

## Survival rates associated with *in vitro* low-temperature storage of kutum (*Rutilus kutum*) eggs

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Received: June 2014

Accepted: November 2015

### Abstract

To study the effects of post-stripping oocyte ageing at low temperatures on the viability of kutum (*Rutilus kutum*) oocytes, unfertilised eggs of five females were stored in ovarian fluid at temperatures of 4 and 7 degree centigrade for 24 hours post stripping (HPS). The stored ova of five female kutum were separately fertilised at 0 (i.e., control eggs fertilised prior to storage), 4, 8, 12, 16, 20, and 24 HPS. The eyeing and hatching rates were recorded as indices of the egg quality. The results indicated that the maximum eyeing and hatching rates of the eggs (92% and 74%, respectively) were observed at 0 HPS, whereas the storage of the eggs at 4 °C for 24 HPS decreased the eyeing and hatching rates to 36% and 28%, respectively. The use of the higher storage temperature resulted in a more rapid decrease in the egg viability: eyeing and hatching rates of 9% and 2%, respectively, were obtained after storage at 7 °C for 24 HPS. The present study demonstrated that stripped kutum eggs that are stored in ovarian fluid at 4 and 7 degree centigrade should be fertilised within 12 and 8 HPS, respectively, to obtain viability rates higher than 50%.

**Keywords:** *Rutilus kutum*, Stripping, Egg storage, Cold temperature, Egg viability

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## Introduction

The difficulties associated with the removal of intercellular water during cooling and the toxicity of cryoprotectants have led to the unsuccessful freezing of fish ova (e.g. Stoss and Donaldson, 1983; Rana, 1995). As a result, the protocols for the short-term preservation of eggs have been more developed. Ovulated oocytes retained in the ovarian or body cavity undergo over-ripening due to gradual morphological, physiological and biochemical changes that negatively affect fertility and larval development (e.g., Nomura *et al.*, 1974; Craik and Harvey, 1984; Formacion *et al.*, 1993; Lahnsteiner, 2000; Bahre Kazemi *et al.*, 2010). Thus, over-ripening of the eggs has been identified as the most important factor that influences the survival of the eggs of many fish species (e.g., McEvoy, 1984; Rime *et al.*, 2004, Policar *et al.*, 2010). After stripping also fish eggs gradually undergo changes that are similar to the effects of over-ripening (Kjorsvik *et al.*, 1990). The time required for the loss of egg viability during short-term preservation has been reported from a few minutes to a few weeks according to the fish species and the storage temperature (e.g., Lahnsteiner *et al.*, 2001; Gisbert and williot, 2002; Rizzo *et al.*, 2003; Niksirat *et al.*, 2007a; Niksirat *et al.*, 2007b) .

Kutum (*Rutilus kutum*), which is a cyprinidae and an endemic fish of the Caspian Sea, is a migratory anadromous fish with a relatively short reproductive

period that starts in early March and ends in late April. Kutum exhibits a great demand and is highly important for commercial and restocking programs in Iran. Thus, the Iranian Fisheries Organisation (Shilat) produces and releases up to 200 million fry into the Caspian Sea annually (Abdolhay *et al.*, 2011). To maximise the efficacy of the mass production of the restocking program for kutum, details on the short-term storage of the eggs deserve to be clarified. This process can not only provide synchronous fertilisation of brood fishes but also be helpful when completely mature male brood fish is unavailable for fertilisation. The asynchronous catching of male and female fishes or the lack of access to completely mature male fish, especially in species such as kutum, are the major limitation factors in restocking programs. Our previous research indicated that kutum eggs can be successfully stored at 4 °C for at least 8 hours after stripping (Samarin *et al.*, 2011). The present study was performed to identify the viable time period for the *in vitro* storage of unfertilised kutum eggs after ovulation at two different cold storage temperatures.

## Materials and methods

### *Fish*

The experimental fish were captured in the Tajan River (36°81'N, 53°16'E) in Sari, Iran, during their upstream migration. To confirm ovulation and for

gamete collection, the fish were anaesthetised with 100 ppm of tricaine methanesulfonate (methylaminobenzoate, MS222) to minimise their stress and ensure safe handling. Of the captured fish, five fishes weighing  $1.3 \pm 0.1$  Kg were selected as the experimental fish and were tagged with coloured tags.

#### *Egg storage in ovarian fluid*

The eggs from each fish were collected separately by abdominal massage. To store the eggs in the ovarian fluid, 14 batches of 7-g aliquots of the oocytes (i.e., approximately 570 eggs) from each of the five selected ovulated females were gently weighed and stored in cell culture plates. In this experiment, the eggs from each female were stored separately. Seven batches of the eggs from each female were fertilised immediately after ovulation (1 batch, fertilisation occurred at 0 HPS) and at 4-h interval up to 24h, one batch was fertilised every 4h. Before fertilisation, the ovarian fluid was removed from the eggs. For fertilisation at each time interval, 2 mL of milt was collected from five males and mixed gently to ensure uniform fertilisation. A volume of 0.2 mL of the mixed milt was used for the artificial egg insemination of each batch. The plates were then transferred to a domestic refrigerator and stored at 4°C in the darkness. The preservation of the seven other egg batches was tested in an incubator at a constant temperature of 7°C.

#### *Incubation and fertility examination*

After fertilisation and elimination of the adhesiveness of the eggs using water for 45 minutes, all of the egg batches were placed in separated 7-l jar incubators with running water at 19°C until the eyeing and hatching stages. The embryos that reached the eyeing and hatching stages were recorded as indices of the egg quality (Lahnsteiner and Weismann, 1999; Goetz and Coffman, 2000; Bonnet *et al.*, 2003). The eyed eggs were examined macroscopically 3 to 4 days after fertilisation, and the number of hatched larvae was counted 7 to 9 days after fertilisation. Both eyeing and hatching percentages were calculated with respect to the total number of eggs fertilised.

#### *Statistical analysis*

The normality of the data was ascertained using the SPSS Software for Windows version 18. The differences between the means of the groups were evaluated using analysis of variance and Duncan's multiple range test. Multiple ANOVA followed by Duncan's test was used to compare the effects of storage at different temperatures. Differences with  $p < 0.05$  were considered statistically significant.

#### **Results**

The eyeing and hatching rates of eggs stored for 12 hours at 4°C were higher than 60% and 50%, respectively.

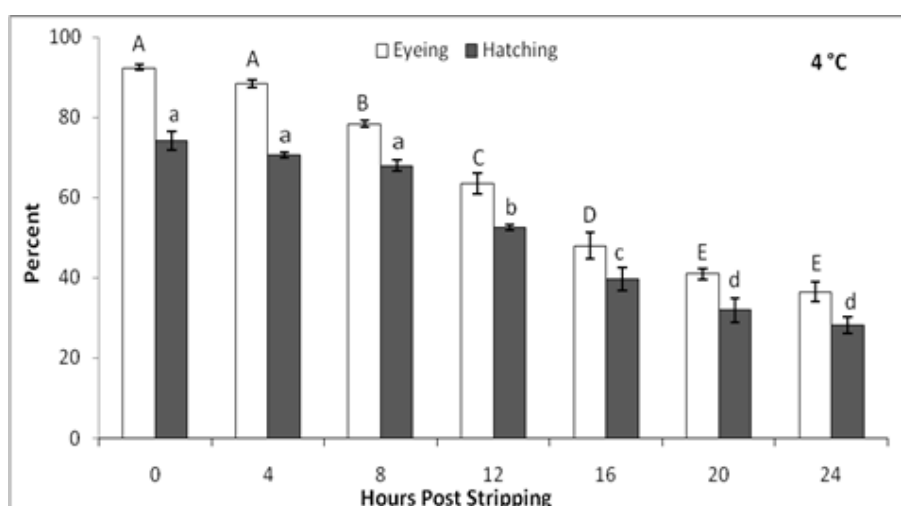


Figure 1: Effects of the *in vitro* storage time on the eyeing and hatching rates of oocytes stored at 4°C. The results shown represent the means  $\pm$  SEM. The differences between means that are denoted with the same alphabetical symbol are not significant.

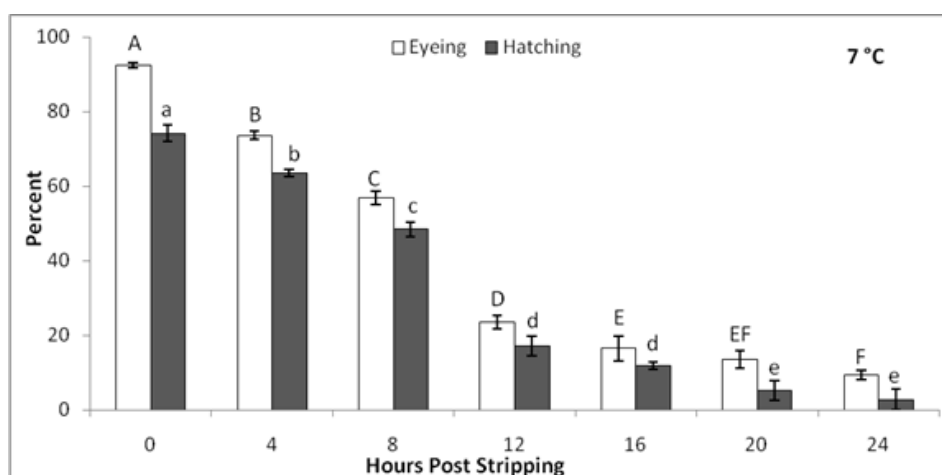


Figure 2: Effects of the *in vitro* storage time on the eyeing and hatching rates of oocytes stored at 7°C. The results shown represent the means  $\pm$  SEM. The differences between means that are denoted with the same alphabetical symbol are not significant.

The eggs stored for a longer period of time in the ovarian fluid exhibited a gradual decrease in the percentages of eyed eggs and hatched larvae: eyeing and hatching rates of  $92.4 \pm 0.6\%$  and  $74.1 \pm 2.2\%$  (mean  $\pm$  SE), respectively, were obtained for the eggs fertilised at 0 HPS, whereas eyeing and hatching percentages of  $36.5 \pm 2.5\%$  and  $28.1 \pm$

$2\%$ , respectively, were obtained for the eggs fertilised at 4°C after 24 HPS (Fig. 1). The above-mentioned trends were also observed with the storage at 7°C, but the values of the eyeing and hatching rates decreased more rapidly to  $9.3 \pm 1.3\%$  and  $2.8 \pm 2.8\%$ , respectively, for eggs fertilised after 24 hours of storage (Fig. 2).

## Discussion

In both thermal regimes used in the present study, the maximum viability rates were obtained when the eggs were fertilised immediately after stripping. The *in vitro* storage of eggs at 7°C resulted in a more rapid decrease in the egg viability compared with that observed with storage at 4°C. In fact, the eggs lost almost their total viability for hatching after one day of storage. Therefore, the viability of kutum oocytes is unstable during short-term storage, and this result is in accordance with earlier studies of other cyprinids (Billard, 1988; Lahnsteiner *et al.*, 2001).

The short-term preservation of salmonid eggs has been reported to be successful due to the occurrence of post-spawning activation only after the oocytes are released into the water. In contrast, short-term preservation is not successful for cyprinid fish species due to the auto-activation that occurs after ovulation (Stoss and Donaldson, 1983). However, the exact causes of the loss of oocyte viability are not well understood (Rizzo *et al.*, 2003). The success of the *in vitro* storage of oocytes appears to be highly dependent on the fish species and the storage temperature, and this dependence is higher for *in vitro* storage compared with the *in vivo* storage of eggs (e.g., Azuma *et al.*, 2003; Bonnet *et al.*, 2003). For example, Curimata (*Prochilodus marggravii*) oocytes can only be successfully stored *in vitro* for one hour at 26°C (Rizzo *et al.*, 2003).

Additionally, during the short-term storage of common carp and grass carp oocytes for 4 hours at 4°C, the fertilisation rate decreased by more than 50% (Lahnsteiner *et al.*, 2001). Sturgeon fish oocytes can retain their viability up to 4 hours at 15-18°C (Gisbert and Williot, 2002). In contrast, salmon and trout oocytes can be successfully stored for a longer time; e.g., oocytes of Caspian brown trout and rainbow trout can be stored up to 2 days (Niksirat *et al.*, 2007a) and 9 days (Bonnet *et al.*, 2003; Niksirat *et al.*, 2007b), respectively. As the abovementioned studies show, the *in vitro* storage of the oocytes of cold-water fish species is more successful than the *in vitro* storage of the oocytes of warm-water fish species. However, it seems that additional reasons contribute to the longer time period during which the eggs of cold-water fish species remain viable. It is possible that other factors, such as the egg size, also affect the successful storage time.

Eggs that were stored at 7°C exhibited a nearly complete loss of egg viability after 24 hours, whereas the hatching rate of eggs stored for 24 hours at 4°C was approximately 30%. Therefore, the present study demonstrated that the egg quality, which was determined by the eyeing and hatching rates, was maintained for a significantly longer time when the eggs were stored at the lower temperature. In some fish species, such as turbot (*Psetta maxima*) (Suquet *et al.*, 1999) and curimata (*Prochilodus*

*marggravii*) (Rizzo *et al.*, 2003), it has been shown that the storage of their eggs at temperatures that the fish do not experience during their normal range of life results in a decrease in the egg viability. In contrast, the storage of unfertilised eggs of kutum at low temperatures showed improved results. Therefore, it is possible that the low-temperature storage of the eggs of only tropical fish species that naturally reproduce at high temperatures is not successful.

In this study, we stored the eggs in the ovarian fluid of fish. We did not attempt to store the eggs in artificial ovarian fluid, although the effect of using artificial ovarian fluid using additional improvements can be examined in the future studies. Based on the results of the present study, unfertilised eggs of kutum can retain approximately 70% and 50% viability after 8 hours of storage in ovarian fluid at temperatures of 4 and 7°C, respectively.

#### Acknowledgements

This study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects “CENAKVA” (No. CZ.1.05/2.1.00/01.0024), “CENAKVA II” (No. LO1205 under the NPU I program)” and POSTDOC JU (No. CZ.1.07/2.3.00/30.0006). We would like to thank the Elsevier Language Editing Service for the English improvement of the text.

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