African Journal of Biotechnology Vol. 10(57), pp. 12309-12314, 28 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.919
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Effects of storage duration and storage temperature on viability of stored ova of kutum (*Rutilus frisii kutum*) in ovarian fluid

Azin Mohagheghi Samarin[1]*, Bagher Mojazi Amiri[2], Mehdi Soltani[3], Rajab Mohammad Nazari[4], Abolghasem Kamali[1] and Mohammad Reza Naghavi[5]

[1]Department of Aquaculture and Fisheries Science, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Fisheries and Environmental Science, Faculty of Natural Resources, University of Tehran, 31585-4314, Karaj, Iran.
[3]Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, 14155-6453, Tehran, Iran.
[4]Nasr Fish Farm, Sari, Iran.
[5]Department of Plant Breeding, Faculty of Agriculture, University of Tehran, 31587-77871, Karaj, Iran.

Accepted 24 June, 2011

Effects of post-stripping oocyte ageing and temperature on egg viability rates were studied in kutum (*Rutilus frisii kutum*). Eggs were retained outside the ovarian cavity (*in vitro* storage) with ovarian fluid at various temperatures of 4, 10, 12 and 26°C. Stored ova of six female kutum were fertilized separately after 0 (control eggs fertilized prior to storage), 2, 4, 6, and 8 h post-stripping (HPS) using fresh and pooled sperm obtained from five males. Eyeing, hatching and eyed egg mortality rates were recorded as an index of egg viability. The results indicated that the maximum eyeing (87%) and hatching (75%) rates of eggs took place at 0 HPS followed by 8 HPS (> 80 and > 70%, respectively) at 4°C. As storage temperature increased, egg viability decreased to 80, 70 and 50% viable at 8 HPS at 4, 10 and 12°C, respectively. The eggs stored at 26°C lost their viability almost completely after 4 HPS. Eyed-egg mortality increased from 13% at 0 HPS to 48.2% and at 4 HPS, at 26°C. This study demonstrated that the *in vitro* storage method is an applicable effective fertilization technique that can be used for restocking programs of kutum within 8 h at temperatures ranging from 4 to 12°C.

Key words: *Rutilus frisii kutum*, egg storage, temperature, ovarian fluid.

INTRODUCTION

Although cryopreservation methods which are especially desirable for gene banking, might eventually be effective for restocking programs because they allow long-term preservation of eggs, currently, they are not practical due to the difficulty of removing intercellular water from oocytes during cooling (Haga, 1982; Harvey and Ashwood-Smith, 1982; Stoss and Donaldson, 1983; Rana, 1995; Lubzens et al., 2005). Instead, short term preservation of eggs has been more developed. Short term preservation has been reported to improve hatchery management, minimize problems resulting from in-breeding and provide synchronous brooder maturation (Bromage and Roberts, 1995). Loss of egg viability because of degradation of egg quality after ovulation is one of the limiting factors for reproduction and mass production of several fish species (Furuita et al., 2003; Rizzo et al., 2003). Ovulated eggs retained in the ovarian

*Corresponding author. E-mail: amohagheghi@hotmail.com. Tel: +98 09123540686. Fax: +98 2166974840.

or body cavity beyond a certain time span undergo over-ripening due to gradual morphological and biochemical changes that negatively affect fertility and larval development (Nomura et al., 1974; Craik and Harvey, 1984; Formacion et al., 1993; Lahnsteiner, 2000). So, over-ripening of the eggs has been identified as the most important factor influencing egg survival of many fish species (McEvoy, 1984; Rime et al., 2004).

On the other hand, after stripping, fish eggs gradually undergo changes similar to over-ripening (Kjorsvik et al., 1990). *In vitro* storage of eggs in the ovarian fluid seems to result in a rapid decrease of egg fertility compared with *in vivo* egg storage. The time interval during which eggs remain viable after stripping differs from species to species and depends largely on temperature. For example, Curimata *Prochilodus marggravii* oocytes could only be successfully stored *in vitro* for one hour at 26°C (Rizzo et al., 2003). Also, during short term storage of common carp and grass carp oocytes for 4 h at 4°C, the fertilization rate decreased by more than 50% (Lahnsteiner et al., 2001). Sturgeon fish oocytes can retain their viability up to 4 h at 15 to 18°C (Gisbert and Williot, 2002; Hajirezaee and Niksirat, 2009). In contrast, salmon and trout oocytes can be successfully stored for more times, even for 2 days in Caspian brown trout (Niksirat et al., 2007a) and for 9 days at 2 to 3°C in rainbow trout (Niksirat et al., 2007b).

Kutum (*Rutilus frisii kutum*) is a cyprinidae endemic to the Caspian Sea. It is a semi-migratory anadromous fish with a reproductive period from early March to late April. There is great demand for kutum, thus, it is highly important for commercial fisheries in Iran. The Iranian fisheries organization (Shilat) (www.shilat.com), currently runs a restocking program aimed at producing and releasing up to 200 million kutum fry annually in the Caspian Sea. Under the program, maturing fish are caught at the river inlets when they return to them for spawning and these fish are used for artificial breeding. Fertilized eggs are transferred to the hatcheries for development to fry and then are released to the sea.

Asynchronous catching of male and female fishes or no accessing to completely mature male fish are the most limitation factors in restocking programs especially for species like kutum when captured from the wild. Among the practical methods currently available for use in restocking programs, *in vitro* egg preservation techniques have been used effectively in various species. It cannot only provide synchronous fertilization of brood fishes, but also can be worthwhile when completely mature male brood fish is unavailable for fertilization. To maximize the efficacy of mass production of kutum for restocking programs, a better understanding of the effects of short-term egg storage is necessary. The objective of this study therefore, was to identify the storage time period during which unfertilized kutum eggs remain viable after stripping when cultured at different temperatures.

MATERIALS AND METHODS

Fish

Experimental fish were captured in the Shazderud River, Babolsar, Iran, during their reproductive migration and then transferred to Sadkin boxes set in the river. The fish were not fed during the experimental period. To confirm that ovulation had occurred and to collect gametes, fish were anaesthetized with 100 ppm tricaine methanesulfonate (methyl-aminobenzoate, MS222) to minimize stress and to make them easy to handle. 30 unovulated females that were expected to ovulate in the near future were captured from the upstream run. The state of ripeness was judged by gentle palpation of the abdomen. Fish for which the eggs could be removed by applying gentle pressure on the abdomen were considered to have already ovulated and were not used. The inspection was carried out 5 h later. Six individuals weighing 1260 ± 53 g (mean ± SEM) were selected as the experimental fish after tagging them with colored tags.

Egg storage in ovarian fluid

The body of each fish was wiped and cleaned with a towel and then the eggs and ovarian fluid were collected separately for each individual. Five batches of 7 g egg aliquots (ca. 570 eggs) from each of the six females were stored with about 1 ml of ovarian fluid per batch in circular plastic plates (7 cm in diameter, Sterilin, U.K.). Each batch from a given female was fertilized with 0.2 ml of mixed milt (obtained from five males) at 0, 2, 4, 6, and 8 h post stripping (HPS) after removing the ovarian fluid at the temperatures of 4, 10, 12 and 26°C. So, four sets of five batches of eggs, one for each temperature, were obtained from each of the six females. For storage at 4°C, the plates were transferred to a refrigerator and kept in the dark; incubators were used to maintain a constant temperature for the 10, 12 or 26°C treatments.

Incubation and fertility examination

All batches of fertilized eggs were transferred to the Shahid Rajayee Hatchery Center and placed in jar incubators with running water at 19°C until the eyeing and hatching stages were reached. The ratio of the number of embryos reaching the eyeing and hatching stages to the number of initially fertilized eggs (eyeing and hatching rates, respectively) were used as indices of egg viability (Lahnsteiner and Weismann, 1999; Goetz and Coffman, 2000; Bonnet et al., 2003). Eyed-egg mortality, which is defined as the percentage of dead eyed-eggs in the total number of eggs that reached the eyed-egg stage, was also used as an index of viability. Eyeing was confirmed macroscopically 3 to 4 days after fertilization as presence/absence of eyes and hatching was examined by counting the number of hatched alevins 7 to 9 days after fertilization.

Statistical analysis

The normality of the data was ascertained using the SPSS software for windows version 18. Differences between the means of the groups were evaluated using analysis of variance and Duncan's multiple range test. In this paper, the control refers to the freshly fertilized eggs with no storage duration and reflects the primary quality of eggs. Multiple ANOVA followed by Duncan's test were also used to compare the effects of storage at different temperatures on eyeing and hatching rates and eyed-egg mortality. $P < 0.05$ was considered to be significant.

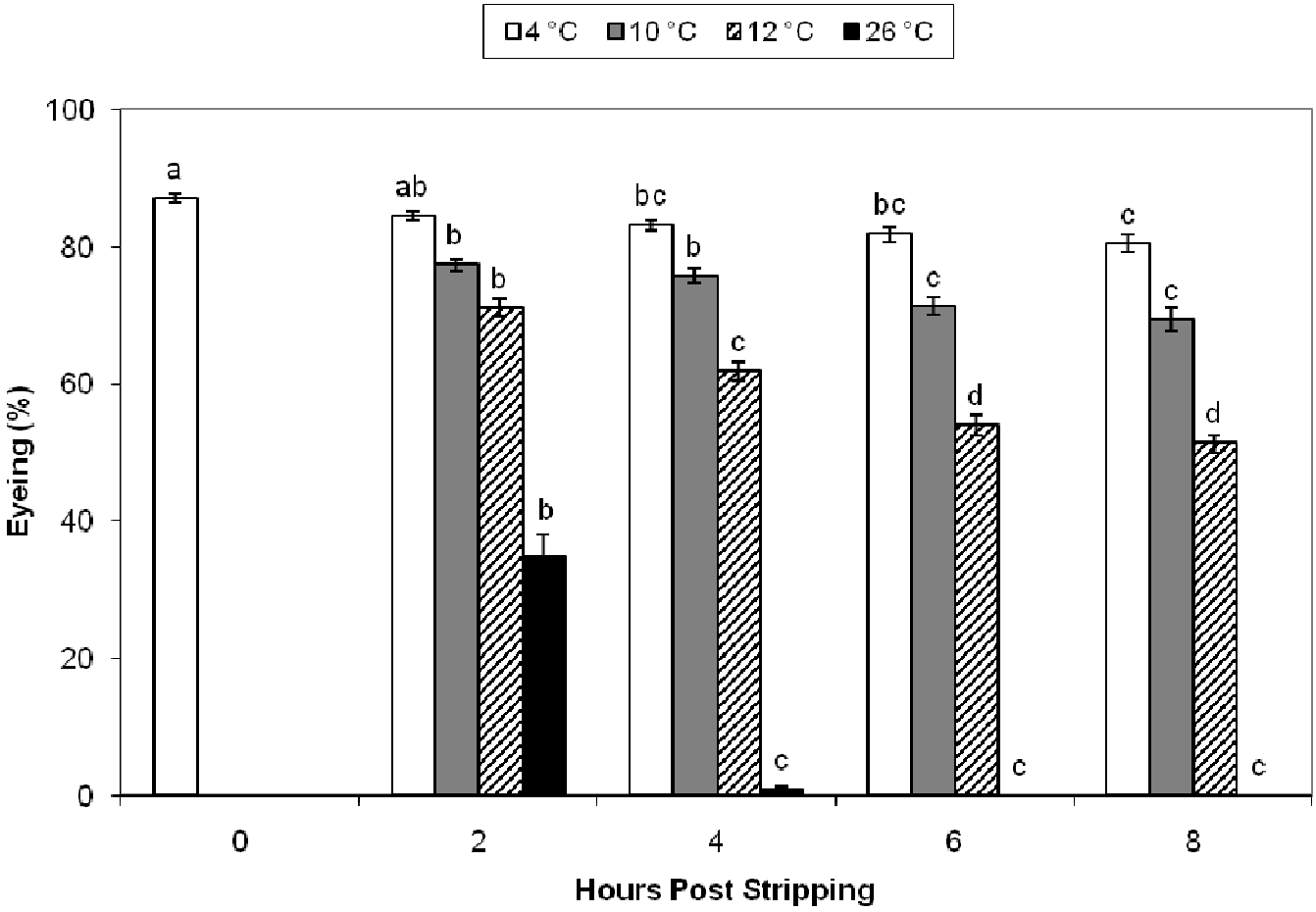

Figure 1. Effects of the egg *in vitro* storage time on eyeing rates shown as mean ± SEM. Means sharing a common alphabetical symbol do not differ significantly.

RESULTS

The eyeing rate remained greater than 80% for the eggs stored in ovarian fluid for 8 h at 4°C (Figure 1). The eggs stored longer at ≥ 10°C exhibited a gradual decrease of eyeing rate and the speed of the decrease was in proportion to the temperature. The eyeing rates decreased from 87.1% at 0 HPS to 69.3% at 10°C and 51.3% at 12°C at 8 HPS, 0% at 26°C at 6 HPS and 80.4% at 4°C at 8 HPS.

The hatching rates showed exactly the same trends as observed for the eyeing rates in the tests performed at 4 to 26°C (Figure 2). It remained greater than around 70% for the eggs stored for 8 h at 4°C. The value decreased from 75.4% at 0 HPS to 53.1% at 10°C and 38.1% at 12°C at 8 HPS, 0% at 26°C at 6 HPS and 68.1% at 4°C at 8 HPS.

Changes in eyed-egg mortality were entirely opposite those of the eyeing and hatching rates (Figure 3). More rapid increases in mortality occurred with increasing temperature. The eyed-egg mortality rates increased from 13.3% at 0 HPS to 15.1%, 23.1 and 25.4% at 4, 10 and 12°C at 8 HPS and 48.2% at 26°C at 4 HPS.

DISCUSSION

The results revealed that the highest eyeing and hatching rates and the lowest eyed-egg mortalities occurred when eggs were fertilized just after stripping, irrespective of temperature; this finding is similar to that reported for several fish species (Azuma et al., 2003; Rizzo et al., 2003; Niksirat et al., 2007a, b). Complete loss of kutum egg viability was observed around 4 HPS at 26°C. Therefore, the viability of kutum oocytes is also unstable during short term storage which is in accordance with earlier studies for the other cyprinids (Billard, 1988; Lahnsteiner et al., 2001).

The time period during which eggs remain viable after stripping, which guarantees egg fertility, has been

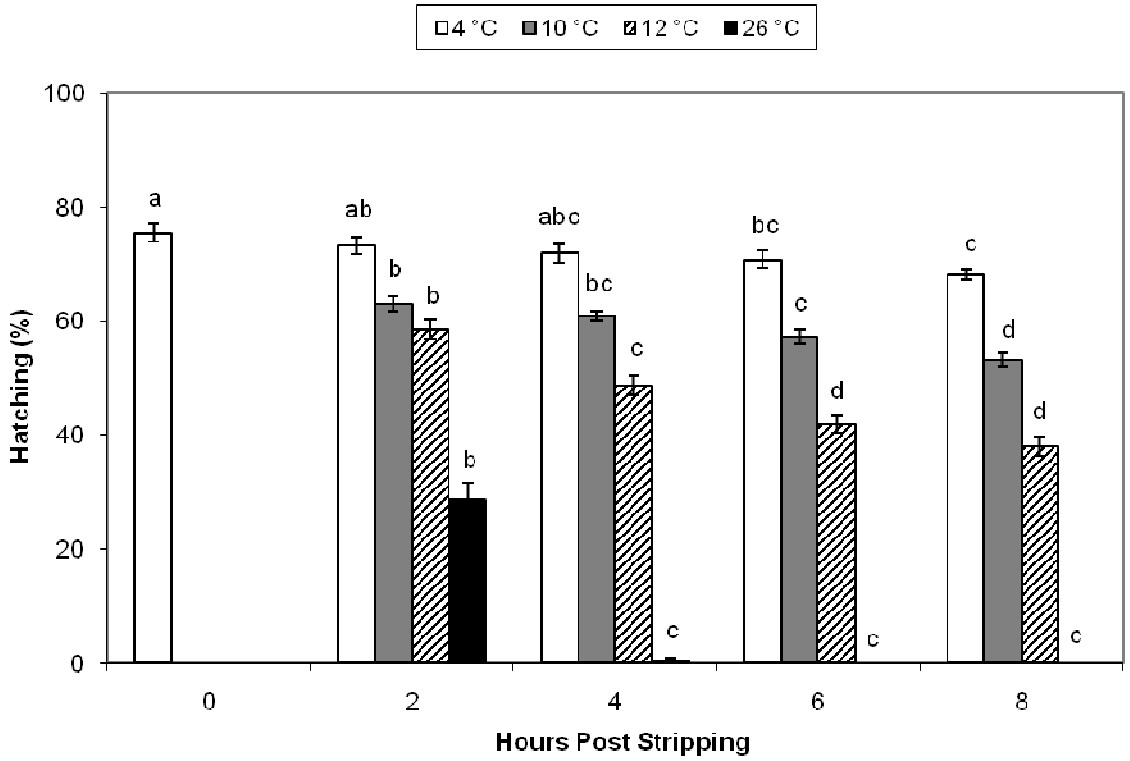

Figure 2. Effects of the egg *in vitro* storage time on hatching rates shown as mean ± SEM. Means sharing a common alphabetical symbol do not differ significantly.

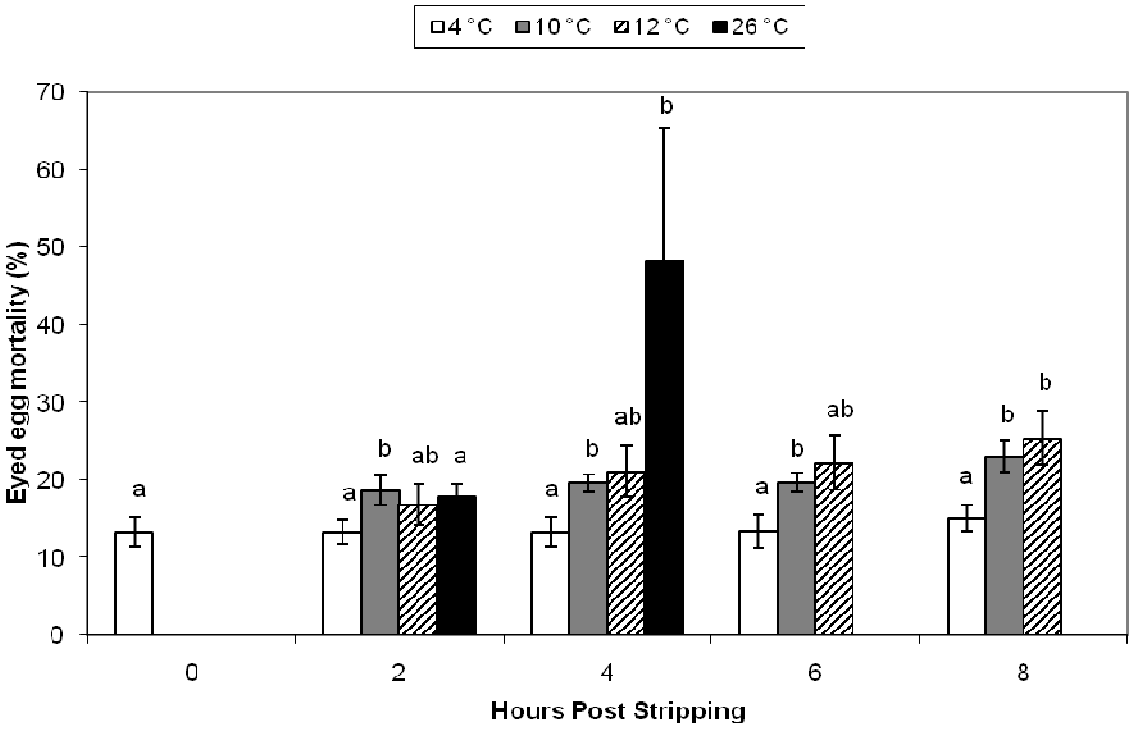

Figure 3. Effects of the egg *in vitro* storage time on eyed egg mortality rates shown as mean ± SEM. Means sharing a common alphabetical symbol do not differ significantly.

reported to be different from some hours to some days for a variety of species. For example, in the curimata *Prochilodus marggravii*, fertilization rate was ~20% for eggs stored *in vitro* for 30 min at 18°C. Then the fertility decreased rapidly and it was recorded close to 0 after 2 h storage, but storage at 18°C caused a drastic reduction in fertilization rates when compared with storage at 26°C (Rizzo et al., 2003). Short-term preservation of cyprinid fish species eggs has been reported not to be successful because of auto activation after ovulation (Stoss and Donaldson, 1983). For instance, storage of common carp and grass carp oocytes in ovarian fluid at 4°C for 4 h, resulted in significant decrease of fertilization rate reaching to 20.7 and 2.8%, respectively (Lahnsteiner et al., 2001). Successful egg storage time has been reported to be almost the same as cyprinids for sturgeon fish species. Gisbert and Williot (2002) observed that ovulated eggs of Siberian sturgeon, *Acipenser baeri* and Sterlet, *Acipenser ruthenus*, stored in ovarian fluid at 15°C retained their viability up to 2 to 4 h, respectively. After such a period of time, eggs became overripe and their fertilizability and hatchability were dramatically reduced. Also, ovulated eggs of Persian sturgeon, *Acipenser persicus*, can be held in ovarian fluid at 18°C up to 3 h without significant loss of fertilization rate and hatchability. After that, fertilization and hatching rates significantly decreased in comparison with eggs fertilized immediately after stripping (Hajirezaee and Niksirat, 2009). Short-term preservation of salmonid eggs in contrast, has been reported to be more successful because of occurrence of posts-pawning activation only after releasing oocytes in the water (Stoss and Donaldson, 1983). For example, after 72 h *in vitro* store of sockeye salmon and pink salmon oocytes in ovarian fluid at 8 to 9°C, fertility around 35% was observed (Withler and Morley, 1968). In addition, fertility of chum salmon eggs was maintained for 40 h when stored *in vitro* in ovarian fluid at 12°C, but decreased to 28% when stored for 82 h (Jensen and Alderdice, 1984). Caspian brown trout eggs could be stored for 2 days *in vitro* at 2 to 3°C when ovarian fluid was used (Niksirat et al., 2007a). Rainbow trout eggs also have been reported to lose complete fertility after 24 h storage at 19°C or 52 h at 10°C (Billard and Gillet, 1981). Azuma et al. (2003) showed that hatching rate of rainbow trout decreased to almost zero after 5 days incubation in artificial coelomic fluid at 10°C. Bonnet et al. (2003) reported ~50% fertilization of rainbow trout eggs stored *in vitro*, for 9 days at 12°C. Also, during 7 to 9 days storage of rainbow trout eggs in ovarian fluid at 1 to 3°C, no significant differences were observed when compared with eggs fertilized prior to storage (Babiak and Dabrowski, 2003; Niksirat et al., 2007b). The exact causes of loss of oocyte viability however, are not yet well known (Rizzo et al., 2003). As shown in these studies, the acceptable post-stripping time for egg storage differs from species to species and also depends on storage temperature. Comparison of these results to the earlier-mentioned reports reveals that kutum eggs have a relatively short time period after stripping during which successful fertilization can occur.

According to the results obtained in this study, the eggs stored at 26°C lost their viability almost completely after 4 HPS. Also, the results demonstrated that the egg quality judged by the eyeing and hatching rates were guaranteed much longer when eggs were stored in the lower temperatures. We did not try to store eggs in ovarian fluid for periods longer than 8 h at temperatures of 4 to 12°C, but this can be examined. Experiments in which eggs are stored *in vitro* for longer than 8 h in ovarian fluid should clarify the exact time of complete infertilability of kutum oocytes at these temperatures too.

Under all thermal regimes, eyed-egg mortalities increased with HPO, but significant differences were not detected in some cases. This is in accordance with the previous study (Niksirat et al., 2007a, b) which reported that mortality of eyed eggs may be considered as a signs of progress of over-ripening caused by time of storage. Although oocyte ageing has been reported to cause several malformations in the larvae of some fish species (Rizzo et al., 2003; Aegerter and Jalabert, 2004; Rime et al., 2004), we did not check it in this study.

Although viability of egg quality among females may be related to nutrition, environmental factors, stress, etc (Bobe and Labbe, 2010) and post ovulatory oocyte ageing has been reported the most important factor affecting egg quality (McEvoy, 1984; Rime et al., 2004). Jensen and Alderdice (1984) reported that differences in fertility observed among females could be partly explained by the holding time in body cavity before *in vitro* storage, especially in experiments (such as this study) performed on fishes captured from the wild. So, we considered the earlier noted comment in this study and tried to minimize the time gap between ovulation and stripping for experimental females as it was possible, that is, 0 to 5 h.

Based on the results of this study, unfertilized eggs of kutum retained their viability up to around 80, 70 and 50% during 8 h of storage in ovarian fluid at temperatures of 4, 10 and 12°C, respectively. Also, the results demonstrated that the egg quality was guaranteed much longer when eggs were stored in the lower temperatures. Therefore, *in vitro* storage method is an applicable effective fertilization technique for restocking programs of this species at least within 8 h at temperatures ranging from 4 to 12°C.

ACKNOWLEDGEMENTS

We would like to express our appreciation to the manager and staff of Shahid Rajayee Hatchery Center for their

collaboration.

REFERENCES

Aegerter S, Jalabert B (2004). Effects of post ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout *Oncorhynchus mykiss*. Aquaculture, 231: 59-71.

Azuma T, Ohta H, Oda S, Muto K, Yada T, Unuma T (2003). Changes in fertility of rainbow trout eggs retained in coelom. Fish. Sci. 69: 131-136.

Babiak I, Dabrowski K (2003). Refrigeration of rainbow trout gametes and embryos. J. Exp. Zool. A Comp. Exp. Biol. 300: 140-151.

Billard R, Gillet C (1981). Ageing of eggs and temperature potentialization of micropollutant effects of the aquatic medium on trout gamets. Cah. Lab. Hydrobiol. Montreau. 12: 35-42.

Billard R (1988). Artificial insemination and gamete management in fish. Mar. Behav. Physiol. 14: 3-21.

Bobe J, Labbe C (2010). Egg and sperm quality in fish. Gen. Comp. Endocr. 165: 535-548.

Bonnet E, Jalabert B, Bobe J (2003). A 3-day *in vitro* storage of rainbow trout (*Oncorhynchus mykiss*) unfertilised eggs in coelomic fluid at 12 ^{o} C does not affect development success. Cybium, 27: 47-51.

Bromage NR, Roberts RJ (1995). Preservation of gametes. In: Bromage NR, Roberts RJ (Eds.), Broodstock Management and Egg and Larval Quality. Blackwell, Oxford. pp. 53-75.

Craik JCA, Harvey SM (1984). Biochemical changes associated with overripening of the eggs of rainbow trout, *Salmo gairdneri*. Aquaculture, 37: 347-357.

Formacion MJ, Hori R, Lam TJ (1993). Overripening of ovulated eggs in goldfish, I: Morphological changes. Aquaculture, 114: 155-168.

Furuita H, Ohta H, Unuma T, Tanaka H, Kagawa H, Suzuki N, Yamamoto T (2003). Biochemical composition of eggs in relation to egg quality in the Japanese eel *Anguilla japonica*. Fish Physiol. Biochem. 29: 37-46.

Gisbert E, Williot P (2002). Influence of storage duration of ovulated eggs prior to fertilization on the early ontogenesis of Sterlet, *Acipenser ruthenus*, and Siberian sturgeon, *A. Baeri*. Int. Rev. Hydrobiol. 87: 605-612.

Goetz FW, Coffman MA (2000). Storage of unfertilized eggs of rainbow trout (*Oncorhynchus mykiss*) in artificial media. Aquaculture, 184: 267-276.

Haga Y (1982). On the subzero temperature preservation of fertilized eggs of rainbow trout. Nippon Suisan Gakk. 48: 1564-1572.

Hajirezaee S, Niksirat H (2009). *In vitro* storage of ova of Persian Sturgeon, *Acipenser persicus* in ovarian fluid: the changes in the pH and osmolality of the ovarian fluid, fertilization and hatching rates. Aust. J. Basic Appl. Sci. 3: 3438-3442.

Harvey B, Ashwood-Smith MJ (1982). Cryoprotectant penetration and super cooling in the eggs of salmonid fishes. Cryobiology, 19: 29-40.

Jensen JOT, Alderdice DF (1984). Effect of temperature on short-term storage of eggs and sperm of chum salmon (*Oncorhynchus keta*). Aquaculture. 37: 251-265.

Kjorsvik E, Mangor-jensen A, Holmfjord I (1990). Egg quality in fishes. Adv. Mar. Biol. 26: 71-113.

Lahnsteiner F, Urbanyi B, Horvath A, Weismann T (2001). Bio-markers for egg quality determination in cyprinid fish. Aquaculture, 195: 331-352.

Lahnsteiner F (2000). Morphological, physiological and biochemical parameters characterizing the overripening of rainbow trout eggs. Fish Physiol. Biochem. 23: 107-118.

Lahnsteiner F, Weismann T (1999). Changes in eggs of brown trout, rainbow trout and grayling during short-term storage. N. Am. J. Aquact. 61: 213-219.

Lubzens Z, Rosenfeld H, Meiri I, Olsson PE, Cerda G, Babin PJ, Admon A, Carnervali O, Rawson D, Zhang T, Dichtl M, Pagelson G (2005). Cryopreservation of fish oocytes: achievements and prospects. In: van Stappen H, Sorgeloos W (Eds.). Larvi 05 – Fish & Shellfish larviculture Symposium, European Aquaculture Society, Special Publication, 36: 296-297.

McEvoy LA (1984). Ovulatory rhythms and overripening of eggs in cultivated turbot *Scophthalmus maximus* (L.). J. Fish Biol. 24: 437-448.

Niksirat H, Sarvi K, Mojazi Amiri B, Karami M, Hatef A (2007a). *In vitro* storage of unfertilized ova of endangered Caspian brown trout (*Salmo trutta caspius*) in artificial media. Anim. Reprod. Sci. 100: 356-363.

Niksirat H, Sarvi K, Mojazi Amiri B, Hatef A (2007b). Effects of storage duration and storage media on initial and post-eyeing mortality of stored ova of rainbow trout *Oncorhynchus mykiss*. Aquaculture, 262: 528-531.

Nomura M, Sakai K, Takashima F (1974). The over-ripening phenomenon of rainbow trout. I. Temporal morphological changes of eggs retained in the body cavity after ovulation. Bull. Jpn. Soc. Sci. Fish. 40: 977-984.

Rime H, Guitton N, Pineau C, Bonnet E, Bobe J, Jalabert B (2004). Post-ovulatory ageing and egg quality: A proteomic analysis of rainbow trout coelomic fluid. Reprod. Biol. Endocrin. 2: 26.

Rizzo E, Godinho HP, Sato Y (2003). Short-term storage of oocytes from the neotropical teleost fish *Prochilodus marggravii*. Theriogenology, 60: 1059-1070.

Rana KJ (1995). preservation of gametes. In: Bromage, N.R., Roberts, R.J. (Eds.), Brood Stock Management and Egg and Larvae Quality. Cambridge University Press, Cambridge. pp. 53-76.

Shilat, Iranian Fisheries Organization (www.shilat.com).

Stoss J, Donaldson EM (1983). Studies on cryopreservation of eggs from rainbow trout (*Salmo gairdneri*) and coho salmon (*Oncorhynchus kisutch*). Aquaculture, 31: 51-65.

Withler FC, Morely RB (1968). Effects of chilled storage on viability of stored ova and sperm of sockeye and pink salmon. J. Fish. Res. Board Can. 25: 2695-2699.