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Chilled storage of unfertilized and fertilized rainbow trout (*Oncorhynchus mykiss*) eggs in sealed polyethylene bags at different temperatures

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ABSTRACT

Three experiments were conducted to study the effect of chilling temperature on unfertilized and fertilized rainbow trout eggs sealed in polyethylene (PE) bags. Experiment 1 showed that after 18 days of storage at 2 °C fertilization capacity of eggs was undiminished. At 7 °C, unimpaired fertilization capacity was maintained for 12 days and at 10 °C for 6 days. This leads to the conclusion that for storage up to approximately 1 week an ordinary household refrigerator will suffice; for more extended storage periods lower temperatures are advised. If PE bags filled with unfertilized eggs were submerged in water during chilling, similar results were obtained, though at a slightly lower level. The second experiment showed that fluctuations in chilling temperature between 2 °C and 10 °C were tolerated by unfertilized eggs as long as sufficient adaptation time was granted. One or two temperature changes within a 12 day period had no impact on fertilization capacity, whereas repeated changes at 2 day intervals caused a reduction in fertilization capacity. The attempt to apply the method of chilled storage of unfertilized rainbow trout eggs in PE bags to fertilized eggs (Experiment 3) indicated that early eyed eggs (14 days incubation at 10 °C) may be stored at 2 °C for 10 days without loss in hatching rate. Reasonable hatching rates may be achieved at 7 °C for about one week and at 10 °C for about 4 days. Storage of fertilized eggs of a more advanced stage of development (21 days incubation at 10 °C) calls for low temperature (2 °C) and storage periods of no > 4 days.

1. Introduction

Rainbow trout do not normally reproduce naturally in Europe. Offspring is produced by stripping eggs and semen from ripe fish during the spawning season and conduct artificial insemination and subsequent incubation until hatching. Rainbow trout are strictly seasonal breeders; therefore eyed eggs are imported to Europe from the southern hemisphere in summer. Reproduction, storage and shipping of eggs thus involves multiple manipulations of gametes and fertilized eggs. The latter are extremely susceptible to mechanical shock, especially from two days after fertilization until the eyed stage (Jensen and Alderdice, 1989). Consequently eggs are usually shipped at the eyed stage and the duration of shipment should preferably not exceed 48 h (Leitritz and Lewis, 1976). Unfertilized ("green") eggs are more resistant to mechanical shock and, due to lower metabolic rate, less prone to smothering (Leitritz and Lewis, 1976). Cryopreservation of eggs is, so far, not possible due to large size, high water content, the mass of yolk and the permeability properties of enveloping membranes (Lubzens et al., 2005). Recent studies are dealing with cryopreservation and hypothermal storage of ovarian germ cells (Lee et al., 2016; Falahatkar et al., 2017).

Chilled storage of unfertilized eggs and fertilized eggs within the first 48 h after fertilization and after eye spots are visible is possible. Shipping of unfertilized eggs has become an option since the availability of cryopreserved semen or semen from milters subjected to a light program providing semen out of season. Only few milters are needed because they may be stripped repeatedly over a period of several weeks (Bueyuekhatipoglu and Holtz, 1984; Pohl-Branscheid and Holtz, 1990). Unfertilized eggs have been stored at low temperature for two days in artificial media (Goetz and Coffman, 2000) and up to 10 days in coelomic fluid (Stoss et al., 1980; Jensen and Alderdice, 1984; Babiak and Dabrowski, 2003; Niksirat et al., 2007; Komrakova and Holtz, 2009; Bahabadi et al., 2011). Komrakova and Holtz (2011) described an efficient system for chilled storage of unfertilized eggs sealed in polyethylene (PE) bags without an air space or oxygen. The present investigation continues from there, attempting to explore to

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what extent eggs stored in this way are able to withstand exposure to different temperatures and temperature fluctuations. Furthermore it is attempted to apply the method of storage to fertilized rainbow trout eggs.

2. Materials and methods

2.1. General procedures

Eggs were obtained by stripping ripe 3 to 4 year old rainbow trout spawners during the peak of the spawning season (December to January) at the experimental farm Relliehausen of Goettingen University (9° 41′ E, 51° 46′ N). To avoid individual effects, strippings, consisting of eggs of sound appearance from several spawners at a time were pooled. Antibiotics (125 IU/g of penicillin-G [Sigma, St. Louis, U.S.A.] and 125 μg/g of streptomycin sulphate [Sigma, Steinheim, Germany]) were added to inhibit bacterial growth. For insemination, throughout the experiment the same batch of frozen-thawed semen was used, consisting of pooled strippings from 7 milters with excellent semen quality. Semen motility was assessed as described in Holtz et al. (1977). Briefly, a tiny drop of semen is placed in a hemocytometer chamber and, while adding tap water, proportion of motile spermatozoa, intensity and duration of progressive motility is viewed at a magnification of 100 ×. Semen with > 70% motile spermatozoa was cryopreserved as 0.1 mL-pellets as described by Holtz (1993). Briefly, freshly stripped semen was extended in a diluent consisting of a 0.6 M sucrose solution supplemented with 10% dimethyl sulfoxide and immediately pellet-frozen on dry ice (− 79 °C), at pellet size 0.1 mL and at least 250 × 106 sperm cells. For insemination, three pellets were dropped into a beaker containing 3 mL of an aqueous 0.12 M $NaHCO_3$ solution at 20 °C. After swirling the beaker until pellets were almost completely liquefied, the content was poured over the eggs that were then gently stirred and, after several rinsings with clear water, transferred to trays in a vertical flow incubator (Veco, Horgen, Switzerland). The incubator was supplied with a constant flow of 1 L/min of aerated water at 10 ± 1 °C and trays were partitioned into segments to accommodate the various treatment groups. Twice weekly non-viable eggs were removed with the aid of an egg picking pipette. After 18 days the number of eyed eggs was recorded and put in relation to the total number of eggs incubated. In Experiments 1 and 2 the proportion of eyed eggs, in Experiment 3 the proportion of hatched eggs served as fertility index. All treatments were repeated five times with separate pools of eggs for each experiment.

2.2. Experiment 1: storage of unfertilized eggs at different chilling temperatures in air and under water

Pooled strippings (eggs and coelomic fluid) from 10 spawners were divided up into 120 batches of 100 ± 10 eggs. Batches were filled into 7 × 3 × 1.4 cm bags of 0.3 mm polyethylene (PE) sheeting. Bags were heat-sealed with a household sealing device without leaving an air space (Komrakova and Holtz, 2011). Forty bags were randomly allocated to each of three laboratory refrigerators set at temperatures 2 °C, 7 °C and 10 °C, respectively. Half the bags were kept under air, the other half submerged in water. From each treatment group eggs of five bags at a time were inseminated with frozen-thawed semen after 3, 6, 12 and 18 days of storage, and incubated until hatching. Batches of 100 ± 10 eggs from the same pool, which had not been subjected to chilled storage, were inseminated with frozen-thawed semen to serve as controls.

2.3. Experiment 2: effect of changes in chilling temperature on unfertilized eggs

Pooled strippings (eggs and coelomic fluid) from 6 spawners were divided up into 40 batches of 100 ± 10 eggs each that were sealed in PE bags as described for Experiment 1. Fifteen bags each were placed into two laboratory refrigerators set at 2 °C and 10 °C, respectively. Five bags remained in the respective refrigerator throughout the 12-day experimental period (Groups 1 and 2). Five bags were exchanged between refrigerators after 4 days and remained there (Groups 3 and 4). Five bags were exchanged between refrigerators after 4 days and returned to the original refrigerator after another 4 days (Groups 5 and 6). The remaining ten bags were placed into the 2 °C refrigerator and moved back and forth between it and the10°C refrigerator at two day intervals. Whereas 5 bags underwent 5 changes (Group 7), the other 5 underwent only 2 changes (Group 8). At the end of the experiment (Groups 1 to 7 after 12 days and Group 8 after 6 days) eggs were inseminated with frozen-thawed pooled semen and incubated to the eyed stage. Control batches of 100 ± 10 eggs each from the same pool that had not been subjected to chilled storage, were inseminated with the same batch of frozen-thawed semen.

2.4. Experiment 3: effect of chilling temperature on 14 and 21 day-old eyed eggs

Freshly stripped eggs pooled from 6 females were inseminated with fresh semen of excellent motility rating (Holtz et al., 1977) pooled from five milters. After 14 and 21 days of incubation at 10 ± 1 °C, batches of 75 ± 5 eggs were gently pipetted to 7x3x1.4 cm PE bags; 5 ± 2 mL of incubator water was added and bags were heat-sealed with no air space remaining. Thirty bags each were distributed to three separate refrigerators set at temperatures 2 °C, 7 °C and 10 °C, respectively. After 4, 7 and 10 days, ten bags at a time were removed from each of the refrigerators. Bags were submerged in water at 10 °C for 20 min to adapt to incubator temperature before they were opened and eggs were released to the tray of an incubator. A corresponding number of egg batches that had remained in the incubator served as controls.

2.5. Statistical analyses

All treatments and untreated controls were repeated five times using pooled batches of eggs for each experiment (technical repetitions). To reveal the impact of treatments on the respective variables, an analysis of variance (F-test, $P < 0.05$) was conducted (SAS version 8) and differences between individual means were tested for significance by Scheffé-test ($P < 0.05$). Data are presented as means ± SEM.

3. Results

3.1. Experiment 1: storage of unfertilized eggs at different chilling temperatures in air and under water

The effect of storage temperature on the fertilization capacity of freshly stripped rainbow trout eggs sealed in PE bags in air or submerged in water, assessed after 3, 6, 12 and 18 days of storage, is depicted in Fig. 1. The data are shown relative to freshly fertilized controls (84.8 ± 2.2%). The effects “storage time” and “temperature”, as well as the interaction “storage time x temperature” were statistically significant ($P < 0.05$, F-test). Scheffé-test revealed that at 2 °C under air, fertilization capacity was maintained essentially constant throughout the 18-day storage period ($P > 0.05$). At 7 °C, fertilization capacity remained constant on days 3, 6 and 12 but dropped to 11.3% on day 18 ($P < 0.05$). At 10 °C, fertilization capacity decreased on days 12 and 18 compared to that on days 3 and 6 (both $P < 0.05$). When egg packages were stored submerged in water at similar temperatures, the tendency was the same, though at a slightly lower level. Across storage days the fertilization capacity at 2 °C was (non-significantly) lower by 12.5%; at 7 °C it was lower by 27.4% and at 10 °C by 42.2%. Differences between eggs stored under water vs. in air, encountered at temperatures of 7 °C and 10 °C, were significant from 6 days of storage onward ($P < 0.05$).