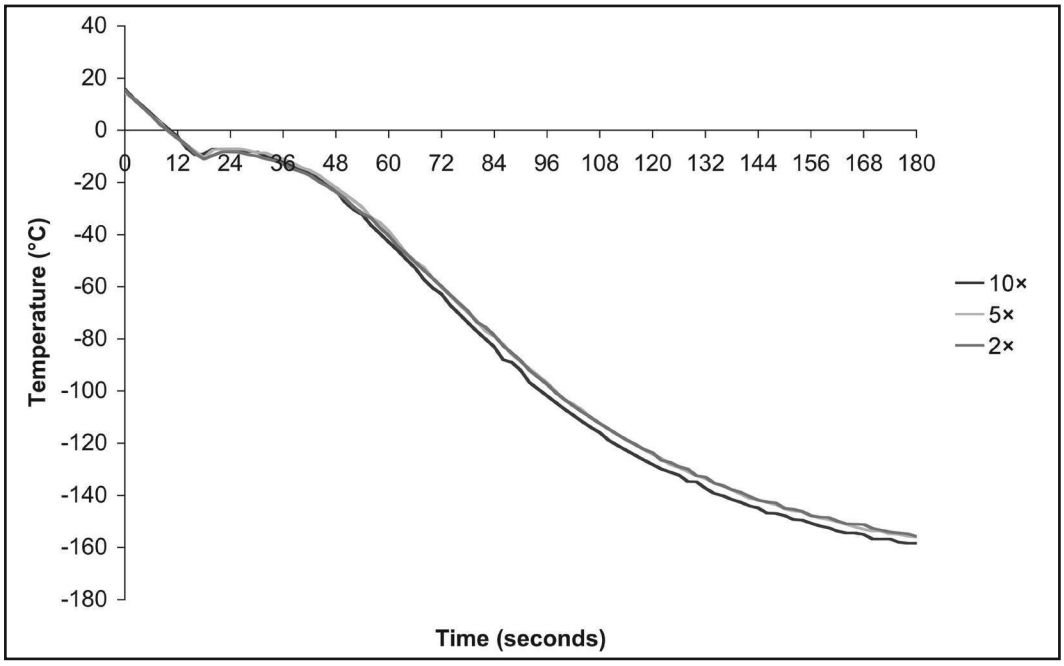
Sperm collection from the grayling through a silicone catheter was only partly successful – of the 12 stripped males sperm was successfully collected only from three individuals. Motility of grayling sperm immediately after collection was 78 ± 10% (N = 12), while that of the marble trout was 83 ± 6% (N = 3).

According to our measurements the cooling rates for the three dilution ratios were 57 ± 1 °Cmin^{-1} for 1:1, 57 ± 0 °Cmin^{-1} for 1:4 and 58 ± 1 °Cmin^{-1} for 1:9 (Figure 1). The highest post-thaw motility (50 ± 0 %) of grayling sperm measured in the first experiment corresponded to the pooled sperm sample with 1:1 dilution ratio (Table 1). In general, significant main effects of dilution ratios ($P < 0.0001$) and sperm samples ($P = 0.0090$) were observed on post-thaw motility of cryopreserved grayling sperm.

In case of the marble trout (Table 2) the highest post-thaw motility (23 ± 6%) was observed in sperm sample 1 at a dilution ratio of 1:9. Significant main effects of the dilution ratio ($P = 0.0110$) as well as the individual sample ($P = 0.0003$) on the post-thaw motility of marble trout sperm were found.

In case of fertilization percentages in the grayling at eyed stage (Table 1.), significant main effects of dilution ratios ($P = 0.0002$) and sperm samples ($P = 0.0285$) were found, however, only dilution

Figure 1. Cooling rates used in the experiments at sperm dilution ratios of 1:1, 1:4 and 1:9 (N=3).



ratios had an effect (P = 0.0008) on the hatch rates. The highest fertilization (74 ± 4 %) and hatch (63±6%) percentages were observed in sample number 10 using the 1:1 dilution ratio. A gradual decrease of fertilization and hatch rates was observed only in sample number 10, whereas, in sample number 11 or in the pooled sample, this decrease was not significant or was not observed at all.

Table 1. Post-thaw motility (a), fertilization at eyed stage (b) and hatch (c) percentages observed using cryopreserved sperm of the Adriatic grayling. All data are given as mean ± SD (N = 3). Values sharing a superscript letter within a column are not significantly different at P < 0.05. Control fertilization: 69 ± 6 %, control hatch: 56 ± 10 %.

Dilution ratio	Sperm sample number		
a	Pool	10	11
1:1	50 ± 0^{a}	43 ± 6^{a}	37 ± 6^{a}
1:4	43 ± 6^{a}	40 ± 10^{a}	30 ± 0^{ab}
1:9	20 ± 0^{b}	4 ± 2^{b}	18 ± 13^{b}
b.			
1:1	63 ± 13^{a}	74 ± 4^{a}	70 ± 2^{a}
1:4	66 ± 11^{a}	62 ± 2^{a}	70 ± 4^{a}
1:9	67 ± 3^{a}	29 ± 13^{b}	53 ± 10^{a}
c.			
1:1	53 ± 12^{a}	63 ± 6^{a}	61 ± 4^{a}
1:4	52 ± 12^{a}	53 ± 0^{a}	59 ± 4^{a}
1:9	53 ± 6^{a}	27 ± 12^{b}	46 ± 8^{a}

In case of the marble trout (Table 2.), the highest percentage of eyed eggs (84 ± 4%) and hatched larvae (70 ± 3%) was observed with sperm sample 3 and dilution ratio of 1:1. A significant main effect of individual samples was observed on the percentage of eyed eggs and hatched larvae (both: P<0.0001), yet, no main effect of the dilution ratio was found.

Sperm-to-egg ratio significantly affected both, fertilization (P = 0.0330) and hatch (P = 0.0455) percentages of grayling eggs. The use of 200 µl of thawed sperm gave the highest fertilization (78 ± 7 %) and hatch (73 ± 7 %) results, although, no significant difference was observed between the 200-µl and 100-µl groups (Table 3).

Table 2. Post-thaw motility (a), fertilization at eyed stage (b) and hatch (c) percentages observed using cryopreserved sperm of marble trout. All data are given as mean ± SD (N = 3). Values sharing a superscript letter within a column are not significantly different at P < 0.05. Control fertilization: 69 ± 6 %, control hatch: 56 ± 10 %.

Dilution ratio	Sperm sample number		
a	1	2	3
1:1	4 ± 6^{a}	3 ± 2^{a}	12 ± 8^{ab}
1:4	13 ± 6^{a}	4 ± 2^{a}	20 ± 0^{a}
1:9	23 ± 6^{b}	5 ± 5^{a}	10 ± 0^{b}
b.			
1:1	40 ± 25^{a}	50 ± 10^{a}	84 ± 4^{a}
1:4	67 ± 6^{a}	15 ± 12^{b}	80 ± 1^{a}
1:9	60 ± 20^{a}	22 ± 7^{ab}	66 ± 17^{a}
c.			
1:1	32 ± 20^{a}	41 ± 10^{a}	70 ± 3^{a}
1:4	57 ± 6^{b}	12 ± 9^{b}	66 ± 3^{a}
1:9	48 ± 16^{ab}	17 ± 5^{ab}	54 ± 13^{a}

Sperm concentration was 9.131 ± 0.415 × 10^{8} spermatozoa per ml, which resulted in 5.687 ± 0.181 × 10^{4} spermatozoa per egg using 200 µl of thawed sperm, 2.545 ± 0.063 × 10^{4} spermatozoa per egg using 100 µl of thawed sperm and 1.256 ± 0.026 × 10^{4} spermatozoa per egg using 50 µl of thawed sperm.

Table 3. Fertilization at eyed stage and hatch percentages using different sperm-to-egg ratios observed using cryopreserved sperm of the Adriatic grayling. All data are given as mean ± SD (N = 3). Values sharing a superscript letter within a row are not significantly different at P < 0.05.

	Sperm-to-egg ratio (spermatozoa egg^{-1})		
	5.69 ± 0.18 × 10^{4}	2.54 ± 0.06 × 10^{4}	1.26 ± 0.03 × 10^{4}
Fertilization	78 ± 7^{a}	72 ± 7^{ab}	60 ± 4^{a}
Hatch	73 ± 7^{a}	67 ± 7^{ab}	57 ± 5^{b}

Control fertilization and hatch results were not counted in this experiment due to human error.

Discussion

In this study, we showed that different sperm dilutions have significant effects on the fertilizing capacity of thawed sperm in the grayling. Dilution ratio 1:1 appears to be the most effective one in this respect, moreover, the highest post-thaw motility and hatch percentages also corresponded with this dilution. This effect was most vividly expressed in post-thaw motility rates, whereas in case of fertilization and hatch percentages the results were more balanced and strongly depended on the individual sample. According to the results, pooling of sperm samples resulted in similar fertilization and hatch rates regardless of the dilution ratio of sperm and observed post-thaw motility. In contrast to our results, a previous study on salmonid species recommended the use of dilution ratios higher than 1:1 (Lahnsteiner et al., 1996b). Only dilution ratio of 1:3 was previously used grayling (Lahnsteiner et al., 1996a).

To the best of our knowledge, this is the first report on the cryopreservation of marble trout sperm. Fertilization and hatch results of marble trout eggs fertilized with cryopreserved sperm show great variation according to the sample used. Drastic reduction of fertilizing capacity of cryopreserved brown trout sperm has been observed by several authors (Labbe & Maisse 2001; Martínez-Páramo et al., 2008) and authors found no correlation between fertilization rates and motility, viability, plasma membrane lipid profile, ATP content, DNA damage, membrane integrity or membrane resistance to osmotic shock.

Nevertheless, effective on-site sperm quality evaluation methods would be necessary to determine the suitability of sperm sample for cryopreservation.

The results obtained in this study also suggest that sperm-to-egg ratio has a significant effect on fertilization and hatch rates. The importance of sperm-to-egg ratios in maximizing fertilization success has been described previously for several salmonid species (Lahnsteiner et al., 1995, Lahnsteiner et al., 1996b). It has to be considered that sperm concentration in the sample used in this experiment was low, i.e. more then 3-6 times lower than that reported for the grayling (Lahnsteiner 2000b). Such a low concentration can in part be explained by the fact that the experiment was conducted relatively late in the spawning season. In a previous study on the grayling, sperm-to-egg ratios of $1.2\text{-}1.6 \times 10^6$ spermatozoa per egg (Lahnsteiner et al. 1996a) were recommended. Results of the present study suggest that much lower sperm-to-egg ratios (as low as 2.5×10^4 spermatozoa per egg) can yield satisfactory hatch percentages. Due to the deficiency of literature on the cryopreservation of grayling sperm our results can contribute to the better understanding of problems associated with fertilization with cryopreserved sperm.

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