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Short-Term Preservation of Grass Carp (*Ctenopharyngodon idella*) Eggs: Effect of Extenders and Storage Duration

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Abstract

The short-term effects of extenders and storage duration on fertilization, eyeing, and hatching rates in short-term stored grass carp eggs was evaluated. Eggs were kept in one of two artificial extenders (Ringer and 300 mM glucose solutions) or in ovarian fluid in 10-cm petri dishes. About 4 ml of artificial extender or coeolomic fluid was added to each petri dish and the dishes were transferred to a dark humid refrigerator for storage at 4ºC for 120, 240, 360, or 480 min. Eggs were fertilized by adding 200 µl milt to each petri dish. The fertilization rate was determined at the gastrula stage. The eyeing rate was defined as the number of eyed eggs divided by the initial number of eggs used for fertilization. The highest fertilization rate (93%) was attained in coelomic fluid stored for 120 min. The highest eyeing rates were in eggs kept in ovarian fluid for 120 or 240 min. The glucose extender was best for longer storage (360 and 480 min). The highest hatching rate was obtained with ovarian fluid at 240 min but the glucose extender was best for storage at 120, 360, and 480 min. Results indicate that glucose solution is the best extender for short-term (2-4 h) storage of grass carp eggs.

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Introduction

Gamete storage in aquaculture has mostly focused on sperm preservation (Leung and Jamieson, 1991), by which spermatozoa are cooled, frozen, and thawed. Similar methods for storage of fish ova have not yet yielded satisfactory results. Preservation of fish eggs have many applications. It can be useful in artificial propagation facilities and genetic studies aimed at overcoming low fecundity. Also, freshly collected and stored eggs can be shipped to other locations for fertilization.

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 other cases, external storage of salmonid eggs in coelomic fluid or artificial media is satisfactory. In general, eggs can be maintained for several days in coelomic fluid, and the duration is related to the holding temperature (Withler and Morley, 1968; Jensen and Alderdice, 1984). In contrast, holding eggs for similar periods in artificial media is less effective (Takano et al., 1973).

It is assumed that differences in fertilization success will occur in relation to species, conditions of storage, extender, and duration between stipping and fertilization. The objectives of this study were to examine the effects of duration on the fertility of eggs retained in different extenders and coelomic fluid under *in vitro* conditions.

Materials and Methods

Broodstock, collection of gametes and ovarian fluid. Grass carp broodstock (4-7 years) were taken from large ponds (5-7 ha). Broodfish suitable for stripping were selected in May and sexes were separately kept in nursery ponds of 0.01 ha. Three days before stripping, the broodfish, still kept separately according to sex, were transferred to the hatchery. Male and female grass carp were kept in separate 4-m^3 hatchery tanks with a water flow of 0.2 l/s, 25-26°C, 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 12 h intervals. Spermiation was induced by one injection of 1.0 mg/kg CPE in Ringer solution. For gamete collection, broodfish were anesthetized in 100 ppm quinaldine sulphate. Males and females were stripped by abdominal massage. Five females were stripped 24 h after the first CPE treatment. Eggs and ovarian fluid were collected separately for each female. Sperm of five males was collected 12 h after CPE treatment. Care was taken to avoid contamination with urine, mucus, blood, or feces.

Storage of eggs in extenders and ovarian fluid. Two extenders and ovarian fluid were used to store the eggs. The first extender was Ringer solution containing 103 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, and 1.1 mM $NaHCO_3$ (Linhart et al., 1991). The second extender was a glucose solution containing 300 mM glucose (Tekin et al., 2003). The eggs of the five females were pooled and 1 g of eggs were gently weighed and placed in 10-cm petri dishes. About 4 ml of extender or ovarian fluid were added to each petri dish. The petri dishes were transferred to a dark humid domestic refrigerator and stored in a constant temperature (4°C) for various durations (120-480 min).

Fertilization. Batches of approximately 1000 eggs (1 g) pooled from five females were fertilized at time 0 (prior to storage; control), and at intervals of 120, 240, 360, and 480 min after stripping. Sperm was stripped from five males at the time of fertilization and examined for motility before use. Only individuals with sperm motility higher than 80% were selected. Sperm from the five males was pooled in the same proportions by volume as that obtained by stripping. For fertilization, the extenders and ovarian fluid were removed from the petri dishes and 200 µl milt was added to the eggs. Eggs were inseminated with diluted or undiluted semen at 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 sperm and eggs were lightly stirred for 30 min, washed with water, and gently transferred to labelled Zuger glass incubators with running water (25-26°C) where they were kept until eyeing (14-16 h) and hatching (4-5 d).

Dead embryos in partially or completely opaque eggs were removed. Fertilization success was determined 3-4 days following fertilization, at the gastrula stage. The eyeing rate was defined as the number of eyed eggs divided by the initial number of eggs used for fertilization. Newly hatched larvae were removed by siphoning and counted. The hatching rate was defined as the number of hatched larvae divided by the initial number of eggs used for fertilization. Each treatment was performed in triplicate for each extender and for each incubation duration (3 extenders x 4 duration times = 12 treatments + 1 control x 3 replicates = 39 incubators).

Statistical analysis. Results are presented as means±SE. Differences between treatment groups were analyzed by repeated analysis of variance (ANOVA). Significant means were subjected to a multiple comparison test (Duncan) for post-hoc comparisons at level of $\alpha = 0.05$. All analyses were carried out using SPSS 10 for Windows Satistical Software Package.

Results

The highest fertilization (93%) rate occured in eggs stored in ovarian fluid for 120 min but the rate decreased to 47.3% after 480 min storage (Fig. 1). Glucose was the best extender for 240, 360, and 480 min storage. Differences between mean fertilization rates were significant ($p<0.05$) when duration was taken into consideration. The interaction between extender and duration was significant ($F = 8.39$).

The best eyeing rates were in eggs stored in ovarian fluid for 120 and 240 min storage but glucose was the best extender for 360 and 480 min storage. Differences between means were significant ($p<0.05$) when duration was considered. The interaction between extender and duration was significant ($F = 5.47$).

The best hatching rates were obtained with ovarian fluid for 240 min, followed by glucose for 120, 360, and 480 min. Differences between mean hatching rates were significant ($p<0.05$) when duration was taken into

consideration. The interaction between extender and duration was significant (F = 1.99).

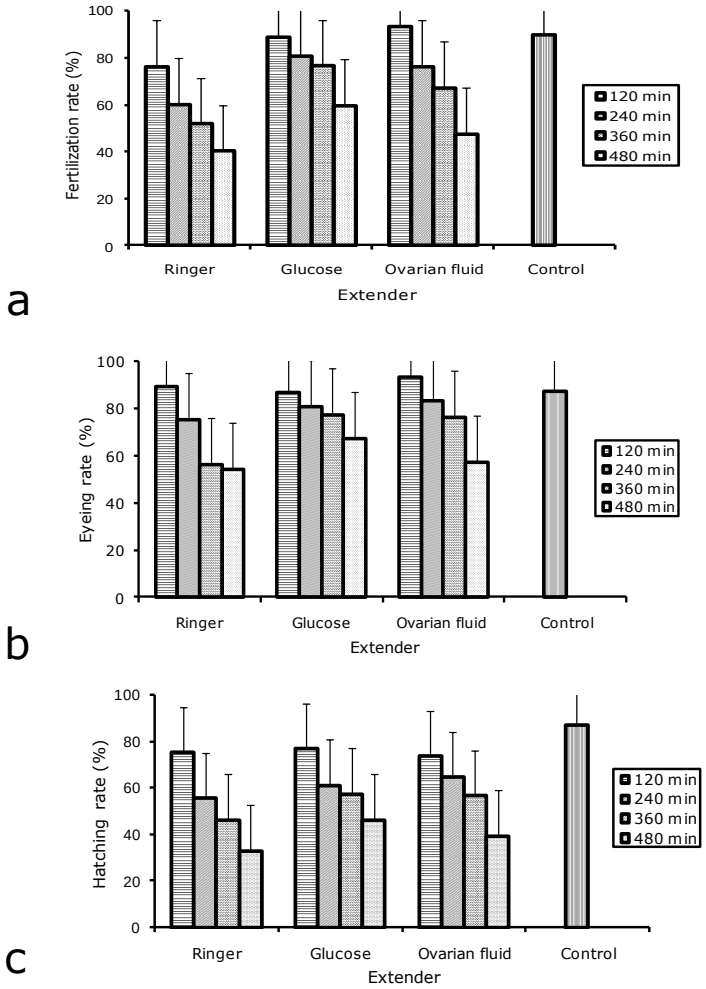

Fig. 1. (a) Fertilization, (b) eyeing, and (c) hatching rates of grass carp eggs after *in vitro* incubation in one of three extenders (Ringer solution, glucose solution, ovarian fluid) for 0 (control), 120, 240, 360, or 480 min prior to fertilization.

Discussion

The Ringer medium, glucose solution, and ovarian fluid enabled extending the storage time of fish eggs at 4°C. Eggs stored in 300 mM glucose had the highest fertilization rates. Thus, we conclude that it is possible to enhance the fertilizing capacity of fish by using a suitable activating media that increases the duration of motility.

Few studies have dealt with the fertilizing capacity and hatching rate ova from warm-water fishes kept in extenders. Ova of the European catfish

(*Silurus glanis*) were sensitive to fresh water, saline solutions, and urine (Linhart and Billard, 1995a). However, variability of ova may be related to the health of the female, time of ovulation, temperature, feeding, and water quality (Horvath, 1978; Linhart and Billard, 1995b). In turbot (*Scophthalmus maximus*), an extender such as turbot artificial ovarian fluid with or without ethylene glycol tetra-acetic acid (EGTA) decreased the storage capacity of ova, and soybean trypsin inhibitor did not significantly change the results compared to dry ova (Suquet et al., 1999).

In spite of successful egg storage in ovarian fluid in the present study, contamination with discarded materials that are often mixed with ovarian fluid during stripping, such as blood, urea, feces, and especially yolk from broken eggs, interferes with fertilization success (Wilcox et al., 1984; Van Heerden et al., 1996). These contaminants may limit the storage capacity of ovarian fluid as an extender medium.

The present study indicates that grass carp (*Ctenopharyngodon idella*) eggs can successfully be preserved for 480 min at 4ºC prior to artificial insemination. Further investigation is needed to determine the viability, survival, and development of larvae produced from short-term stored eggs.

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