

In situ and ex situ preservation of Prussian carp oocytes

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Abstract. The decline of ova viability after ovulation is one of the limiting factors in controlled reproduction of several fish especially in warm water fish. The purpose of this study was evaluating the viability of Prussian carp oocytes retained within the ovarian cavity (in situ storage) and outside of the ovarian cavity (ex situ storage) at 21°C and 4°C for 20, 30, 60 and 120 min. Experiments were performed with 20 female fish (10 females for each experimental group) *Carassius gibelio* (Bloch, 1782) induced to spawn with 0.5 mg kg⁻¹ Ovaprim (sGnRH+Dompridon). Prussian carp oocytes could be successfully stored for 2 h in situ at 21°C and 4°C without significant decrease in eyed embryo and hatching rate ($P > 0.05$). The percentage of eyed embryo in kept eggs outside the ovarian cavity at 21°C and 4°C for all durations was significantly different compared to control group ($P < 0.05$). There was not significantly different in deformed larvae percent during in situ and ex situ storage at 21°C and 4°C as compared with control.

Key Words: oocyte, in situ, ex situ storage, Prussian carp.

Introduction. Fish spermatozoa from various species have been successfully cryopreserved, but cryopreservation has not been successful for unfertilized eggs or fertilized eggs at any stage of development because of their large size, complexity, yolk content and relative impermeability to cryoprotectants (Zhang et al 1993). Oocyte viability refers to the time period during which fertilization of ovulated oocytes remains possible once they have been emitted by the female (Legendre et al 1996). The fertilizability of mature ovulated ova gradually decrease and then disappears altogether when they are retained in the body cavity of the female, kept in coelomic fluid or in water, or under in vitro conditions (Rothbard et al 1996). Fertility of fish gametes undergoing external fertilization is limited to a few seconds or minutes in water because oocytes are activated and undergo cortical reaction leading to micropyle closure (Hart 1990). Short term preservation aimed at increasing post spawning gamete longevity may improve hatchery management, minimizing problems resulting from inbreeding and asynchronous brooder maturation (Bromage & Roberts 1995). Ovulated oocytes retained in the ovarian cavity undergo over ripening due to gradual morphological and biochemical changes that negatively affect fertility and larval development (Formacion 1993). As over-ripening of eggs will inevitably eventually occur in fish bloodstocks, it is important to obtain and fertilize eggs at the correct time after ovulation. In fish hatcheries, over ripening represents a problem when ovulated eggs are not stripped on time, especially for those fish whose eggs can only be obtained by manual stripping and fertilized artificially (Kjorsvik et al 1990). Unlike salmon and trout, oocytes of the few warm water fishes examined to date lose their viability within a few hours of storage in the ovarian cavity (Espinach et al 1984; Rizzo et al 2003).

After ovulation, the ova remain arrested in metaphase II of meiosis until they become activated. Activation removes this dormant stage and is associated with a number of physiological changes (Ginsburg 1968; Linhart et al 1995b). The post-ovulation storage of ova in the ovarian cavity (in vivo) of common carp *Cyprinus carpio* (Linnaeus, 1758) is usually very short, ranging from 1–6 h (Horvath 1978; Suzuki 1980;

Jahnichen 1981; Marcel 1981). Ova stripped after ovulation and stored in vitro progressively lose their fertility after 4–6 h at temperatures of 15–20°C (Jahnichen 1978; Kiselev 1980; Marcel 1981; Zlabek & Linhart 1987). Koi carp (*Cyprinus carpio* Linnaeus, 1758) ova stored without an extender for 6 h at a stable temperature of 20°C had more than 50% hatching rate (Rothbard et al 1996). The fertility of tench *Tinca tinca* (Linnaeus, 1758) ova stored in vitro for 1, 5.5 and 8.5 h at 13°C significantly decreased to 45.4, 10.1 and 0.6%, respectively, as calculated by hatching rate (Linhart & Billard 1995a).

The aim of this study was to evaluate the effect of in situ and ex situ Prussian carp ova storage on its eyed embryo and hatching capacity and malformed larvae percent.

Material and Method. Broodstocks (15 mature males and 20 mature females) were obtained from reared hatchery at Nahar Khoran, Gorgan, during the spawning season of Prussian carp and transferred to the place of experiment and acclimated for 2 weeks in 1000 L tanks. The experiment was carried out at the aquaculture Center of Agricultural Science and Natural Resources, University of Gorgan, Iran.

Gametes collection. To stimulate fish, we injected intraperitoneally: 0.5 mL kg⁻¹ b.w. Ovaprim (sGnRHa+dompridon). Milt samples were collected during the 2010 spawning season from 15 sexually mature two-years-old male Prussian carps (mean TW: 43.6 ± 9.84 g). Semen samples were collected by massage from the anterior portion of the testis towards the genital papilla. Care was taken to avoid contamination of the semen with water, mucus, blood cells, faeces or urine. Semen of each male was collected by using 2 cc polyethylene syringes and sperm batches were stored in a refrigerator at 4°C until use for fertilization. At fertilization, sperm of all males was pooled in the same proportions by volume as that obtained by stripping. These mixtures were used for the fertilization assay.

Also, females (mean TW: 57.23 ± 8.61 g) were injected with 0.5 mg kg⁻¹ Ovaprim and 12 hours after injection treatment half of them were stripped and their eggs were collected in a bowl. The other half females (10 females) were kept in tank at 21°C (5 females) and in refrigerator at 4°C (5 females).

Storage of ovulated oocytes. Recently obtained eggs and females were subjected to one of the following storage treatments:

- Group 1 (in situ storage at 21°C): 5 ovulated, not stripped females (i.e. with their oocytes retained in the ovarian cavity) were kept together in one 300 L tank with aeration and were stripped 20, 30, 60 and 120 min after ovulation.
- Group 2 (in situ storage at 4°C): 5 ovulated, not stripped females were kept in 2 aquariums (with 80 L aerated water) and were stripped 20, 30, 60 and 120 min after ovulation.
- Group 3 (ex situ storage at 21°C and 4°C): The all of female ova were pooled. This treatment had 8 treatments and three replicates for each treatment. For each replicate 300 mg (about 300) eggs were placed in Petri dish (10 cm diameter). Half of Petri dishes were placed into a refrigerator (exposure to 4°C) and half of them held at 21°C.
- Control: Batches of about 300 ova (300 × 3 replicates) were fertilized immediately after egg collection and was considered as control group.

Fertilization and incubation. After storage for above time in the two test temperatures and in situ and ex situ conditions, undiluted sperm (0.02 mL) was added to oocytes, and water (10 mL) at ambient temperature (21°C) was used to activate the gametes, allowing fertilization and adhesion of eggs to the Petri dishes, then transferred to aquarium (containing 80 liters aeration water) and were placed in the bottom of them. The eggs were placed in a monolayer to ensure adequate oxygen supply. The percentage of eyed embryos, hatching rate and percentage of malformed larvae were recorded.

Statistical analysis. Data analysis of variance (two-way ANOVA) was done with Duncan test for the comparison of mean values resulting from the various treatments at

a significance level of $P < 0.05$. Before analysis by ANOVA, data was used for normality of data distribution and homogeneity of variance. Results are presented as mean \pm SD. Statistical analyses were performed with SPSS 16 for windows statistical package.

Results and Discussion

Effects of ex situ and in situ storage on eyed embryo and hatching rate. In general, there was a significant decline ($P < 0.05$) in the rate of eyed embryo and hatching rate over the period of ex situ oocyte storage. Results showed 64% decline in the rate of eyed embryo at 4°C and 70% decrease at 21°C as compared with controls after 120 min in this experimental group. Also, at 60 min, the eyed embryo rate had decreased by over 19% at 4°C and 23% at 21°C (Table 1). The percentage of hatching of stored ova in ex situ condition decreased with increasing time at 4°C and 21°C ($P > 0.05$). Temperature had significantly effect on eyed embryo and hatching rate; so that these parameters at 4°C were greater than 21°C (Table 1).

Table 1

Effects of ex situ (out of ovarian cavity) storage, temperature and time on eyed embryos and hatching percent (Mean \pm SD) in Prussian carp ova (*C. gibelio*)

Treatments	Eyed embryo	Hatching
Control	79.47 \pm 2.38 ^a	89.52 \pm 3.10 ^a
ex situ- 4°C- 20 min	71.62 \pm 4.25 ^b	84.46 \pm 2.89 ^b
ex situ- 4°C- 30 min	62.53 \pm 5.48 ^d	76.61 \pm 4.53 ^d
ex situ- 4°C- 60 min	45.30 \pm 3.64 ^f	70.83 \pm 5.64 ^f
ex situ- 4°C- 120 min	15.73 \pm 6.20 ^h	62.71 \pm 6.23 ^h
ex situ- 21°C- 20 min	66.82 \pm 4.56 ^c	80.25 \pm 2.67 ^c
ex situ- 21°C- 30 min	52.64 \pm 5.37 ^e	72.39 \pm 3.24 ^e
ex situ- 21°C- 60 min	35.60 \pm 7.82 ^g	66.84 \pm 4.19 ^g
ex situ- 21°C- 120 min	9.60 \pm 2.80 ⁱ	54.60 \pm 3.18 ⁱ

In oocytes stored in situ at 21°C and 4°C, the eyed embryo and hatching percent at 20, 30, 60 and 120 min of storage were statistically similar to those of the controls (Table 2). In situ storage at 21°C and 4°C had not drastic reduction in eyed embryo and hatching rates as compared with control (Fig. 2).

Effect of egg storage in situ and ex situ on malformed larvae rate. The percentage of malformed larvae had not significantly different in all treatments in situ and ex situ storage after 20, 30, 60 and 120 min storage (Figs 1-2).

Table 2

Effects of in situ (in ovarian cavity) storage, temperature and time on eyed embryos and hatching percent (mean \pm SD) in Prussian carp ova (*C. gibelio*)

Treatments	Eyed embryo	Hatching
Control	79.47 \pm 2.38 ^a	89.52 \pm 3.10 ^a
in situ- 4°C- 20 min	78.46 \pm 5.3 ^a	88.29 \pm 4.67 ^a
in situ- 4°C- 30 min	77.19 \pm 4.23 ^a	88.42 \pm 5.81 ^a
in situ- 4°C- 60 min	77.52 \pm 5.80 ^a	87.76 \pm 3.94 ^a
in situ- 4°C- 120 min	76.64 \pm 4.12 ^a	86.39 \pm 5.48 ^a
in situ- 21°C- 20 min	77.29 \pm 3.71 ^a	87.67 \pm 4.58 ^a
in situ- 21°C- 30 min	77.37 \pm 3.82 ^a	87.81 \pm 6.27 ^a
in situ- 21°C- 60 min	76.58 \pm 6.74 ^a	86.26 \pm 3.37 ^a
in situ- 21°C- 120 min	76.61 \pm 4.19 ^a	86.45 \pm 5.64 ^a

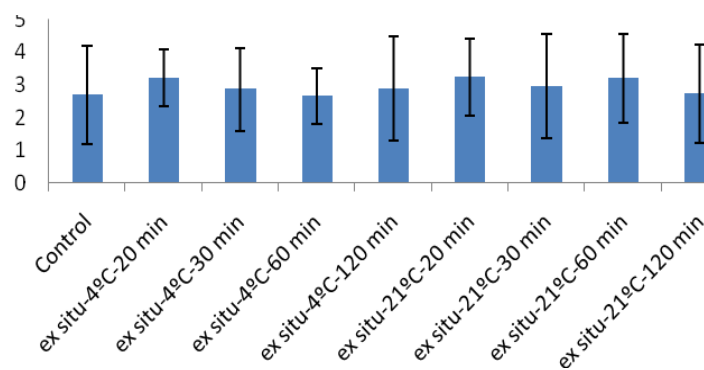


Fig. 1. Larvae malformation in ex situ storage of Prussian carp eggs.

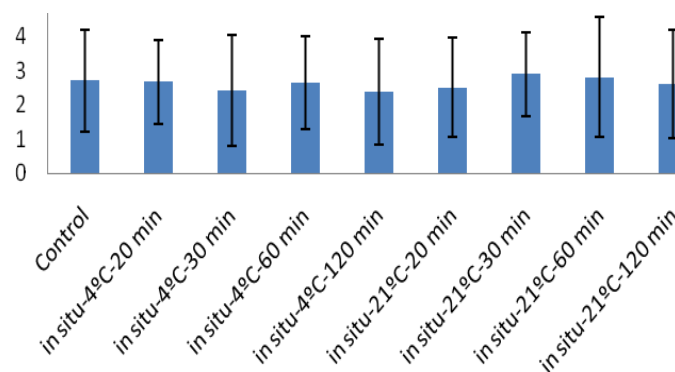


Fig. 2. Larvae malformation in the case of in situ storage of Prussian carp eggs.

Effect of time of storage, holding condition and temperature on eyed embryo, hatching and malformed larvae percent. Results shown that the effect of ex situ storage time on eyed embryos and hatching rate was significant. In this status, storage temperature had significant effect on eyed egg rate ($P < 0.05$). Eyed egg and hatching rate decreased with increasing time (20, 30, 60 and 120 min) but malformed larvae rate had not changes with time in all treatments. Highest eyed embryo and hatching rate between treatments of ex situ storage was observed in 20 min storage and 4°C and lowest eyed embryo and hatching rates was observed in 120 min storage at 21°C. Also, any significant difference was not observed in eyed egg, hatching and malformed larvae percent in ovarian cavity storage at 21°C and 4°C and 20, 30, 60 and 120 min storage.

In this work, the viability of *C. gibelio* was assessed through eyed embryo and hatching rates evaluated after in situ and ex situ storage. Percentage of eyed eggs and hatching in ex situ holding decreased rapidly as storage time increased and it was recorded close to 9.6%, 54.6% and 15%, 62% after 120 min ex situ storage at 21°C and 4°C respectively, similar to reports in several teleost species (Mollah & Tan 1983; Espinach et al 1984; Linhart & Billard 1995b; Linhart et al 2001; Rizzo et al 2003). Thus, in our experiments, *C. gibelio* oocytes could only be successfully stored for 1 h in ex situ treatment at 4°C. The highest eyed eggs and hatching rate were obtained when stripping and fertilization were performed immediately after ovulation, as in the *Rhamdia sapo* (Valenciennes, 1840) *Prochilodus platensis* (Valenciennes, 1837) and *Prochilodus marggravii* (Walbaum, 1792) (Espinach et al 1984; Fortuny et al 1988; Rizzo et al 2003). Another study on storage of common carp ova (Rothbard et al 1996; Urbanyi et al 1998), without any extender, showed a time-related negative effect of decreased temperature on fertilization and hatching rate. However, variability of ova may be related to the health of the female, time of ovulation, temperature, feeding and quality of water (Horvath 1978; Linhart & Billard 1995a). A decrease in temperature had a positive effect on the fertilizing potential of eggs through time in this experiment. In another study using common carp eggs (Urbanyi et al 1998) and no extender, a decrease in temperature had a negative effect on fertilization capacity and hatch through time. These results were not agreed with our study.

More research on storage has been done with eggs of coldwater fishes than with eggs of warm water fishes. Eggs of chum salmon *Oncorhynchus keta* (Walbaum, 1792) had greater viability when stored undiluted (without addition of extender solutions) at 9 to 15°C than at 3 to 6°C (Jensen & Alderdice 1984). Of the warm water fishes, eggs of European catfish *Silurus glanis* (Linnaeus, 1758) stored undiluted at 19°C for 3.5 h had greater hatching (54%) than did control eggs stored for 0 h (35%), while eggs stored at 8°C yielded no hatching (Linhart & Billard 1995b). Eggs of common carp stored between 0 and 24°C for 60 min without an extender exhibited time dependent and temperature-dependent sensitivity (Urbanyi et al 1998).

In our observations (Table 2) eyed embryo and hatching rate in ovarian cavity storage is better than ex situ holding and offer a potential for extending the storage time of Prussian carp ova. Although, storage time can increase if we use of low temperature. These results did not confirm the results of Rizzo et al (2003) in *Prochilodus marggravii*.

Kjorsvik et al (1990) reported that after stripping, fish eggs gradually undergo changes similar to over ripening. On the other hand, Formacion et al (1993) stated that oocyte over ripening may be evaluated on the basis of the decline in fertilization and hatching rates. In addition, Lahnsteiner (2000) reported that eyeing rate in rainbow trout eggs, retained inside of body cavity, decreased with the progress of over ripening process. Therefore, decline of eyed eggs may be considered as a sign of progress of over ripening caused by time of storage. Furthermore, the increase in storage duration may result in bacterial infection by *Pseudomonas fluorescens* (Holcomb et al 2005), for example.

Oocyte over ripening may be evaluated on the basis of the decline in fertilization and hatching rates (Formacion et al 1993). Since fertility and deformed larvae rate are strongly correlated, the fertilization rate alone may be sufficient to indicate the subsequent performance of embryos and larvae (Springate et al 1984). The rate of deformed larvae in Prussian carp oocytes stored in situ and ex situ at 21°C and 4°C

(present study) had not significant different in all of keeping time that was not similar to findings in the catfish *R. sapo* (Espinach et al 1984) and *P. marggravii* (Rizzo et al 2003). Morphological and biochemical alterations during oocyte over ripening may result from breakdown of yolk proteins, loss of small organic molecules through oocyte membranes, and dephosphorylation of proteins and lipids (Craik & Harvey 1984). In carps, a disturbance in the aerobic respiration process occurs that leads to the production of lactic acid, which accumulates in the ovarian fluid and reduces its pH, with ultimate loss of oocyte membrane integrity (Linhart et al 1995). Although the eye eggs and hatching rate in *C. gibelio* (in ovarian cavity storage) were not lower than control group after 2 h storage.

Conclusions. Results shown that storage of Prussian carp artificial ova out of ovary and without extender is not very appropriate for short-term storage of Prussian carp eggs, but storage of oocytes of this species in ovarian cavity is appropriate for longer duration than 20, 30, 60 and 120 min storage. Also, maintain Prussian carp ova at 4°C had percentage of eyed embryo and hatching greater than 21°C in ex situ storage. Storage duration out of ovary had significant effect on hatching performance, but holding status (ex situ and in situ), temperature and time had not significantly influence on malformed larvae.

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