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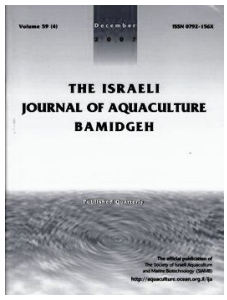

Effect of Temperatures and Storage Periods on Fertilizing and Hatching of Short-Term Preserved Scaly Carp (*Cyprinus carpio*) Eggs

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Abstract

Preserved fish eggs can be used in many applications including artificial propagation and genetic studies. The aim of this study was to determine the effects of different temperatures and storage periods on fertilization and hatching success of short-term preserved scaly carp (*Cyprinus carpio*) eggs. Eggs were kept in 4 ml ovarian fluid in 10-cm petri dishes that were stored in a refrigerator at 4°C, 8°C, or 12ºC for 30, 60, or 90 min. The stored eggs were fertilized by adding 200 µl milt to each petri dish. The highest fertilization (98%) and hatching (80%) rates were obtained with eggs stored for 30 min at 8ºC and 12ºC respectively, indicating that this combination was best for short-term preservation of scaly carp eggs.

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Introduction

The practical use of fish gametes is limited by their rapid and continuous decline in viability once released or removed from gonads. Mature ova may remain arrested for weeks in the second meiotic metaphase until ovulation and activation (Sohrabnezhad et al., 2006) but, after ovulation, storage of eggs for later use and prolonged viability become crucial issues (Rothbard et al., 1996). Eggs must be fertilized within a certain period of time after ovulation to produce viable embryos and this time varies between species from hours to weeks (Hobby and Pankhurst, 1997). Fertilization gradually diminishes, then disappears altogether, when mature ovulated eggs are retained in the body cavity of the female or kept *in vitro* in water. Fertility of eggs is retained much longer in saline water and, especially, in ovarian fluid (Dettlaff et al., 1993).

Preservation of fish eggs can have many applications such as in artificial propagation. Also, freshly collected and stored eggs can be shipped to other locations for fertilization (Bozkurt et al., 2010). Gametes stored for short terms can be transported from the hatchery to distant incubation facilities for delayed fertilization. Attempts have been made to preserve unfertilized eggs in artificial medium rather than in ovarian fluid. Unfertilized rainbow trout eggs can be stored in modified Cortland medium buffered with Hepes or Tris for 24 or 48 h at 12-13ºC (Goetz et al., 2000). However, *in vitro* storage of rainbow trout eggs in a medium that was very similar to ovarian fluid for three days at 12ºC resulted in a dramatic decrease of subsequent developmental success compared to eggs held in ovarian fluid (Bonnet et al., 2003). Unlike shellfish eggs and embryos, finfish ova and embryos are large, contain a large amount of yolk, and are covered with a relatively thick chorion. Because of their complex organic matrix, uniformity of penetration of conventional cryoprotectants, consistent cooling rates, freezing processes, and cryopreservation of finfish ova and embryos are often unsuccessful (Chao and Liao, 2001). Further, inadequate dehydration and toxicity of cryoprotectants tend to freeze fish ova (Suquet et al., 1999).

Ovulated oocytes retained in the ovarian cavity undergo over-ripening due to gradual morphological and biochemical changes that negatively affect fertility and gamete quality (Formacion et al., 1993). However, in certain cases, eggs can be retained after ovulation in the abdominal or ovarian cavity with no loss of fertility (Springate et al., 1984). In general, eggs can be maintained for several days in ovarian fluid, the storage period being related to the holding temperature (Jensen and Alderdice, 1984). Storage temperature has a significant affect on fertilization success (Jensen and Alderdice, 1984) and is a major factor that affects the viability of fish gametes in *in vitro* studies. Viability can be prolonged by maintaining gametes and embryos close to 0°C to reduce their metabolic rate. However, the ability to tolerate such a low temperature varies among species (Leung and Jamieson, 1991).

Development of a technique to preserve scaly carp (*Cyprinus carpio*) eggs for short periods would improve the efficiency of reproduction management. The goal of the present study was to determine the effects of different storage temperatures and periods on the fertilizing and hatching capacities of short-term preserved scaly carp eggs retained in ovarian fluid under *in vitro* conditions.

Materials and Methods

Artificial propagation of scaly carp under hatchery conditions. Ten mature males and five mature females were were kept in separate 4-m^3 hatchery tanks in 23-24°C water with a flow of 0.2 l/s and 6-7 mg O_2/l. Ovulation was induced by injections of 0.5 and 2.0 mg/kg carp pituitary extract (CPE) in Ringer solution at a 12-h interval. Spermiation was induced by one injection of 1.0 mg/kg CPE in Ringer solution. To collect gametes, broodfish were anesthetized in 100 ppm quinaldine sulphate. Males and females were stripped by abdominal massage. Females were stripped 12 h after the second CPE treatment and their eggs and ovarian fluids were pooled. Sperm was collected 12 h after CPE treatment. Care was taken to avoid contamination with urine, mucus, blood, or feces.

Storage of eggs in ovarian fluid. The pooled eggs were stored in pooled ovarian fluid. About 4 ml ovarian fluid was placed in each of thirty (treatments were performed in triplicate) 10-cm petri dishes and 1 g eggs were gently weighed and added. The petri dishes were transferred to three refrigerators for storage at 4°C , 8°C, or 12°C for 30, 60, or 90 min.

Fertilization. Batches of approximately 1000 pooled eggs (1 g) were fertilized at time 0 (prior to storage; control), and 30, 60, and 90 min after stripping. Sperm was stripped at the time of fertilization and examined for motility before use. Only sperm with ≥80% motility was used. Sperm from the five males was pooled in the same proportion (by volume) as obtained by stripping. For fertilization, ovarian fluid was removed from the petri dishes and 200 µl semen was added to the eggs (approximately 2 x 10^5 spermatozoa per egg) according to the dry fertilization technique using a solution of 3 g urea and 4 g NaCl in 1 l distilled water. The eggs were stirred 1 h, then treated with tannic acid solution (0.5 g/l) for 30 s to remove stickiness. Finally, the eggs were washed with water and gently transferred to labeled Zuger glass incubators with running water (23-24°C) where they were kept until eyeing (14-16 h) and hatching (4-5 d). Fertilization success was determined 3-4 days following fertilization, at the gastrula stage.

Statistical analysis. Data are expressed as means±standard deviations. Relative quantities were transformed by angular transformation, and metric data were tested for normality. Data were analyzed by one-way multifactorial variance (ANOVA) with subsequent Tukey's b test.

Results

The highest fertilization (98%) and hatching (80%) rates were obtained in eggs stored at 8ºC for 30 min (Fig. 1). Differences between all mean fertilization rates within the same storage temperature groups were significant ($p<0.05$). Also differences between all mean hatching rates within the same storage temperature groups were significant ($p<0.05$) when duration was taken into consideration. The interaction between storage temperature and period was significant ($F = 6.17$).

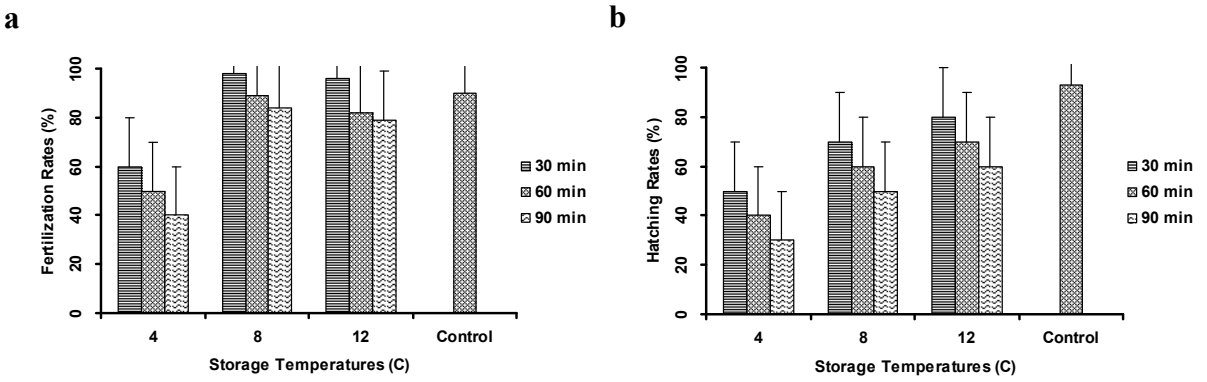

Fig. 1. (a) Fertilization and (b) hatching rates of scaly carp eggs after *in vitro* incubation in ovarian fluid at 4ºC , 8ºC, or 12ºC for 30, 60, or 90 min prior to fertilization.

Discussion

Fish eggs and embryos can be sensitive to chilling (Gwo, et al., 1995; Hagedorn et al., 1996) caused by direct or indirect chilling injury. The extent of damage is related to the cooling rate as well as the temperature and period at which the cells were exposed to low temperature (Dinnyes et al., 1998; Lahnsteiner et al., 2003). Several theories exist for the mechanism of chilling sensitivity, but they differ according to species and cell type. At low temperatures, microtubules are depolimerized, and cellular processes such as cell division in oocytes can be irreversibly disrupted. Plasma membranes can suffer lateral phase separation, proteins can be denatured due to the destabilization of hydrophobic

bonds, and cell membranes can shrink relative to the intracellular space, potentially resulting in stress or damage to membranes (Arav et al., 1996; Dinnyes et al., 1998).

The present study indicates that scaly carp eggs can be preserved for 90 min at 8ºC and 12ºC. Lower preservation temperatures may increase chilling injury. Long holding times can decrease fertilization and hatching rates. In the present study the highest fertilization and hatching rates at each tested temperature were obtained when eggs were stored for the minimum period - 30 min.

Further investigation is necessary to standardize techniques for increasing the duration of storage that is possible at refrigerator temperatures.

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