New Chapter

Evaluation of Extenders for Refrigerated Storage of Koi Carp and Goldfish Sperm

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Problems and Approaches to Gamete Storage

Refrigerated storage of sperm can be an effective tool to improve genetic research and lengthen the time between collection of sperm and use for fertilization of eggs. Extenders, usually consisting of a salt solution with added organic compounds, are used to dilute and preserve sperm. By adjusting the osmolality of an extender, sperm can be held immotile, and activation of eggs can be delayed (e.g., Glenn and Tiersch 2002) lengthening their useable lifetime. The extent of extender dilution can alter sperm concentration and thus affect fertilization. An activating solution (an extender with low osmolality) has been occasionally used with sperm and eggs of freshwater fish to lessen the degree of osmotic shock that can occur when solutions of different osmolality are mixed (Cognie et al. 1989, Drokin et al. 1994). The pH of an extender can also affect motility and fertilization (Roubaud et al. 1984). Extenders are occasionally used with cryoprotectants for additional protection during cold storage, and various osmolalities, pH, and dilutions have been used with different degrees of success. Studies of refrigerated and cryopreserved sperm of common carp *Cyprinus carpio* have dealt with survival and motility (e.g., Sneed and Clemens 1956, Withler 1980). Many of the reports addressing extenders and refrigerated storage in carp and other species are hard to find, inconsistent in reporting of results and conclusions (Rana 1995), and presented in several languages (see References). In addition, experimental methods are often dissimilar and difficult to repeat. This chapter is intended to show some of the problems and opportunities encountered when choosing an extender for refrigerated storage and cryopreservation. We chose to illustrate the process with a literature review and experimental work addressing common carp and goldfish.

Common carp have been selectively bred for more than 200 yr to produce domesticated ornamental varieties called koi carp. Superior koi possess distinctive coloration and markings, and individuals can be valued at greater than US$50,000. Traits of koi depend on environment, bloodline, and type of feed. Goldfish *Carassius auratus* are hardy, fast growing, easily maintained, and spawn readily in captivity. Millions of goldfish are bred each yr for sale as ornamental fish and baitfish. Improvement of procedures for selective breeding of koi and goldfish could increase the quality and value of these fish and expand our understanding of the heredity of colors and patterns. The demand for breeding husbandry of koi and goldfish will increase as the demand for ornamental ponds increases in private homes and businesses,

Like many other species of fish, koi and goldfish can be bred naturally in ponds or artificially through the use of fresh, refrigerated or cryopreserved sperm. Natural propagation of carp (sometimes stimulated by hormonal injections) has proved to be economical and efficient for commercial and research purposes. However, artificial spawning can increase the number of fry and provide greater control of specific broodstock crosses, and thus can be more economical and efficient than natural spawning. Artificial spawning using stored sperm can reduce the number of male broodstock required, thereby maximizing space in the hatchery. Sperm from one male can fertilize eggs from several females over time (even after the death of a valuable male).

Glenn III, D. W., R. P. Lang and T. R. Tiersch. 2011. Evaluation of Extenders for Refrigerated Storage of Koi Carp and Goldfish Sperm. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 107-124.

Fertilization capacity of common carp eggs with refrigerated sperm has been found to equal that obtained with fresh sperm (Hulata and Rothbard 1979), and sperm can be collected off-site and transported to the laboratory or hatchery for artificial propagation.

Study of artificial breeding with refrigerated sperm requires examination of how extenders and sperm interrelate. The storage of sperm can be increased by dilution in extenders which are used to supply ionic and osmotic conditions appropriate to prevent activation during storage and handling. Osmolality and dilutions interact with extenders to affect motility and storage. The effects of potassium and osmolality on motility has been studied in sperm of freshwater cyprinids including goldfish (Morisawa et al. 1983). Frog Ringer's solution was used to store common carp sperm for 30 d at 3-5°C (Sneed and Clemens 1956), and sperm from common carp, crucian carp *Carassius carassius*, and dace *Tribolodon hakonesis* were held in 300 mOsmol/Kg NaCl (Morisawa et al. 1983). Sperm of common carp was stored at 4°C for 12 d in extenders containing various amounts of KCl, NaCl, glucose, and DMSO (Chen et al. 1992).

The present study evaluated Hanks' balanced salt solution (HBSS), calcium-free (C-F) HBSS (Tiersch et al. 1997), and solutions of NaCl and $NaHCO_3$ for refrigerated storage of goldfish and koi sperm. Hanks' balanced salt solution has been used for storage of sperm of marine fishes such as *Cynoscion nebulosus* (Wayman et al. 1996) and *Pogonias cromis* (Wayman et al. 1997), and freshwater fish such as channel catfish *Ictalurus punctatus* (Christensen and Tiersch 1996). We sought to test the effect of dilution rates and osmolality changes with these extenders on motility and fertilizing ability of refrigerated sperm. This chapter begins with a literature review of extenders tested for refrigerated storage of common carp to illustrate the wide range of protocols and formulations available, and then provides the experiments used to test a panel of extenders. The research objectives were to evaluate the: 1) relationship among osmolality of body fluids, extender and sperm activation; 2) effect of extender osmolality on refrigerated storage; 3) effect of sperm dilution on refrigerated storage, and 4) fertilization with stored sperm.

A Review of Extenders and Sperm Refrigeration in Common Carp

Extenders have been used in most studies with sperm storage (Table 1). Undiluted sperm often quickly loses fertilizing ability due to contamination with urine (Tiersch et. al. 1997), lack of oxygen and nutrients for storage, and bacterial contamination (Belova 1982, Billard et al. 1995). Ribonucleic acid levels decreased in undiluted sperm over 24 hr when stored at 4-6 ^{o}C, decreasing fertilization (Nedovesova 1983). Suspension in extenders can reduce these problems, although excessive dilution can itself reduce quality (Paniagua-Chavez et al. 1998). Optimum osmolality and dilution must be studied for each extender, and several have produced high motility, fertilization and hatch (summarized in Appendix, end of chapter). Some extenders used for common carp are frog Ringer's solution (FR), phosphate buffer (PB), Alsever's solution (A), Cortland's fluid (C), and modified Cortland's fluid (mC). Motility was sometimes prolonged with addition of antibiotics and supplemental oxygen (Saad et al. 1988). Stirring was not recommended due to sperm fragility, and storage at lower concentrations was suggested (Belova 1981). Samples were stored at 0 to 33 ^{o}C (Musselius 1951, Bhowmick and Bagchi 1971), most commonly at ~4 ^{o}C, over a range of 3.5 to 720 h. Motility and fertilization were not different from fresh controls (Hulata and Rothbard 1979, Jahnichen 1981, Rothbard et al. 1996). Overall, refrigerated storage requires optimization of osmolality, dilution rate, container type, and temperature to maintain fertilization rates comparable to control samples.

Table 1. Extenders used for refrigerated storage of sperm from common carp *Cyprinus carpio*.

Ingredients (g/L)	Frog Ringer's solution	Phosphate buffer	Not specified	Not specified	Alsever's solution	Cortland's fluid	Diluent for freezing bull's semen	Milk-yolk diluent
NaCl	6.50				4.00	7.25		
KCl	0.14					0.38		
$CaCl_2$	0.12							
$CaCl_2 \cdot 2H_2O$						0.23		
$C_6H_5O_7Na_3 \cdot 2H_2O$					8.00		720.00	
$NaHCO_3$	0.20					1.00		
NaH_2PO_4	0.01			15.00				
$NaH_2PO_4 \cdot H_2O$						0.41		
$Na_2HPO_4 \cdot 12H_2O$		20.00						
$Na_3C_6H_5O \cdot 2H_2O$			30.00					
KH_2PO_4		2.00						
$MgSO_4 \cdot 7H_2O$						0.23		
$C_6H_{12}O_6$	2.00							
yolk				200.00			200.00	100.00
milk								900.00
fructose							12.50	
glucose					20.50	1.00		
streptomycin							1.00	
penicillin							1.00×10^6 IU	
pH		7.40	7.60					
Reference	Sneed and Clemens 1956	Sneed and Clemens 1956	Sneed and Clemens 1956	Kossmann 1973	Moczarski 1973	Moczarski 1973	Moczarski 1973	Moczarski 1973

Table 1. continued.

Ingredients (g/L)	Modified Cortland's fluid	Ringer's fluid	Not specified	Rinsing solution	Saline solution	Salt	Na-citrate	Not specified
NaCl	1.88	6.00		4.00	7.31	2-100.00		
KCl	7.20							14.91
$CaCl_2$	0.23				0.01			
$NaHCO_3$	1.00							
$C_6H_5O_7Na_3$							2-100.00	
$NaH_2PO_4 \cdot H_2O$	0.41							
$MgSO_4 \cdot 7H_2O$	0.23							
ethanol			10-20.00					
urea				3.00				
glucose	1.00							
streptomycin					50.00			
bipenicillin					5.00×10^4 IU			
Tris HCl								4.73
Tris					2.42			
pH					8.00			8.00
Osmolality								380
Reference	Moczarski 1973	Moczarski 1973	Moczarski 1973	Hulata and Rothbard 1979	Saad et al. 1988	Magyary et al. 1991	Magyary et al. 1991	Redondu-Muller et al. 1991

Table 1. continued.

Ingredients (g/L)	D-15	D-16	D-17	D-19	D-20	D-21	Calcium-free Hanks' balanced salt solution
NaCl	8.00	10.00	9.00	9.00	8.00	8.00	8.00
KCl	0.50	0.50	0.50	1.00	1.00	2.00	0.40
$NaHCO_3$							0.35
Na_2HPO_4							0.06
KH_2PO_4							0.06
$MgSO_4 \cdot 7H_2O$							0.20
glucose	15.00	15.00	15.00	15.00	15.00	15.00	1.00
Osmolality							305
Reference	Chen et al. 1992	Chen et al. 1992	Chen et al. 1992	Chen et al. 1992	Chen et al. 1992	Chen et al. 1992	Glenn 1998[1]

[1]Koi carp.

A Research Approach to Gamete Storage

Fish Maintenance and Gamete Collection

For these experiments, mature male and female koi and goldfish were maintained at the Aquaculture Research Station of the Louisiana Agricultural Experiment Station, Baton Rouge. Only broodstock exhibiting spawning characteristics were selected. Spawning condition was indicated by free-flowing sperm in males, and swelling of the urogenital region in females. Female goldfish were selected for injection based on the presence of rounded bellies, swollen vents and spawning behavior in the presence of males. Female koi were catheterized to assess condition of oocytes. If nuclei were located near the periphery in a majority of the eggs (observed by naked eye), the females were considered to be more likely to respond to hormonal treatment (described below). Fish were not fed for 2 d prior to spawning. For collection of gametes, fish were anesthetized with tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, Washington, USA), and the urogenital papilla was wiped dry. Sperm were hand-stripped and collected into a syringe to avoid contamination with blood and feces.

To stimulate spawning, females received single injections of luteinizing hormone-releasing hormone ethylamide (Peninsula Laboratories, Inc., Belmont, California, USA), at 10 μg/Kg, and of metoclopramide, a dopamine antagonist (Sigma Chemical Corp., St. Louis, Missouri, USA), at 20 mg/Kg (Rothbard 1994). Males did not receive this treatment. Eggs were collected after injection between 9 and 12 hr for koi and 6 to 9 hr for goldfish in a dry bowl, coated with vacuum grease (Dow Corning Corp., Midland, Michigan, USA) to avoid adhesion to the bowl. Eggs were fertilized on separate 100 x 15 mm disposable Petri dishes (Baxter Healthcare Corp., McGaw Park, Illinois, USA) and placed in a recirculating system until percent eyed embryos and hatch were evaluated (koi and goldfish eggs are naturally adhesive when placed into water and activated). Petri dishes were placed in a mesh screen and held vertical to increase oxygen circulation and to avoid sediment accumulation.

Percent Estimation of Sperm Motility

Although subjective, motility is the method most commonly used to evaluate quality of fish sperm, which are typically not motile before dilution in water. Once activated, carp sperm swim rapidly for a short time (usually for 30-40 sec) (Billard et al. 1995). A 2-μL sample of semen from each male was examined using dark-field microscopy at 200-x magnification immediately following activation with 20 μL of deionized water. Percent motility took into account initial movement and duration of the motility within each activated sample. Sperm vibrating in place were not considered to be motile.

Osmolality and Sperm Activation

Osmolality influences the initial activation and duration of sperm motility. By determining the osmolality of body fluids, the environment for non-motile sperm can be better understood. Blood samples from 33 koi (male and female) and 4 goldfish (male) were collected and allowed to clot. Plasma (10 μL) was used to determine osmolality with a vapor-pressure osmometer (model 5500, Wescor Inc., Logan, Utah, USA) to aid in preliminary formulation of extenders. Osmolality was also determined from 10 μL samples of seminal plasma from koi (n = 20) and goldfish (n = 9).

The effect of osmolality on motility was evaluated using dilution of 2-μL sperm samples with 20 μL of graded test solutions (Bates et al. 1996). The solutions were prepared from C-F

HBSS by use of reagent-grade chemicals (Sigma Chemical Corp.): 8.00 g of NaCl, 0.40 g of KCl, 0.20 g of $MgSO_4 \cdot 7H_2O$, 0.06 g of Na_2HPO_4, 0.06 g of KH_2PO_4, 0.35 g of $NaHCO_3$, 1.00 g of glucose, and sufficient deionized water to yield the desired osmotic pressure. Removal of $CaCl_2 \cdot 2H_2O$ from the standard formulation of HBSS was necessary to avoid a gelatinous condition from forming which suppressed motility as observed in the sperm of razorback sucker *Xyrauchen texanus* (Tiersch et al 1997). Sperm from five koi males were diluted at 1:10 (sperm:C-F HBSS) (305 mOsmol/kg). Test solutions ranged from 50-391 mOsmol/Kg in increments of ~25 mOsmol/kg. Sperm from five goldfish were diluted at 1:7 (sperm:C-F HBSS) (310 mOsmol/kg) prior to the experiment. Test solutions ranged from 18-416 mOsmol/kg in increments of ~20 mOsmol/kg. The osmolality of the sperm activation medium was determined by a 10-μL sample taken immediately after motility estimation from the microscope slide and analyzed by osmometer. Percent motility was used to determine the osmolality at the threshold activation point (defined as 10% motile sperm) and the complete activation point (highest osmotic pressure yielding the highest percent of motile sperm).

Extender Osmolality and Refrigerated Storage

Maintenance of sperm in a non-motile state (allowing activation when diluted in water) for long periods is affected by the osmotic pressure of the surrounding environment. Sperm samples (0.2 mL) from six koi males were aliquotted into 50-mL disposable plastic beakers (B2722-50A, American Scientific Products, Illinois, USA). The extender (C-F HBSS) was prepared at six osmolalities: 209, 245, 270, 308, 357, or 397 mOsmol/Kg and added to the beakers with sperm at a final dilution of 1:7 at a final volume of 1.6 mL. An undiluted control (0.2 mL) was also included for each male. All beakers (42 total) were stored at 4 ^{o}C. Motility was assessed daily until sperm could no longer be activated.

Sperm Dilution and Refrigerated Storage

Sperm dilution with an extender can prolong fertilizing ability through time. Based on the osmolality storage experiment, C-F HBSS with an osmolality of 305 mOsmol/Kg was chosen for this study. Six dilution ratios (1:0, 1:1, 1:3, 1:7, 1:15 and 1:20) were tested. Sperm samples (1.2 mL) from five koi males were collected. A sperm sample (0.2-mL) from each male was added to 4 mL of C-F HBSS (yielding a dilution ratio of 1:20) and was aliquotted into 50-mL disposable plastic beakers. An additional 0.5-mL sample from each male was placed into beakers to serve as undiluted controls. The remaining 0.5 mL of undiluted sperm from each male was used for serial dilutions with C-F HBSS. All beakers were stored at 4 ^{o}C and contained 0.5 mL of sperm-extender medium. Motility was assessed daily until sperm could no longer be activated.

Based on the goldfish activation studies, solutions of C-F HBSS, NaCl, and $NaHCO_3$ were prepared at 310 mOsmol/Kg for use as extenders. The NaCl solution contained 1g of NaCl in 100 mL of deionized H_2O (18 MΩ/ cm; Barnstead Nanopure D4741 ion-exchange system, Dubuque, Iowa). The $NaHCO_3$solution contained 1.5 g of $NaHCO_3$ in 100 mL of deionized H_2O. Sufficient deionized water was added to obtain an osmolality of 310 mOsmol/kg. Sperm (0.1 mL) was aliquotted into 15-mL centrifuge tubes (Baxter Scientific Products, McGaw Park, Illinois, USA; C3920-15). Extender (0.7 mL) was added to centrifuge tubes containing sperm from each of five males. Undiluted sperm (0.1 mL) from each male was used as a control. The first day of storage was designated as “0 d”. The samples were held in a refrigerator at 4 °C, and were mixed by manual inversion at 24-hr intervals. Motility was assessed daily.

Fertilization Capacity with Stored Sperm

Eggs from female koi (n = 2) were hand-stripped into dry, greased bowls and dispensed (0.25 mL) into 100 x 15 mm disposable Petri dishes. One day prior to stripping of eggs, fresh sperm from 6 males was collected and maintained diluted (1:7) with C-F HBSS (306 mOsmol/Kg). On the day of egg collection, fresh sperm was collected undiluted. Sperm-extender media was added undiluted to eggs (0.20 mL) or diluted (0.20 mL) and activated immediately following stripping of eggs. Water (10 mL) at 23 °C was added to initiate motility and activate eggs. Eggs were incubated in a recirculating system at 23 °C. The concentration of spermatozoa of four males was estimated by hemacytometer to be 1.3×10^9 sperm per mL.

Eggs from goldfish (n = 5) were hand stripped immediately following dilution of sperm with extenders (C-F HBSS, NaCl, and $NaHCO_3$), and were placed in 100 x 15 mm disposable Petri dishes. Sperm-extender media (0.02 mL) or undiluted sperm (0.003 mL) was added to 0.1 mL of eggs. Water (10 mL) at 29 °C was added to initiate motility and activate eggs. Sperm-extender media was prepared 30 min prior to stripping of eggs, and was added and activated immediately following collection of eggs. Inseminated eggs were incubated in a recirculating system at 29 °C. The percentage of eyed embryos at 24 h was calculated by dividing the number of eyed eggs (recognizable by black pigmentation) by the total number of eggs in the Petri dish.

Recommended Statistical Analysis

All percent motility values were arcsine-square-root transformed before statistical analysis. The osmotic pressures of blood plasma and seminal fluid were compared using a Student's t-test assuming equal variances (Microsoft Excel 5.0, Microsoft Corp.). In sperm activation with different osmolalities, the threshold activation point was compared to the complete activation point using a paired Student's t-test (Excel 5.0). In the koi osmolality and dilution studies, a repeated measures analysis of variance (SAS 6.10, SAS Institute Inc., Cary, North Carolina, USA) was used to test the effects of osmolality (209, 245, 270, 308, 357, 397 mOsmol/Kg, or undiluted) and dilution (1:0, 1:1, 1:3, 1:7, 1:15 or 1:20) on motility over time. In the goldfish storage study, a repeated measures analysis of variance was used to test the effect of extender (C-F HBSS, NaCl, and $NaHCO_3$) and time on sperm motility. In the goldfish fertilization study, a two-way analysis of variance was used to test the effect of extender (C-F HBSS, NaCl, and $NaHCO_3$) and individual female (n = 5) on percent eyed embryos. For all analyses, Duncan's multiple range test was used to determine if significant differences ($P < 0.05$) existed among treatment means.

Results for Gamete Storage

Osmolality and Sperm Activation

Koi sperm motility decreased as the osmolality of C-F HBSS increased (Figure 1). The threshold activation point (10% motile sperm) occurred at 252 mOsmol/Kg, and the complete activation point (highest observed motility) occurred at 162 mOsmol/kg. Osmolality at complete activation was significantly higher ($\underline{P} < 0.0001$) than at threshold activation. The osmolality that prevented activation was higher than the osmolality of seminal fluid (mean ± SD: 266 ± 19 mOsmol/Kg) but lower than the osmolality of blood plasma (286 ± 7 mOsmol/Kg). The osmotic pressures of these fluids were significantly different ($P < 0.0001$). In the zone of incomplete activation, a reduction of 10 mOsmol/Kg increased motility by ~10%.

Goldfish sperm motility also decreased as osmotic pressure increased (Figure 1). threshold activation occurred at 253 mOsmol/kg, and complete activation occurred at 179 mOsmol/Kg ($P \leq 0.0001$). The osmolality of seminal fluid (253 ± 23 mOsmol/Kg) was lower than the osmolality of the blood plasma (274 ± 5 mOsmol/Kg).

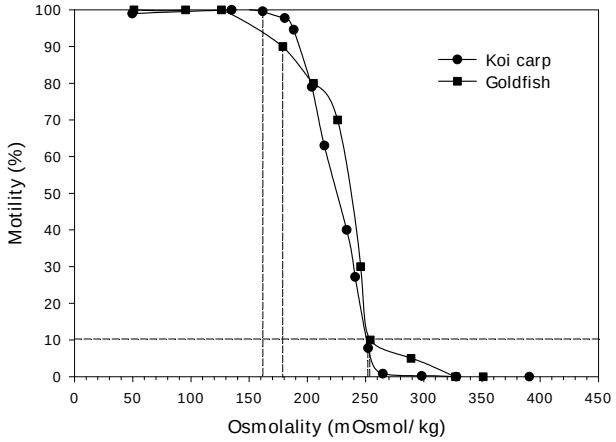

Figure 1. Percent sperm motility of koi (circles) and goldfish (squares) across a range of C-F HBSS osmolalities. Threshold activation is indicated by dashed horizontal lines; complete activation is indicated by dashed vertical lines. Each point represents the mean value for five fish.

Extender Osmolality and Refrigerated Storage

Motility was assessed for 26 d (Figure 2) and the osmolality of C-F HBSS influenced storage time of koi sperm at refrigerated temperatures. At 5 d, the motility of sperm stored at 270, 245, and 308 mOsmol/Kg was significantly higher ($P = 0.0001$) than that of sperm stored undiluted or at 209, 357, or 397 mOsmol/Kg. Samples at 270 mOsmol/Kg maintained motility (>1%) for 19 d. Undiluted sperm lost motility within 3 d.

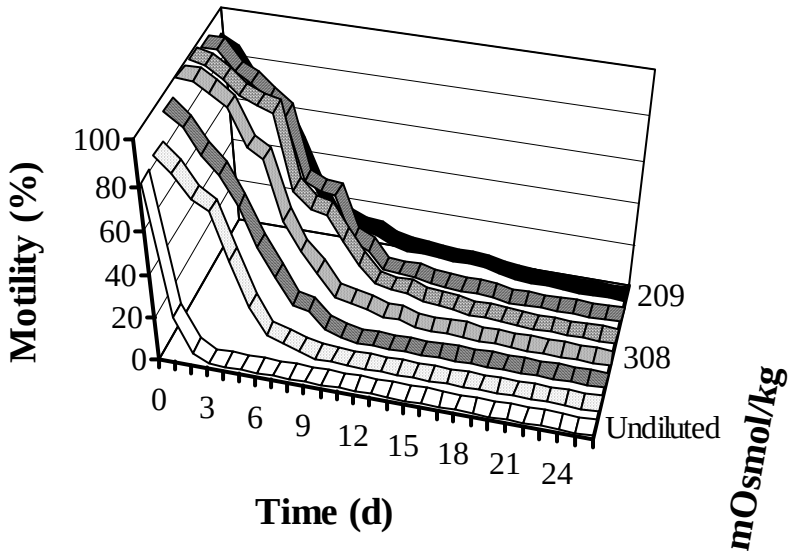

Figure 2. Percent motility of sperm from koi (n = 6) was monitored daily for 26 d. Sperm were stored undiluted or diluted 1:7 in C-F HBSS at six osmolalities. At 5 d, sperm stored at 270, 245, and 308 mOsmol/Kg had significantly higher ($P = 0.0001$) motility.

Sperm Dilution and Refrigerated Storage

Dilution ratios in C-F HBSS did not affect motility of refrigerated koi sperm. Motility was assessed for 12 d (Figure 3), and no significant differences were found over time (P = 0.7640). The dilution ratios of 1:0 and 1:20, although not significantly different, yielded consistently lower motility.

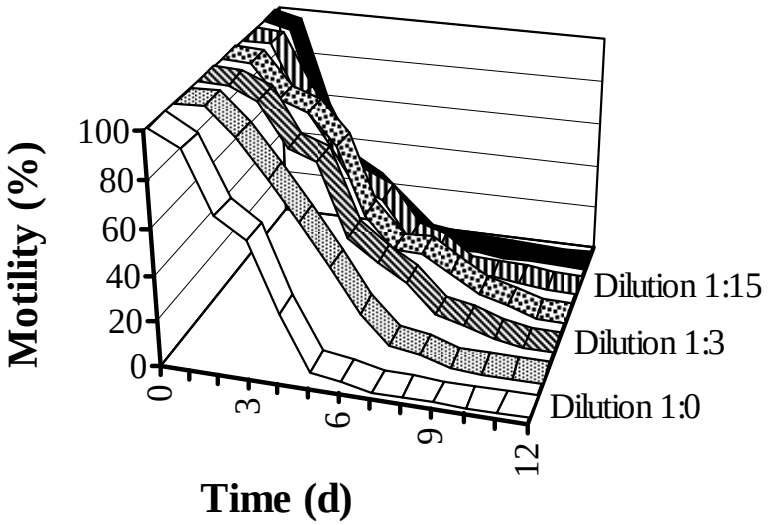

Figure 3. Percent sperm motility of koi (n = 5) was assessed for 12 d after dilution (v:v) in C-F HBSS (305 mOsmol/kg). No significant difference was found among dilutions.

Storage in extenders had a significant effect on goldfish sperm motility (P = 0.0001). Motility of sperm diluted in C-F HBSS at 3 d was 57 ± 52%, although there was much variation at this time: 3 samples had 95% motility and 2 had 0% motility (Figure 4). Motility of sperm diluted in NaCl was 46 ± 32% at 3 d. Motility was not observed in undiluted sperm samples at 4 d. Sperm cells failed to fully suspend in solutions of $NaHCO_3$, and motility ceased within 1 d. No motility occurred in any activated sperm following 6 d.

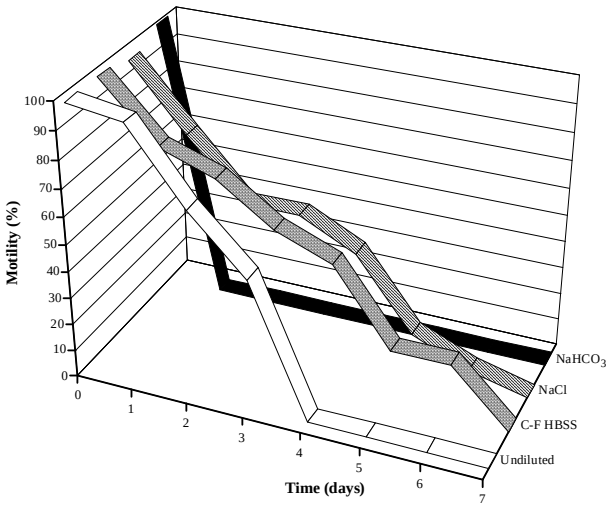

Figure 4. Percent motility for goldfish (n = 5) sperm samples in three extenders (C-F HBSS, NaCl, and $NaHCO_3$) assessed at 24-hr intervals. Samples in C-F HBSS had higher motility after 7 d. Samples in NaCl, $NaHCO_3$, and dry (undiluted) were significantly lower after 7 d.

Fertilization Capacity with Stored Sperm

Fertilization capacity with koi eggs was similar when using undiluted (90 ± 7%) or diluted sperm (92 ± 4%). Fertilization capacity with goldfish eggs was lowest (39 ± 18%) when using sperm diluted with NaCl ($P = 0.0200$). There was no significant difference ($P = 0.1026$) in percent eyed embryos among eggs fertilized with sperm suspended in C-F HBSS (49 ± 21%), $NaHCO_3$ (49 ± 17%), and the undiluted control (48 ± 28%).

Observations for Gamete Storage Procedures

Refrigeration of sperm offers several advantages including use for hybridization and crossbreeding which can be performed in the hatchery. To increase motility and fertilization capacity, an overall evaluation of extenders and their effects on sperm is necessary. As indicated herein, extenders can be evaluated by reviewing the interaction between osmolality and dilution. Blood and seminal plasma can be useful to predict osmolalities at which the extender will maintain non-motile sperm. The osmotic pressure of seminal plasma of koi reported in a previous study was 273 ± 5 mOsmol/Kg (Lubzens et al. 1993). In another study, blood plasma of common carp was reported to be 302 ± 5 mOsmol/Kg and seminal plasma was 302 ± 5 mOsmol/Kg (Morisawa et al. 1983). These values were similar or higher than those from this study, possibly due to different broodstock or environmental influences such as water quality, and indicate that values should be measured for the particular stocks under study.

Sperm activation is an important consideration in the selection and preparation of extenders for sperm storage. Extenders should be prepared at sufficient osmotic pressure to inhibit sperm motility during storage. Sperm of goldfish become motile at spawning because of a reduction of the osmolality (Morisawa et al. 1983). Activation of goldfish sperm should occur at osmotic pressures below that of the seminal plasma (317 mOsmol/Kg). In our study, motility was initiated at osmolalities as high as 271 mOsmol/Kg. The osmolality of extenders prepared for refrigerated storage of goldfish sperm should exceed this value to maintain viability and fertilizing ability over time.

Several studies have reported sperm storage of cyprinids such as the common carp (Sneed and Clemens 1956, Hulata and Rothbard 1979, Saad et al. 1988), silver carp *Hypophthalmichthys molitrix* and bighead carp *Hypophthalmichthys nobilis* (Chen et al. 1992). Various extenders have been used to improve motility and fertilization capacity of refrigerated sperm. Sperm quality is characterized by the ability to fertilize eggs, and subsequent normal development of embryos and fry. Bacterial activity could have reduced sperm motility over time in the present study. Fertilization rates comparable to those of fresh sperm have been maintained in common carp sperm stored at 4 ^{o}C for 16 d with antibiotics and supplemental oxygen (Saad et al. 1988). The present study also show that it is possible to store goldfish sperm in C-F HBSS (310 mOsmol/kg) at 4 °C for as long as 4 d without a significant decrease in motility. Further research is needed to explain why high variation in motility occurred among samples.

Motility of goldfish sperm was retained for 6 d in samples diluted in NaCl, although with a lower rate of fertilization compared to sperm suspended in C-F HBSS, which reduces its value as an extender for extended refrigerated storage. Compensation for low fertilization capacity may be achieved by use of a larger amount of sperm during insemination (Saad et al. 1988). Increased fertilization in common carp was obtained by mixing of sperm and eggs before activation of gametes (Billard et al. 1995), although such methods have not been reported for goldfish. Sperm samples did not remain in suspension in the $NaHCO_3$ solution, making it unsuitable as an

extender. Furthermore, goldfish gamete production occurred in all injected males and 50% of injected females. Intramuscular dosing of metoclopramide and synthetic luteinizing hormone-releasing hormone were used to stimulate gamete production in koi (Rothbard 1994), a method not previously evaluated for goldfish.

Storage of fish sperm in extenders such as C-F HBSS would benefit commercial propagation of koi carp and goldfish. Refrigerated storage is essential for shipping of samples and establishment of cryopreservation programs and germplasm banks for all species. The literature database for this work can be broadly distributed across journals, disciplines and countries. We made our own translations for all of the reviewed studies, but this capability may not be available to all researchers. In addition, a high level of variation in experimental methods and reporting makes direct comparison of results problematic. Thus, decisions on extender choice remain largely empirical for most situations, and currently need to be established on a per-species, per-laboratory basis. This study can provide a general model for short-term gamete storage of freshwater fish species. However, eventual standardization of approach and reporting criteria would greatly facilitate this work and allow cross-laboratory comparisons.

Acknowledgments

This work was supported in part by funding from Louisiana Sea Grant College Program and the United States Department of Agriculture. We thank W. Wayman, R. Malone, and C. Figiel for advice, and J. Huner and L. Beecher for supplying broodstock. We also thank R. Becker of Louisiana Sea Grant for administrative support. Approved by the Director of the Louisiana Agricultural Experiment Station as manuscript number 2011-244-5433.

References

Bates, M. C., W. R. Wayman, and T. R. Tiersch. 1996. Effect of osmotic pressure on the activation and storage of channel catfish sperm. Transactions of the American Fisheries Society 125:798-802.

Belova, N. V. 1981. Ecological-physiological peculiarities of semen of pond carps. II. Change in the physiological parameters of spermatozoids of some carps under the influence of environmental factors. Journal of Ichthyology 21:70-81.

Belova, N. V. 1982. Ecological and physiological properties of the semen of pond cyprinids III. Physiological-biochemical parameters of the semen of some cyprinid species. Journal of Ichthyology 22:63-79.

Bhowmick, R. M. and M. M. Bagchi. 1971. A note on the preservation of sperms of carps. Journal of the Inland Fisheries Society of India 3:119-120.

Billard, R., J. Cosson, G. Perchec and O. Linhart. 1995. Biology of sperm and artificial reproduction in carp. Aquaculture 129:95-112.

Chen, S., X. Liu, D. Lu, L. Zhang and J. Fang. 1992. Short-term preservation of spermatozoa from common carp, silver carp and bighead carp. Freshwater Fisheries 3:3-7 (our translation from Chinese, with English abstract).

Christensen, J. M., and T. R. Tiersch. 1996. Refrigerated storage of channel catfish sperm. Journal of the World Aquaculture Society 27:340-346.

Cognie, F., R. Billard and N. H. Chao. 1989. La cryoconservation de la laitance de la carpe, *Cyprinus carpio*. Journal of Applied Ichthyology 5:165-176 (our translation from French, with English summary).

Drokin, S. I., S. I. Ochkur, B. B. Dzuba and E. F. Kopeika. 1994. New aspects in technology of cryopreservation of sperm of carp and silver carp. *In* High Performance Fish. Proceedings of an International Fish Physiology Symposium. D. D. MacKinlay, editor. Vancouver, Canada. Pp. 358-359.

Glenn III, D. W. 1998. Effect of osmolality, extender and temperature on gamete storage of koi carp (*Cyprinus carpio*) Master's thesis. Louisiana State University, Baton Rouge, Louisiana, USA, 202 pp.

Glenn, D. W. and T. R. Tiersch. 2002. Effect of extenders and osmotic pressure on storage of eggs of ornamental common carp *Cyprinus carpio* at ambient and refrigerated temperatures. Journal of the World Aquaculture Society 33:254-267.

Guest, W. C., J. W. Avault, and J. D. Roussel. 1976. Preservation of channel catfish sperm. Transactions of the American Fisheries Society 105:469-474.

Hulata, G. and S. Rothbard. 1979. Cold storage of carp semen for short periods. Aquaculture 16:267-269.

Jahnichen, H. 1981. Kurzzeitkonservierung von karpfensperma als weitere moglichkeit der rationalisierung der kunstlichen vermehrung von karpfen (*Cyprinus carpio* L.). Zeitschrift fur die Binnenfischerei der DDR 28:244-245 (our translation from German).

Kiselev, I. V. 1980. Quality of gametes and factors influencing them prior to fertilization. *In:* Biological Foundations of Fertilization and Incubation of Sticky Fish Eggs. Naukova Dumka Press, Kie. Pp. 150-178 (our translation from Russian).

Kossmann, H. 1973. Versuch zur konservierung des karpfenspermas (*Cyprinus carpio*). Archiv fur Fischereiwissenschaft 24:125-128 (our translation from German, English summary).

Lubzens, E., S. Rothbard and A. Hadani. 1993. Cryopreservation and viability of spermatozoa from the ornamental Japanese carp (nishikigoi). Israeli Journal of Aquaculture/Bamidgeh 45:169-174.

Magyary, I., R. Szabo, T. Kovacs and L. Horvath. 1991. Effect of different Na-citrate concentrations on motility of common carp sperm. Halaszat 3:142-144 (our translation from Hungarian, with English summary).

Moczarski, M. 1973. Trials with preservation of carp semen. Roczniki Nauk Rolniczych 73-82 (our translation from Polish, with English summary).

Morisawa, M., K. Suzuki, H. Shimizu, S. Morisawa, and K. Yasuda. 1983. Effects of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. Journal of Experimental Biology 107:95-103.

Musselius, V. A. 1951. How to store the fingerlings of carp and determine their quality. Rybnoe Khozyayistvo 8:51-53 (our translation from Russian).

Nedovesova, Z. P. 1983. Behavior of nucleic acids of the spermatozoids in bream *Abramis brama* L., and carp, *Cyprinus carpio* L., in ejaculates stored *in vitro* and during the ontogenesis of the males. Hydrobiological Journal 19:61-66.

Paniagua-Chavez C., J. Buchanan and T. R. Tiersch. 1998. Effect of extender solutions and dilution on motility and fertilizing ability of eastern oyster sperm. Journal of Shellfish Research 17:231-237.

Rana, K. 1995. Preservation of gametes. *In* Broodstock Management and Egg and Larval Quality. N. R. Bromage and R. J. Roberts, editors. Blackwell Science, Inc., Cambridge, Massachusetts, USA. Pp. 53-75.

Redondo-Muller, C., M. P. Cosson, J. Cosson and R. Billard. 1991. *In vitro* maturation of the potential for movement of carp spermatozoa. Molecular Reproduction and Development 29:259-270.

Rothbard, S. 1994. Cloning of Nishiki-goi, Japanese ornamental (koi) carp. Israeli Journal Aquaculture-Bamidgeh 46:171-181.

Rothbard, S., I. Rubinshtein and E. Gelman. 1996. Storage of common carp, *Cyprinus carpio* L., eggs for short durations. Aquaculture Research 27:175-181.

Roubaud, P., C. Gillet and R. Billard. 1984. Influence du pH du milieu au cours de la fecondation sur la survie embryonnaire de la carpe commune (*Cyprinus carpio*). Canadian Journal of Zoology 62:851-861 (our translation from French, English abstract).

Saad, A., R. Billard, M. C. Theron and M. G. Hollebecq. 1988. Short-term preservation of carp (Cyprinus carpio) semen. Aquaculture 71:133-150.

Sneed, K. E. and H. P. Clemens. 1956. Survival of fish sperm after freezing and storage at low temperatures. Progressive Fish-Culturist 18:99-103.

Tiersch, T. R., W. R. Wayman, C. R. Figiel Jr., O. T. Gorman, J. H. Williamson and G. J. Carmichael. 1997. Field collection, handling, and storage of sperm of the endangered razorback sucker. North American Journal of Fisheries Management 17:167-173.

Wayman, W. R., R. G. Thomas and T. R. Tiersch. 1996. Cryopreservation of sperm of spotted seatrout (*Cynoscion nebulosus*). Gulf Research Reports 9:183-188.

Wayman, W. R., R. G. Thomas and T. R. Tiersch. 1997. Refrigerated storage and cryopreservation of black drum (*Pogonias cromis*) spermatozoa. Theriogenology 47:1519-1529.

Withler, F. C. 1980. Chilled and cryogenic storage of gametes of Thai carps and catfishes. Canadian Technical Report of Fisheries and Aquatic Sciences 948:1-15.

Zell, S. R., M. H. Bamford and H. Hidu. 1979. Cryopreservation of spermatozoa of the American oyster, *Crassostrea virginica* Gmelin. Cryobiology 16:448-460.

Appendix. Review of reports addressing refrigerated storage of sperm of common carp *Cyprinus carpio*. Abbreviations: EG, ethylene glycol; DMSO, dimethyl sulfoxide; G, glycerol; PG, propylene glycol.

Extender[1]	Dilution (sperm: extender)	Temperature (^{o}C)	Storage Time (hr)	Comments	Reference
not specified	not specified	0-2, 2-6	200	Motility at 150 h was 100% (0-2 ^{o}C) and 0% (2-6 ^{o}C). Fertilization was >50%. Control had 27% fertilization.	Musselius 1951
FR, PB with 6% glycerin, sodium citrate dihydrate with 6% glycerin	not specified	3-5	720	Stored in 1-mL vials. Decline in motility after 10 d. Phosphate and Na-citrate had motility for >1 wk at 3 ^{o}C. Penicillin (1000 U/mL) interfered with motility.	Sneed and Clemens 1956
egg-yolk-citrate, sodium citrate, PB, Holtfreter's solution plus 1% glycerin, FR plus 1% glycerin	not specified	0-5, 28-33	72	Thawed at room temperature for 5-10 min before estimating motility. Three extenders were unsatisfactory. Sperm in Holtfreter's solution motile for 50 hr at 0 ^{o}C. In water, sperm were motile for 2 min. Sperm in frog Ringer's motile for 72 h at 0-5 ^{o}C. At 28-33 ^{o}C, sperm in frog Ringer's solution were motile for 4.5-6 hr.	Bhowmick and Bagchi 1971
sodium phosphate and yolk	not specified	4	144	Sperm activated with Woynarovich solution. Undiluted sperm gelled after 144 hr. Addition of yolk extended motility (70% at 24 hr). Other combinations of extenders did not aid in sperm storage.	Kossmann 1973
A, mC, C, Ringer's fluid, milk-yolk, ethanol, diluent for freezing bull's semen. 5-20% EG, DMSO, G, or PG was added.	1:1	2, 4	552	Sperm stored in ampoules. Sperm stored in Alsever's solution with EG for 1-19 d at 2 ^{o}C.	Moczarski 1973

[1]See Table 1 for list of chemicals.
[2]Koi carp.

Appendix. cont.

Extender[1]	Dilution (sperm: extender)	Temperature (^{o}C)	Storage Time (hr)	Comments	Reference
rinsing solution	5:3	0-5	45	Sperm stored in glass tubes. No difference in fertilization or hatch when stored diluted (91%) or undiluted (91%), or with fresh sperm.	Hulata and Rothbard 1979
glucose-yolk diluent	1:0, 1:1, 1:3	2	10-36	Fertilization similar to control. 90% of fry had abnormalities with stored sperm. Stored at 18-25 ^{o}C undiluted for 40 hr maintained motility longer when activated by 5% glucose or sodium diphosphate (pH 8.4) than water (pH 6.8 or 8.4).	Kiselev 1980
not specified	not specified	2-9, ice	3.5	Stored in 1-mL disposable plastic syringes. Motility was 100% at 3.5 hr (2-9 ^{o}C). Motility was 2% at 24 h, For ice, sperm motility was 90% at 2.5 hr.	Withler 1980
not specified	not specified	2-8	216	Motility lasted 180 h. At 168 hr, fertilization (82%) and hatch (75%). Sperm volume:egg number was 200-250,000:1.	Belova 1981
not specified	not specified	2-5	24-48	Stored in beakers and activated by water and Woynarovich solution. No difference in motility or fertilization between cooled and fresh sperm. .	Jahnichen 1981
not specified	not specified	6-8, 2-4	10-12, 120	Significant hydration in semen for prolonged storage. After storage, reduction of fat and protein in the dry matter and change in the ratio of lipid fractions.	Belova 1982

[1]See Table 1 for list of chemicals.
[2]Koi carp

Appendix. cont.

Extender[1]	Dilution (sperm: extender)	Temperature (°C)	Storage Time (hr)	Comments	Reference
not specified	not specified	4-6	24	During storage, ribonucleic acid content decreased and deoxyribonucleic acid content remained the same.	Nedovesova 1983
saline solution	1:10	4	480	Stored in 5-mL aliquots in 50-mL flasks (0.5 cm thick). Washed or centrifuged before adding extender. Motility without dilution in antibiotics was 0% at 6-8 d. Motility and fertilization with dilution and antibiotics was ~100% at 8 d. Variability was high beyond 8 d. Oxygen improved fertilization for 6 d. Hatch from stored sperm (15 d) not different from fresh sperm. Dilution did not improve motility or fertilization. Extender was used to activate gametes (2.63 g NaCl, 0.37 g KCl, and 2.42 g Tris, per L, pH 8.0). Sperm volume:egg number was 10^{-2} to 10^{-8}:200.	Saad et al. 1988
solution with Na-citrate or NaCl	1:10	0-4	Na-citrate reactivated at 198; NaCl at 15	Higher concentrations of both extenders were used for storage. Na-citrate provided longer duration of motility and storage.	Magyary et al. 1991
solution with KCl and Tris HCl	1:150	2	10	Dealt with potential to move, not motility directly. Seminal fluid was unable to maintain motility. Motility was optimum between 3.73-14.91 g/L KCl. Similar results with NaCl media at 7.31-8.77 g/L.	Redondo-Muller et al. 1991

[1]See Table 1 for list of chemicals.
[2]Koi carp

Appendix. cont.

Extender[1]	Dilution (sperm: extender)	Temperature (°C)	Storage Time (hr)	Comments	Reference
D-15, D-16, D-17, D-19, D-20, or D-21. DMSO was added at 2, 4, 6, 8, 10, 12, or 14%.	1:1, 1:2, 1:4, or 1:8.	2-4	312	Extender, D-19, and 6-8% DMSO gave best results. Optimal dilution was 1:1. Motility at 12 d was 5-15%.	Chen et al. 1992
not specified	not specified	5-9 (~7), 20	5	Sperm stored in 100-mL glass beakers. No difference in fertilization rates between stored and fresh sperm with either egg storage temperature. Volume of sperm to number of eggs was 0.1 mL:200-400.	Rothbard et al. 1996[2]
C-F HBSS	1:0, 1:1, 1:3, 1:7, 1:15, or 1:20	4	288	Sperm stored in 50-mL disposable plastic beakers. No difference in motilities among dilutions was observed. A dilution of 1:7 was easier to evaluate motilities. An activation curve with C-F HBSS showing 10% motility (252 mOsmol/kg) and 100% motility (162 mOsmol/kg). Different concentrations of C-F HBSS (397, 357, 308, 270, 245, or 209 mOsmol/kg) were evaluated to determine optimum osmolality over time. Extender osmolality of 270, 245, and 308 mOsmol/kg had higher motilities at 5 d (total 26 d). Undiluted control lost motility within 3 d.	Glenn 1998[2]

[1]See Table 1 for list of chemicals.
[2]Koi carp