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Effect of Extenders and Osmotic Pressure on Storage of Eggs of Ornamental Common Carp *Cyprinus carpio* at Ambient and Refrigerated Temperatures

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Abstract

The eggs of ornamental (koi) common carp *Cyprinus carpio* were stored at ambient temperature (~22–25 C) and at refrigerated temperatures (0–20 C) in extenders at different osmolalities. The treatments evaluated were dry (control), calcium-free Hanks' balanced salt solution (C-F HBSS), salt (NaCl), synthetic ovarian fluid (SOF), and Kurokura #2 (K2). In the first study, eggs were placed in extenders at osmolalities ranging from 130 to 450 mOsmol/kg and were fertilized after 2 h. The percentage of eyed embryos (our measure of fertilization capacity) was calculated 24 h later, and percent hatching was calculated at 60 h. Fertilization capacity of eggs suspended in C-F HBSS (28%) or SOF (37%) was highest ($P = 0.0001$) at 250 mOsmol/kg, while eggs stored dry (control) had a fertilization capacity of 24%. Fertilization capacity of eggs suspended in NaCl (40%) or K2 (39%) was highest ($P = 0.0001$) at 200 mOsmol/kg. The percent of eyed embryos and percent hatch were found to be positively correlated ($r = 0.9914$). In the second study, eggs were stored in these extenders with the most effective osmolality from the previous study to evaluate percent eyed embryos and hatching over time. Samples of eggs were fertilized at every hour for 7 h. Eggs in the extenders C-F HBSS and SOF yielded the highest ($P = 0.0001$) percent eyed embryos during 7 h. Percent hatch of these eggs was not significantly different ($P = 0.1258$) among treatments at each time interval. Eggs stored in the extenders SOF, C-F HBSS, and NaCl had higher fertilization capacity ($P = 0.0271$) at 7 h than did the dry control. Eggs were also stored at refrigerated temperatures in these four extenders at the most effective osmolalities from the first study. A dry control (no extender) was also compared. The third study compared quality of eggs stored for 0, 2, 4, or 6 h in each of the extenders at 5 C or at ambient temperature (~22–25 C). Eggs suspended in C-F HBSS had significantly higher fertilization capacity at ambient temperature over time than did eggs stored in NaCl, SOF, K2, or the dry control. Eggs suspended in C-F HBSS and the dry control had significantly higher fertilization capacity at 5 C over time than did eggs stored in NaCl, SOF, or K2. Eggs held dry had higher hatch at ambient temperatures ($P = 0.0001$) and at 5 C ($P = 0.0002$) over time than did eggs stored in any extender. At 6 h, fertilization capacity with eggs in C-F HBSS or K2 was higher than with NaCl, SOF, or the dry control. The fourth study used C-F HBSS (250 mOsmol/kg) as the extender to evaluate fertilizing and hatching ability during storage at temperatures 0, 5, 10, 15, 20, or 25 C. Eggs were fertilized after 0, 1, 3, 6, 9, or 12 h of storage. Eggs stored at 15 C had significantly higher fertilization capacity ($P = 0.0001$) than at any other temperature. Eggs stored at 15 C and 10 C had significantly higher hatch ($P = 0.0001$) than at any other temperature. Fertilization capacity at 12 h was significantly higher in eggs stored at 10 C (33%) or 15 C (29%) than at any other temperature. Storage of koi carp eggs in C-F HBSS at refrigerated temperatures extended fertilizing ability for as long as 12 h compared to storage in NaCl, SOF, K2, or the dry control.

Common Carp *Cyprinus carpio* have been selectively bred for more than 200 years to produce ornamental color variants known as koi carp, or koi. These are highly valued for distinctive colors and patterns and have sold for as much as US $60,000 each. They are easily cultured and have recently gained popularity in the United States. However, koi carp bred outside of Japan typically do not have the bloodlines that bring high prices. Research on gametes

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of koi carp can improve breeding stocks and husbandry techniques (Jhingran and Pullin 1988; Horvath et al. 1992).

The limiting factor for storage of fish gametes is the ability of the gametes to function normally in fertilization and development. Short-term storage can aid in artificial spawning of fish and the transport of gametes, improving accessibility to specific seedstocks. The majority of papers on gametes of common carp have dealt with refrigerated storage and cryopreservation of sperm (Saad et al. 1988; Babiak et al. 1997). Sperm from common carp have shown a loss of viability within 6 h when stored at 28 to 33 C (Bhowmick and Bagchi 1971). Sperm has also been stored at 4 C for as long as 16 d with a 20% reduction in fertilization ability (Saad et al. 1988).

Research on fish sperm has received more attention than has the storage conditions of eggs before fertilization. Ovulated oocytes held in the ovary or *in vitro* progressively lose the capacity for fertilization and embryonic development (Linhart et al. 1995). More research on storage has been done with eggs of coldwater fishes than with eggs of warmwater fishes. Eggs of chum salmon *Oncorhynchus keta* had greater viability when stored undiluted (without addition of extender solutions) at 9 to 15 C than at 3 to 6 C (Jensen and Alderdice 1984). Of the warmwater fishes, eggs of European catfish *Silurus glanis* 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 and Billard 1995). 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). Studies with warmwater fishes typically have not addressed storage of eggs in extenders or at refrigerated temperatures perhaps because normal egg development occurs at spring and summer temperatures.

Storage of eggs can facilitate seedstock production, preserve genetic diversity, improve selective breeding and hybridization, and expand research opportunities. Eggs of the common carp stripped and stored undiluted (without an extender) gradually lost the ability to be fertilized after 4 to 6 h at temperatures of 15 to 20 C (Renard et al. 1987; Linhart et al. 1995). At higher storage temperatures (24–30 C), fertilization (morula stage) was 0% after 1.5 to 2 h (Jahnichen 1978). In other studies, koi carp eggs stored without an extender for 6 h at 22 to 24 C had ~50% survival of larvae (Rothbard et al. 1996), and common carp eggs stored at 20 to 24 C for 3 h had 96% fertilization (Kiselev 1980).

With the addition of an extender solution, storage at reduced temperatures can delay progressive declines in gamete viability. However, there is a lack of research regarding storage of eggs in extender solutions and different temperatures prior to fertilization. Eggs of the tilapia *Sarotherodon mossambicus* stored in coelomic fluid at 20 C for 19 h yielded 35% fertilization, while at temperatures below 18 C, the fertilizing ability was decreased after 1.5 h (Harvey and Kelley 1984). Eggs of the common carp stored at different salinities and osmotic pressures for 9 h (Renard et al. 1987) had spontaneous elevation of the chorion, eliminating fertilization (Renard et al. 1990). This was also observed with eggs of common carp in hypotonic and isotonic Ringer's solution (Yamamoto 1961). The reaction was delayed (4–6 h) when common carp eggs were stored in ovarian fluid (Billard et al. 1986; Renard et al. 1987). Nevertheless, parthenogenetic development in mature common carp eggs stored in ovarian fluid was found to begin with or without fertilization soon after stripping (Withler 1980).

The value of extenders for storage of koi carp eggs has not been studied, and systematic studies of extenders, osmotic pressures, and temperatures have not been performed for koi carp. This study sought to prolong the viability of koi carp eggs with the use of extenders at ambient and refrigerated

temperatures. The objectives at ambient temperatures were to: 1) evaluate the effect on the percentage of eyed embryos and hatch of four extenders at osmotic pressures of 130 to 450 mOsmol/kg; and 2) evaluate storage of eggs in these extenders at selected osmotic pressures for 7 h. The objectives at refrigerated temperatures were to: 1) compare the effects of four extenders at ambient (~22–25 C) and refrigerated (5 C) temperatures; and 2) evaluate the effects of storage in a single extender across a range of temperatures from 0 C to 25 C.

Materials and Methods

Fish Maintenance and Gamete Collection

Mature male and female koi carp (2–3 yr of age and ~1 kg each) were maintained at the Louisiana Agricultural Experiment Station, Aquaculture Research Station in Baton Rouge, Louisiana, USA. Fish were not fed for 2 d prior to spawning. Broodstock received a single intramuscular injection (behind the dorsal fin) of 10 μg/kg of synthetic luteinizing hormone-releasing hormone ethylamide (Peninsula Laboratories, Inc., Belmont, California, USA) and 20 mg/kg of metoclopramide, a dopamine antagonist (Sigma Chemical Corp., St. Louis, Missouri, USA), to stimulate spawning (Rothbard 1994). For the ambient temperature study, sperm were collected between 11 and 16 h after injections, and eggs were collected between 9 and 23 h. For the refrigerated temperature study, sperm were collected between 12 to 15 h after injections, and eggs were collected between 9 to 20 h. Following anesthesia with tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, Washington, USA), the genital pore was dried, and sperm or eggs were expelled by hand stripping. Sperm were collected in 3-mL syringes to avoid contamination with feces and water; eggs were collected in dry bowls coated with vacuum grease (Dow Corning Corp., Midland, Michigan, USA) to avoid adhesion (Tiersch et al. 1994).

Study 1: Evaluation of the Effect of Extender Osmolality

The osmolality of extender solutions was evaluated for the effect on fertilization capacity (development to stage of eye pigmentation) and hatch. Optimum osmolality was determined for each of the following extenders: calcium-free Hanks' balanced salt solution (C-F HBSS) (Tiersch et al. 1994), salt (NaCl), synthetic ovarian fluid (SOF) (Bongers et al. 1994), and Kurokura #2 (K2) (Kurokura et al. 1984). The volume of deionized water was varied to produce the desired osmotic pressures as measured with a vapor pressure osmometer (model 5500, Wescor Inc., Logan, Utah, USA). Eggs with no extender (dry) served as a control to allow direct comparisons with other studies. Ovarian fluid (10-μL aliquots) was collected from egg samples of koi carp not used in this study ($N = 39$) and from others used in this study ($N = 5$). This fluid was analyzed in a vapor pressure osmometer to measure osmolality for comparison with extender data. Four samples were also used for measurement of pH.

The eggs were stored for 2 h at ambient temperature (22–25 C) in extenders (aliquotted at 0.15 mL) at specific osmolalities. For each female ($N = 5$), 17 mL of eggs were separated into eight 0.25-mL aliquots (~200 eggs) with an additional replicate for each extender at 130, 200, 250, 300, 325, 350, 400, or 450 mOsmol/kg. Eggs (dry) from two Petri dishes were also fertilized at 0 h (2–15 min after stripping) and at 2 h. Eggs were stored in 68 separate 100 × 15-mm disposable plastic Petri dishes (Baxter Healthcare Corp., McGaw Park, Illinois, USA) with tops.

Sperm were collected in 3-mL syringes and refrigerated for 1 to 12 h until the females ripened. Sperm from each of two males (not pooled) were used to fertilize eggs from each female. Eggs were fertilized at 2 h, roughly the time at which eggs begin to lose fertility (Suzuki 1980; Linhart et al. 1995). Undiluted sperm (0.02 mL) was add-

ed, and water (10 mL) at ambient temperature (~27 C) was used to activate the gametes, allowing fertilization and adhesion of eggs to the Petri dishes. The eggs were placed in a monolayer to ensure adequate oxygen supply. After 3–5 min, the Petri dishes were placed in a mesh screen and incubated in a recirculating system (~27 C).

Screens were held vertically in the water column to increase circulation until percent eyed embryos and hatch were evaluated. The percentage of eyed embryos at 24 h was calculated by dividing the number of eyed eggs (in which eyes were recognizable by a black pigment) by the total number of eggs in the Petri dish. Percent hatch of fertilized eggs was calculated by subtracting the remaining unhatched embryos in the Petri dish after 60 h from the total eyed eggs at 24 h and dividing that number by total eyed eggs at 24 h.

To test correlation of fertilization capacity and hatching, percent hatch of the total number of eggs was calculated by subtracting the remaining unhatched embryos in the Petri dish from the total eyed eggs at 24 h and dividing that number by the original number of eggs in the Petri dish.

Study 2: Evaluation of Extenders at Ambient Temperature

Extenders were evaluated for their effect over time on fertilization capacity and hatch. Eggs and sperm were collected as described above. Extenders were prepared at the osmolalities yielding the highest percent eyed embryos in Study 1: C-F HBSS at 250 mOsmol/kg (pH, 8.0), NaCl at 200 mOsmol/kg (pH, 5.5), SOF at 250 mOsmol/kg (pH, 7.5) and K2 at 200 mOsmol/kg (pH, 7.5). Eggs with no extender (dry) served as a control.

For each female (N = 5), 20 mL of eggs were separated into eight 0.25-mL aliquots with an additional replicate for each time interval. Eggs were stored in 80 separate 100 × 15-mm disposable Petri dishes with tops in extenders aliquotted at 0.15 mL. Sperm from five males were refrigerated for 1 to 12 h until the females ripened. Sperm from each of two males (not pooled) were used to fertilize eggs from each female (a total of five males were used). The tops were removed and undiluted sperm (0.02 mL) was added to a treatment set (four extenders and a control) of Petri dishes hourly for 7 h. Water (10 mL) at ambient temperature was added to activate the gametes, allowing fertilization and adhesion to the Petri dishes. After 3–5 min, the Petri dishes were placed in a mesh screen and incubated as described above until percent eyed embryos and hatch were evaluated.

Study 3: Evaluation of Extenders at Ambient Temperature and 5 C

Four extenders were evaluated for the effect on fertilization capacity and hatch at ambient temperature (22–25 C) and at 5 C. Eggs with no extender (dry) served as a control for egg quality and fertilization capacity. The osmolality of each extender was optimized previously in Study 1. For each female (N = 5), 17.5 mL of eggs were separated into five 0.25-mL groups (~200 eggs) with an additional replicate for each extender (aliquotted at 0.15 mL) at storage times of 0, 2, 4, or 6 h and temperatures of 5 C or 22–25 C. At 0 h (2–15 min after stripping), only ambient temperature was evaluated. To ensure adequate oxygen supply, eggs were placed in a monolayer in each Petri dish. Sperm from five males were collected into 3-mL syringes and refrigerated for 1 to 20 h until the females ovulated. Sperm samples were not pooled. Methods for the collection, incubation, and fertilization capacity of the gametes were the same as described above.

Study 4: Evaluation of Extender at Different Temperatures

The extender yielding the highest percent eyed embryos after storage at 5 C from the study above (C-F HBSS) was evaluated for the effect on fertilization capacity and hatch at six temperatures. Eggs (from 5 females)

and sperm (from 5 males) were collected as described above. The extender was evaluated across a range of temperatures (0, 5, 10, 15, 20, and 25 C) and storage times (1, 3, 6, 9, and 12 h). The extender was aliquotted at 0.15 mL in 62 separate 100 × 15-mm disposable Petri dishes with 0.25 mL of eggs in each to assess fertilization capacity and hatch, yielding two replicates per female per time interval per temperature. Eggs from two Petri dishes were also fertilized at 0 h (2–15 min after stripping). Undiluted sperm (0.02 mL) was used to fertilize the eggs with the addition of 10 mL of water at ambient temperature after storage.

Petri dishes were stored in separate low-temperature incubators ($N = 6$) constructed from standard compact refrigerators based on Tiersch and Tiersch (1993). An external hydraulic-action thermostatic controller (model 1609-101, White-Rodgers Division, Emerson Electric Co., St. Louis, Missouri, USA) was installed on the side of each refrigerator to control the temperatures. A type-T thermocouple (#TMTSS-040G-12, Omega Engineering Inc., Connecticut, USA) and 5-channel datalogger (OM-550, Omega) were used to record temperatures which were reported as the mean ± SD at 3-min intervals. Petri dishes were removed from the incubators at specified times. Eggs were fertilized and placed in a mesh screen and incubated as described above until percent eyed embryos and hatch were evaluated.

Statistical Analyses

All values for percent eyed embryos and hatching were arcsine square-root transformed before statistical analysis. In Study 1 for each extender (C-F HBSS, NaCl, SOF, or K2), a two-factor analysis of variance (SAS 6.10, SAS Institute Inc., Cary, North Carolina, USA) was used to test the effect of osmolality (130, 200, 250, 300, 325, 350, 400, or 450 mOsmol/kg) and individual female ($N = 5$) on fertilization capacity. A corresponding separate two-factor analysis of variance was performed for hatch. A correlation coefficient was calculated for eyed embryos and hatching percentages (SAS 6.10). In Study 2, a three-factor analysis of variance (SAS 6.10) was used to test the effect of extender (C-F HBSS, NaCl, SOF, or K2), individual female ($N = 5$), and time (0, 1, 2, 3, 4, 5, 6, or 7 h) on fertilization capacity. A separate three-factor analysis of variance was performed for hatch. A two-factor analysis of variance (SAS 6.10) was performed at each time interval (0, 1, 2, 3, 4, 5, 6, or 7 h) to test the effect of extender (C-F HBSS, NaCl, SOF, or K2) and individual female ($N = 5$) on fertilization capacity. In Study 3 for each time (2, 4, or 6 h), a three-factor analysis of variance (SAS 6.10) was used to test the effects of extender (C-F HBSS, NaCl, SOF, or K2), individual female ($N = 5$), and temperature (ambient or 5 C) on fertilization capacity. A three-factor analysis of variance was used to test the effects of extender (C-F HBSS, NaCl, SOF, or K2), individual female ($N = 5$), and time (0, 2, 4, or 6 h) on fertilization capacity for storage at ambient temperatures. A separate three-factor analysis of variance was performed for hatching. A three-factor analysis of variance (SAS 6.10) was used to test the effects of extender (C-F HBSS, NaCl, SOF, or K2), individual female ($N = 5$), and time (2, 4, or 6 h) on fertilization capacity for storage at 5 C. A separate three-factor analysis of variance was performed for hatching. A two-factor analysis of variance for each time interval (2, 4, or 6 h) was used to test the effects of extender (C-F HBSS, NaCl, SOF, or K2) and individual female ($N = 5$) on fertilization capacity at 5 C. In Study 4, a three-factor analysis of variance was used to test the effect of temperature (0, 5, 10, 15, 20, or 25 C), time (1, 3, 6, 9, or 12 h), and individual female ($N = 5$) on fertilization capacity. A separate three-factor analysis of variance was performed for hatching. A two-factor analysis of variance for each time interval (1, 3, 6, 9, or 12 h) was used to test the effect of temperature (0, 5, 10,

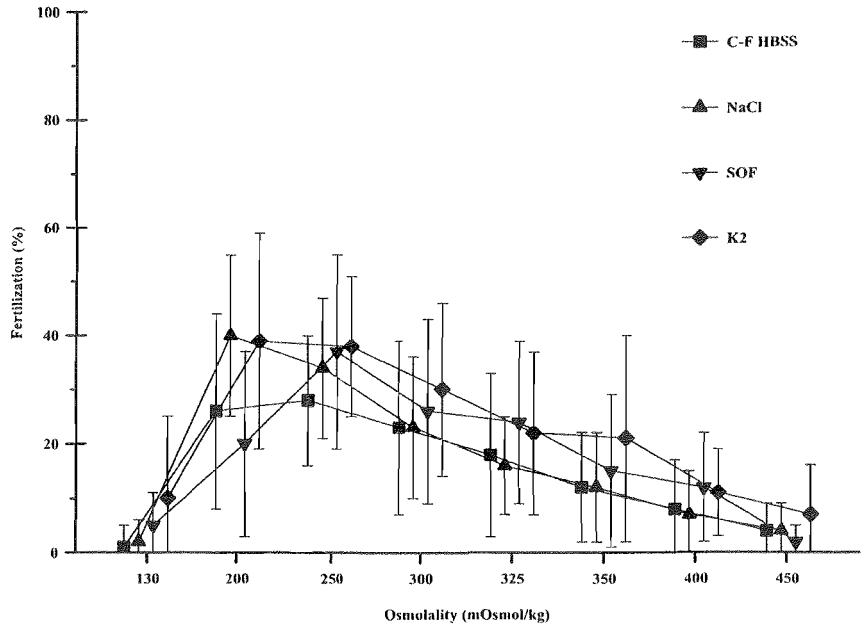

FIGURE 1. *Koi carp eggs from five females were stored at ambient temperature (22–25 C) for 2 h to evaluate the effects of extender and osmolality on fertilization capacity. Percent eyed embryos at 24 h after insemination was calculated by dividing the number of eyed eggs by the total number of eggs. Eggs suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) or synthetic ovarian fluid (SOF) had significantly higher fertilization capacity at 250 mOsmol/kg than at 130, 200, 300, 325, 350, 400, or 450 mOsmol/kg. Eggs suspended in salt (NaCl) or Kurokura #2 (K2) had significantly higher fertilization capacity at 200 mOsmol/kg than at any other osmolality. Fertilization capacity of eggs held dry (control) was 24 ± 11%. Mean ± standard deviation is shown for each treatment.*

15, 20, or 25 C) and individual female (N = 5) on fertilization capacity. For all tests, Duncan's multiple range test was used to determine if significant differences (at $P \leq 0.05$) existed among the means.

Results

Study 1: Evaluation of the Effect of Extender Osmolality

Eggs stored for 2 h at osmolalities ranging from 130 to 450 mOsmol/kg were compared for percent eyed embryos and percent hatch. Eggs suspended in C-F HBSS had higher (P = 0.0001) fertilization capacity (mean ± SD: 28 ± 12%) at 250 mOsmol/kg than at 130, 200, 300, 325, 350, 400, or 450 mOsmol/kg (Fig. 1). The fertilization capacity of the dry (control) eggs was 58 ± 23% at 0 h and 24 ± 11% at 2 h. Eggs suspended in NaCl had significantly higher (P = 0.0001) fertilization capacity with osmolalities of 200 mOsmol/kg (40 ± 15%) and 250 mOsmol/kg (34 ± 13%) than at 130, 300, 325, 350, 400, or 450 mOsmol/kg. Eggs suspended in SOF had significantly higher (P = 0.0001) fertilization capacity (37 ± 18%) at 250 mOsmol/kg than at 130, 200, 300, 325, 350, 400, or 450 mOsmol/kg. Eggs suspended in K2 had higher (P = 0.0001) fertilization capacity (39 ± 20%) at 200 mOsmol/kg than at any other osmolality.

The results of percent hatch paralleled the results of percent eyed embryos (Fig. 2). Hatching of eggs suspended in C-F HBSS was significantly higher (P = 0.0001) at osmolalities above 130 mOsmol/kg. Hatching for the dry (control) was 97 ± 5%. Hatching of eggs suspended in NaCl was significantly higher (P = 0.0008) at osmolalities above 130 mOsmol/kg. Hatching of eggs suspended in SOF was not significantly different (P = 0.0854) among osmolalities. Hatching of eggs suspended in K2 was not significantly different (P = 0.8393) among osmolalities. Percent eyed

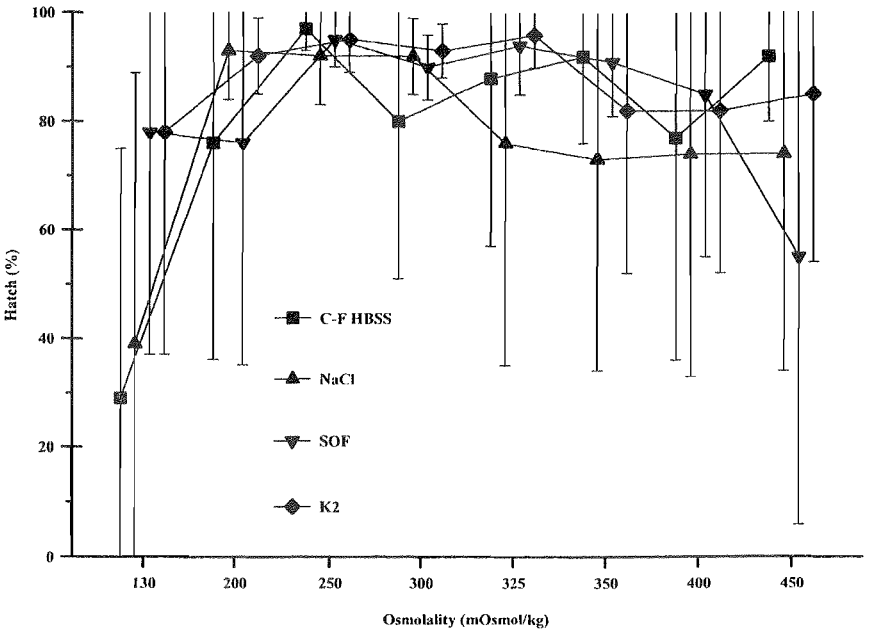

FIGURE 2. *Koi carp eggs from five females were stored at ambient temperature (22–25 C) for 2 h to evaluate the effects of extender and osmolality on hatching. Percent hatch of fertilized eggs at 60 h after insemination was calculated by subtracting the remaining unhatched embryos from the total eyed eggs at 24 h and dividing that number by the total number of eyed eggs at 24 h. Eggs suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) or synthetic ovarian fluid (SOF) at all osmolalities above 130 mOsmol/kg were not significantly different in percent hatch. Eggs suspended in salt (NaCl) or Kurokura #2 (K2) were not significantly different in percent hatch at any osmolality. Hatch of eggs held dry (control) was 97 ± 5%. Mean ± standard deviation is shown for each treatment.*

embryos and hatch were found to have a positive correlation coefficient (y = 0.938786x + 0.005559, where y = percent hatch and x = percent eyed embryos; r = 0.9914).

The osmolality of the ovarian fluid samples (N = 44) was 290 ± 13 mOsmol/kg (mean ± SD). This was higher than the osmolality determined for each extender to maximize fertilization capacity and hatch. The pH of ovarian fluid was 8.8 ± 0.25 (N = 4).

Study 2: Evaluation of Extenders at Ambient Temperature

As time increased, the percent eyed embryos decreased for all extenders (Fig. 3). Between 0 and 7 h, eggs stored in C-F HBSS and SOF had significantly higher (P = 0.0001) percent eyed embryos than did eggs stored in NaCl, K2, or the dry control. The interactions between extenders, time, and individual females were all significant (P = 0.0001). At 0 h, eggs suspended in NaCl had significantly lower (P = 0.0006) percent eyed embryos (39 ± 32%) than did any other treatment including dry (control) eggs (46 ± 30%). At 1 h, eggs stored in C-F HBSS, SOF, or dry had significantly higher (P = 0.0001) percent eyed embryos than did eggs stored in NaCl or K2. At 2 h, eggs stored in C-F HBSS (35 ± 24%) had higher (P = 0.0002) percent eyed embryos than did any other treatment. At 3 h, eggs stored in C-F HBSS, SOF, or dry had significantly higher (P = 0.0001) percent eyed embryos than did eggs stored in NaCl or K2. At 4 h, eggs stored in SOF had higher (P = 0.0091) percent eyed embryos than did all other treatments. At 5 h, eggs stored in SOF and C-F HBSS had higher (P = 0.0423) percent eyed embryos than did eggs stored in NaCl, K2, or dry. At 6 h, eggs stored in C-F HBSS had higher (P = 0.1429) percent eyed embryos than did any other treatment. At 7 h, eggs stored in SOF

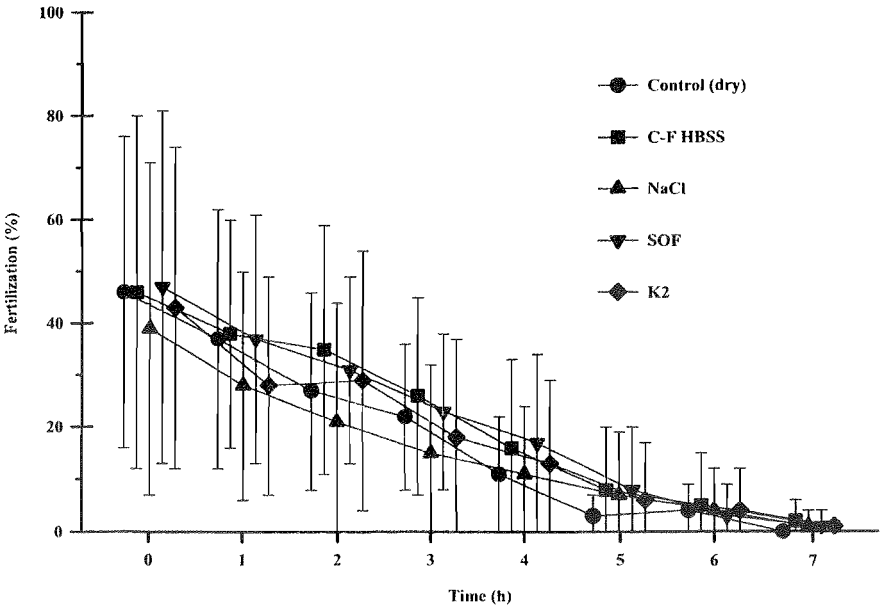

FIGURE 3. *Koi carp eggs from five females were stored at ambient temperature (22–25 C) for as long as 7 h to evaluate the effects of extender and storage time on fertilization capacity. Percent eyed embryos at 24 h after insemination was calculated by dividing the number of eyed eggs by the total number of eggs. Eggs suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) or synthetic ovarian fluid (SOF) had significantly higher percent eyed embryos (*P *= 0.0001) than did eggs in salt (NaCl), Kurokura #2 (K2), or the control (dry) over 7 h. Mean ± standard deviation is shown for each treatment.*

(1 ± 3%), C-F HBSS (2 ± 4%), and NaCl (1 ± 3%) had higher (P = 0.0271) percent eyed embryos than did eggs stored in K2 or the control. Fertilization capacity among the treatments decreased in variability over 7 h. The percent hatch of these eggs was not significantly different (P = 0.1258) among extenders between 0 and 7 h (Fig. 4). Hatching among the treatments increased in variability over 7 h. Percent eyed embryos and hatch were found to have a positive correlation coefficient ($y = 0.971604x - 0.003431$, where y = percent hatch and x = percent eyed embryos; r = 0.9982).

Study 3: Evaluation of Extenders at Ambient Temperature and 5 C

As storage time increased, fertilization capacity decreased at ambient and refrigerated temperatures (Fig. 5). At 2, 4, and 6 h, percent eyed embryos was significantly higher (P = 0.0001) at ambient temperature than at 5 C. For ambient temperature, eggs suspended in C-F HBSS had significantly higher fertilization capacity than did eggs suspended in NaCl, SOF, K2, or left dry (control) at all time intervals. Fertilization capacity with eggs stored in C-F HBSS decreased after 2 h as did fertilization capacity of eggs stored in NaCl, SOF, or K2, or dry. Fertilization capacity at 0 h was significantly higher (P = 0.0001) than at 2, 4, and 6 h. Fertilization capacity among the treatments decreased in variability over time.

At 5 C, eggs suspended in C-F HBSS or left dry had significantly higher (P = 0.0001) fertilization capacity than did eggs suspended in NaCl, SOF, or K2 at 2, 4, and 6 h. Percent eyed embryos at 2 h were significantly higher (P = 0.0001) than at 4 or 6 h. Percent eyed embryos at 2 h for eggs at 5 C was significantly higher (P = 0.0001) with no extender (9 ± 8%) than with C-F HBSS, NaCl, SOF, or K2. At 4 h, eggs had higher (P = 0.0006) fertilization capacity when suspended in C-F HBSS or left dry than with NaCl, SOF, or K2. At 6 h, eggs had higher (P = 0.0517) fertilization capacity when suspended in C-F HBSS

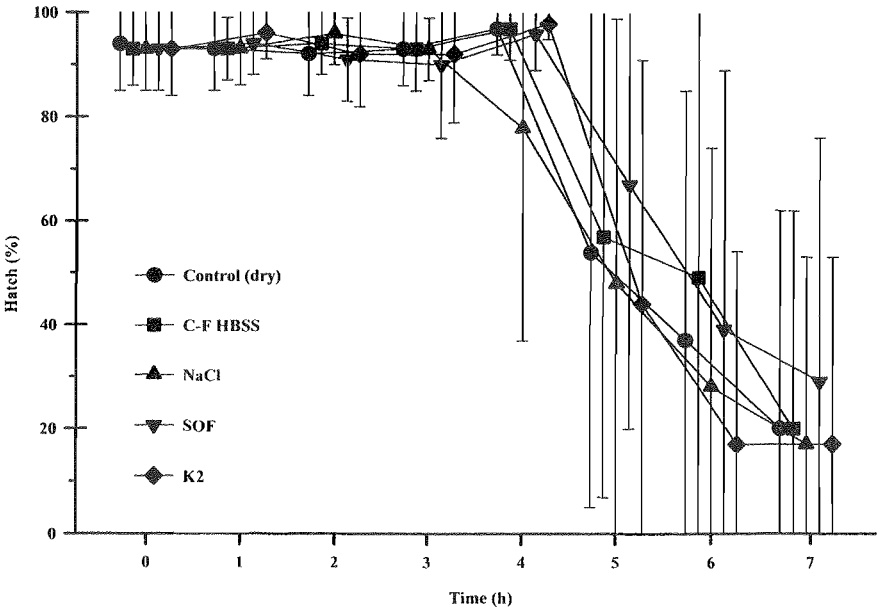

FIGURE 4. *Koi carp eggs from five females were stored at ambient temperature (22–25 C) for as long as 7 h to evaluate the effects of extender and storage time on hatching. Percent hatch of fertilized eggs at 60 h after insemination was calculated by subtracting the remaining unhatched embryos from the total eyed eggs at 24 h and dividing that number by the total number of eyed eggs at 24 h. There was no significant difference (*P *= 0.1258) among extenders over 7 h. Variability in the treatments increased after 4 h of storage. Mean ± standard deviation is shown for each treament.*

or K2. Variability among the treatments was similar over time.

Dry eggs at ambient temperature had higher (P = 0.0001) percent hatch than did eggs suspended in C-F HBSS, NaCl, SOF, or K2 at 0, 2, 4, and 6 h (Fig. 6). Percent hatch at 0 h was significantly higher (P = 0.0001) than at 2, 4, and 6 h. Dry eggs at 5 C had higher (P = 0.0002) hatch than did eggs suspended in C-F HBSS, NaCl, SOF, or K2 at 2, 4, and 6 h. Percent hatching at 2 h was significantly higher (P = 0.0001) than at 4 or 6 h. Variability among the treatments and temperatures was similar over time.

Study 4: Evaluation of Extender at Different Temperatures

From the studies above, it was found that eggs suspended in C-F HBSS consistently produced the highest fertilization capacity and comparable hatching rates to all the treatments over time at temperatures of 22 to 25 C and at 5 C. Thus, C-F HBSS was chosen for study across a range of temperatures. The six experimental incubators held constant temperatures with little variation: 1 ± 1 C; 5 ± 2 C; 10 ± 1 C; 15 ± 2 C; 20 ± 1 C, and 25 ± 2 C (mean ± SD). Eggs stored at 15 C had significantly higher (P = 0.0001) fertilization capacity over time than did eggs stored at 0, 5, 10, 20, or 25 C. The percentage of eyed embryos was significantly different (P = 0.0001) at 1, 3, 6, 9, or 12 h (Fig. 7). Eggs stored at 15 C or 10 C had significantly higher (P = 0.0001) hatch over time than did eggs stored at 0, 5, 20, or 25 C. Percent eyed embryos and hatch among treatments decreased with longer storage times (Fig. 8).

Fertilization capacity of dry eggs stored at 25 C for 0 h was 64 ± 35%. At 1 h, eggs stored in C-F HBSS had significantly higher (P = 0.0001) fertilization capacity (65 ± 34%) at 20 C than at 0, 5, 10, 15, or 25 C. At 3 h, fertilization capacity was significantly higher (P = 0.0001) for eggs stored at 20 C (65 ± 26%) or 15 C (66 ± 24%). Eggs stored at 15 C had significantly higher

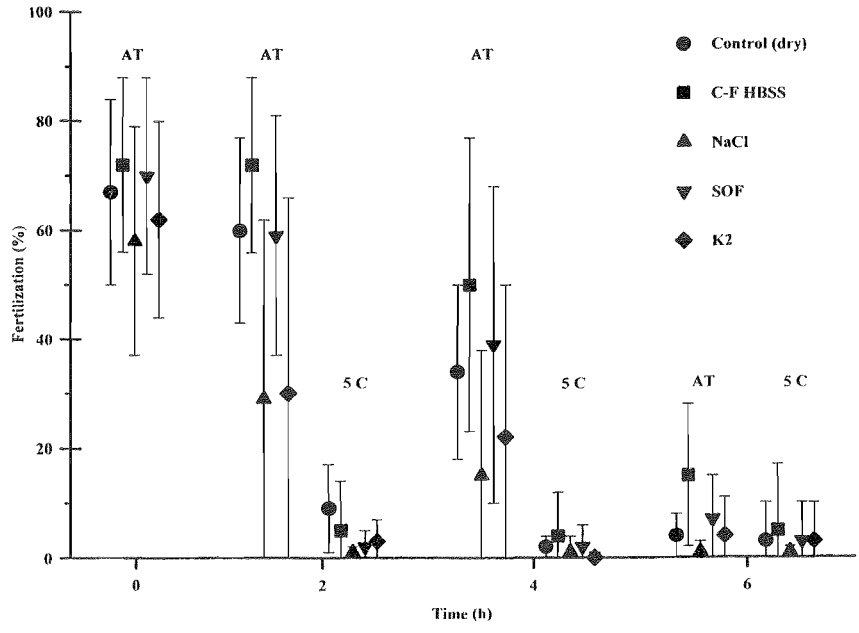

FIGURE 5. *Koi carp eggs from five females were stored for as long as 6 h at ambient temperature (AT; 22–25 C) or at 5 C to evaluate the effects of storage time and temperature on fertilization. Percent eyed embryos at 24 h after insemination was calculated by dividing the number of eyed eggs by the total number of eggs. Eggs stored at ambient temperatures had significantly higher fertilization capacity (*P = 0.0001*) than did eggs stored at 5 C for 2, 4, and 6 h. At ambient temperatures, eggs stored in calcium-free Hanks' balanced salt solution (C-F HBSS) had significantly higher fertilization capacity than did eggs stored in salt (NaCl), synthetic ovarian fluid (SOF), Kurokura #2 (K2), and dry (control) at 0, 2, 4, and 6 h. At 5 C, eggs stored in C-F HBSS or held dry had significantly higher fertilization capacity than did eggs stored in NaCl, SOF, and K2 at 2, 4, and 6 h. Fertilization capacity decreased at each temperature over time. Mean ± standard deviation is shown for each treatment.*

(P = 0.0001) fertilization capacity at 6 h (67 ± 16%) and 9 h (41 ± 28%). At 12 h, fertilization capacity was significantly higher (P = 0.0001) for eggs stored at 10 C (33 ± 26%) or 15 C (29 ± 29%).

Discussion

Short-term storage of fish eggs requires protocols to maintain fertilizing and hatching ability over time. Osmotic pressure and extenders influence the ability of the eggs to become fertilized after storage (Renard et al. 1987). The osmotic pressure of the dilution medium can be responsible for the elevation or activation of the chorion (Renard et al. 1987). The osmolality of common carp ovarian fluid was reported to be 305 mOsmol/kg (Plouidy and Billard 1982), higher than that of this study (290 ± 13 mOsmol/kg), possibly due to use of common carp instead of koi carp or to environmental influences such as water quality, and indicates that values should be measured for particular stocks. By evaluating the environment surrounding the eggs, adjustments can be made to the extender osmolality to prevent unintended activation. This method has been useful with sperm of freshwater fish, in which lowering of the osmolality of the extender will activate the sperm (Bates et al. 1996).

In previous studies, common carp eggs did not remain fertile after ovulation when suspended in extenders (Yamamoto 1961; Renard et al. 1987). Many factors other than extender type affect the fertilizing capability of eggs when stored. Egg quality could be related to the health of the female and time of ovulation, and could also be influenced by temperature, pH, and oxygen levels (Horvath 1978; Horvath and Peteri 1980). In these studies, we attempted to

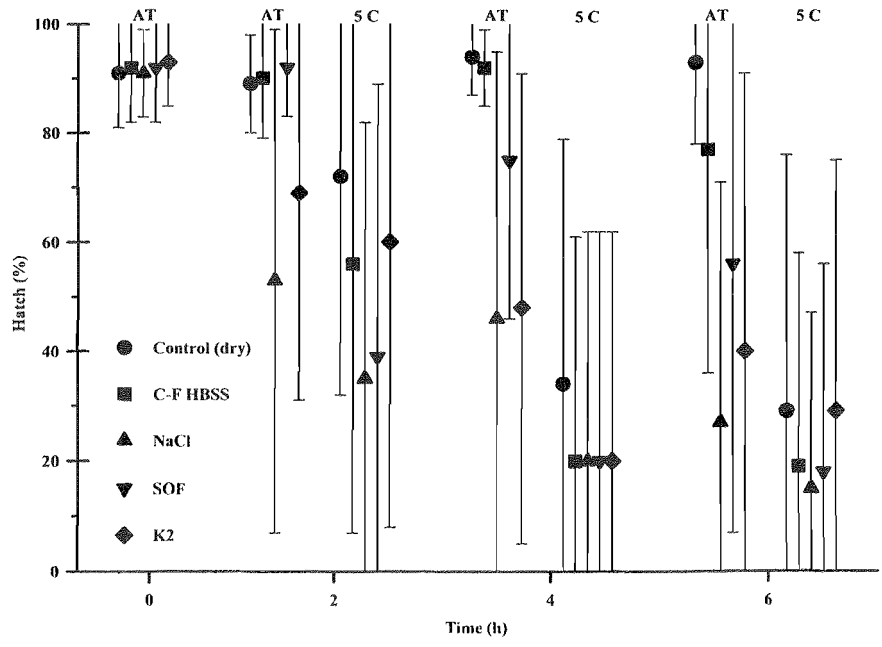

FIGURE 6. *Koi carp eggs from five females were stored for as long as 6 h at ambient temperature (AT: 22–25 C) or at 5 C to evaluate the effects of storage time and temperature on hatching. Percent hatch of fertilized eggs at 60 h after insemination was calculated by subtracting the remaining unhatched embryos from the total number of eyed eggs at 24 h and dividing that number by total number of eyed eggs at 24 h. Eggs held dry (control) at ambient temperatures and at 5 C had a higher percent hatch over time than did eggs stored in calcium-free Hanks' balanced salt solution (C-F HBSS), salt (NaCl), synthetic ovarian fluid (SOF), and Kurokura #2 (K2). Hatching decreased at each temperature over time. Mean ± standard deviation is shown for each treatment.*

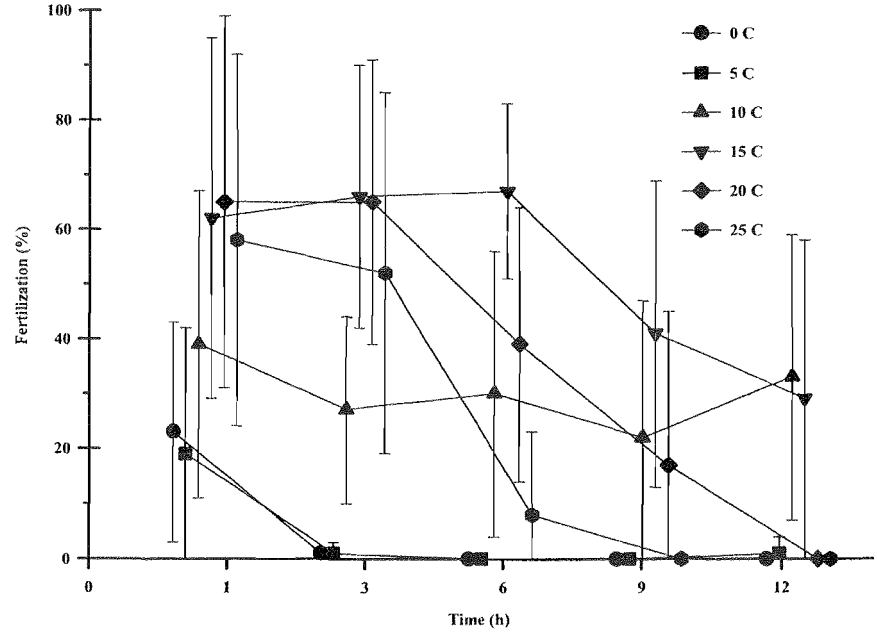

FIGURE 7. *Koi carp eggs from five females were stored in calcium-free Hanks' balanced salt solution for as long as 12 h at 0, 5, 10, 15, 20, or 25 C. Percent eyed embryos at 24 h after insemination was calculated by dividing the number of eyed eggs by the total number of eggs. Eggs stored at 15 C had significantly higher fertilization capacity over time than did eggs stored at other temperatures. At 0 h of storage, the fertilization capacity for the control (dry) was 64 ± 35%. Mean ± standard deviation is shown for each treament.*

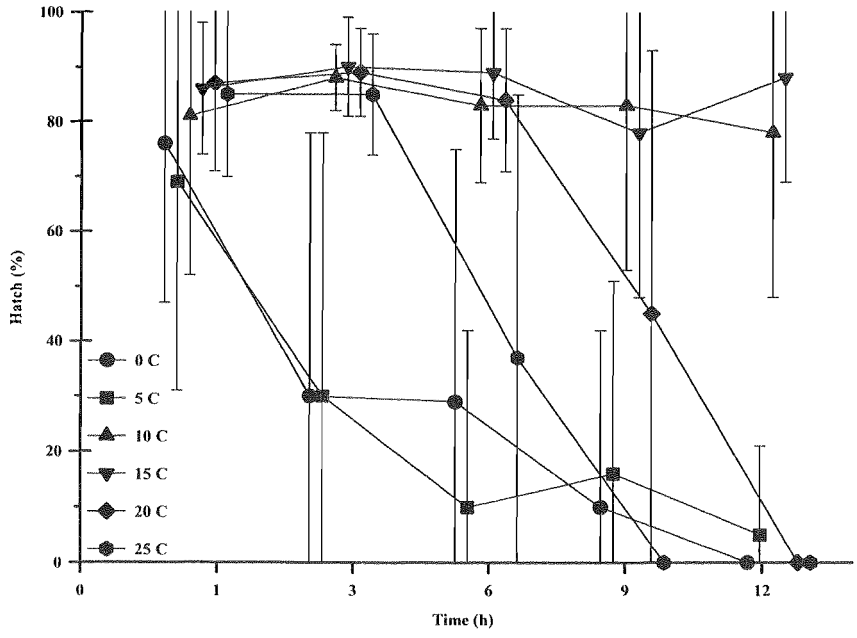

FIGURE 8. *Koi carp eggs from five females were stored in calcium-free Hanks' balanced salt solution for as long as 12 h at 0, 5, 10, 15, 20, or 25 C. Percent hatch of fertilized eggs at 60 h after insemination was calculated by subtracting the remaining unhatched embryos from the total eyed eggs at 24 h and dividing that number by total eyed eggs at 24 h. Eggs stored at 15 C or 10 C had significantly higher hatch over time than did eggs stored at other temperatures. At 0 h of storage, the percent hatch for the control (dry) was 90 ± 10%. Mean ± standard deviation is shown for each treament.*

minimize variation in some of these parameters. The pH of ovarian fluid was found to be 8.8 for koi carp broodstock, comparable to the pH of ovarian fluid of 8.5 reported for common carp (Plouidy and Billard 1982). The optimum pH for fertilization in common carp was reported to be between 7.2 and 9.6 (Roubaud et al. 1984). All extenders used in the present study were within this range except for the salt solution (200 mOsmol/kg) which had a pH of 5.5, possibly explaining the poor fertilization capacity and hatch for eggs stored in this solution.

Research on storage of fish eggs at refrigerated temperatures has been confined to evaluation of undiluted (dry) eggs. In the past, eggs stored at refrigerated temperatures have had reduced fertilizing potential over time due to cold shock (Leung and Jamieson 1991). Fertilization capacity and hatch of koi carp eggs in this study as well as in Urbanyi et al. (1998) also decreased through time. Eggs of common carp were stored undiluted in a preliminary study at 2 to 9 C yielding no fertilization (Withler 1980). Eggs of koi carp stored undiluted for 7 h had decreased survival at 6 to 9 C (~1%) or 12 to 24 C (~2%) compared to the control (~30%) at 0 h (Rothbard et al. 1996). The fertilization rate (determined at morulation) of common carp eggs stored undiluted was found to be 86% after 10 h at 3 to 5 C, and to be 82% after 18 h at 14.5 to 18 C (Zlabek and Linhart 1987).

In the present study, extenders (specifically C-F HBSS) preserved the fertilizing capability of koi carp eggs at lower temperatures compared to the control (dry). Osmolality and ions of extenders were found to play an important role in the activation of common carp eggs (Renard et al. 1987). Koi carp eggs suspended for 2 h in C-F HBSS (250 mOsmol/kg) at ambient temperature exhibited higher fertilization capacity than did the dry control. A decrease in temperature had a positive effect on the fertilizing potential of eggs through time. In another study using common carp eggs (Urbanyi et al. 1998) and no extender, a de-

crease in temperature had a negative effect on fertilization capacity and hatch through time. These results indicate that the use of extender solutions offers potential for extending the storage time of fish eggs at ambient or refrigerated temperatures. However, variability in results exists and could be related to the health of the female, timing of ovulation, or temperature and condition of the recirculating water (Horvath 1978; Horvath and Peteri 1980).

Percent fertilization from prior studies was calculated at the stage of morulation, about 4 to 6 h after insemination (Jahnichen 1978; Zlabek and Linhart 1987). Parthenogenetic development (without fertilization) in the eggs of common carp can continue to the morula or gastrula stage after which the eggs deteriorate (Withler 1980). Embryogenesis has also been found to cease spontaneously in eggs after the morula stage, while the quantity of embryos at the eyed stage and before hatching was found to be similar in the present study. Therefore, fertilization capacity could be best estimated using later stages of larval development, and should be supported with percent hatch data. However, percent hatch is subject to errors unrelated to fertilizing capability such as disease or low dissolved oxygen. In the present study, fertilization capacity was calculated well after the gastrula stage, when eyed eggs appeared, avoiding the inclusion of embryos arresting at earlier stages of development, and percent hatch was measured to complement the data for percent eyed embryos. The high degree of variation in hatching we observed beyond 4 h of egg storage might be related to abnormal larval development. In addition, percent hatch of eggs had a direct relationship to percent eyed embryos in each experiment. This could benefit research by allowing the collection of only one data set (percent eyed embryos or hatch) to evaluate egg quality.

Throughout this study, eggs in extenders such as C-F HBSS had an equal or higher percentage of eyed embryos than did the dry control. This shows that extenders can be used with koi carp eggs to prolong fertilizing ability and prevent premature activation. The short-term storage of koi carp eggs at refrigerated temperatures offers benefits for research and breeding. Eggs from different stocks (from disease-free environments) could be transported among local hatcheries. Koi carp, due to their hardiness and fecundity in captivity, could serve as a model for other warmwater species to study the fertilizing potential of stored eggs. This study shows that eggs can be stored in an extender at 15 C for as long as 12 h with a loss of ~50% fertilization capacity compared to fresh eggs, while dry controls were reduced to this level within 4.5 h. Future research should address the specific conditions that affect egg activation, and extenders that further maintain the fertilizing potential of eggs over time.

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