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ARTICLE

Development of a Sperm Cryopreservation Protocol for Redside Dace: Implications for Genome Resource Banking

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Abstract

Populations of Redside Dace *Clinostomus elongatus* have declined in many areas across the species' North American range. Therefore, the development of sperm cryopreservation technology would provide an invaluable means of preserving genetic diversity in populations that are in imminent danger of extirpation. We developed cryopreservation protocols by testing the effects of diluent (buffered sperm motility-inhibiting saline solution [BSMIS]; BSMIS + glycine; sucrose; and Hanks' balanced salt solution [HBSS]), cryoprotectant (dimethyl sulfoxide [DMSO]; propylene glycol [PG]; N,N-dimethylacetamide [DMA]; and methanol), freezing rate (1, 5, and 10°C/min), and male-to-male variation on sperm quality. Incubating sperm in extenders affected motility; BSMIS + glycine + methanol, BSMIS + glycine + PG, and HBSS + methanol were the only treatments for which motility was not significantly different from that of fresh sperm. Sperm frozen with sucrose had higher motility than sperm frozen with BSMIS + glycine, and sperm frozen with DMSO had higher motility than sperm frozen with methanol. Freezing rates were evaluated for BSMIS + glycine, HBSS, and sucrose; all diluents were frozen with DMSO. The effect of freezing rate was not significant for BSMIS + glycine or for HBSS, but an effect was detected for sucrose, with sperm frozen at 5°C/min or 10°C/min having higher motility than sperm frozen at 1°C/min. The effect of extender was not significant at 1°C/min or 5°C/min, but an effect was detected at 10°C/min such that sperm frozen with sucrose had the highest motility. Male-to-male variability was evaluated by using sucrose + DMSO and a freezing rate of 10°C/min. For these males, the sperm motility recovery index ranged from 6.67% to 79.27%, and the sperm velocity recovery index ranged from 21.37% to 57.33%. Our findings demonstrate that cryopreservation of Redside Dace sperm in a sucrose + DMSO extender at a freezing rate of 10°C/min is adequate for preserving genetic diversity via sperm banks.

The Redside Dace *Clinostomus elongatus* is a small cyprinid fish that is native to headwater streams in the basins of the Mississippi River and Lakes Erie, Huron, Michigan, and Ontario (Parker et al. 1988). Redside Dace typically occur in pools and slow-flowing sections of relatively small streams (stream width of 1–10 m and depth of 0.3–2.0 m) with clear, flowing water and overhanging flora (McKee and Parker 1982; Parker et al. 1988). These fish live up to 4 years of age, reaching sexual

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maturity in their second or third year (Parker et al. 1988; COSEWIC 2007). Female Redside Dace grow faster and are generally larger than males of a given age (Koster 1939; Schwartz and Norvell 1958; McKee and Parker 1982). Redside Dace have highly conspicuous, ornamental red stripes on their sides; the stripes are present year-round but become more intense during the spawning season, with males being more ornamented than females (Beausoleil et al. 2012).

Redside Dace are external fertilizers and exhibit a nonresource-based mating system (i.e., males provide no parental care; Pitcher et al. 2009). Spawning occurs in gravel riffles between mid-May and early June (Pitcher et al. 2009; Beausoleil et al. 2012). During the spawning season, females have been reported to carry between 409 and 1,971 eggs (Koster 1939; Parker et al. 1988), which are normally deposited in the nests of cooccurring minnow species such as the Common Shiner *Luxilus cornutus* and Creek Chub *Semotilus atromaculatus* (COSEWIC 2007). During a spawning event, sperm competition is intense, as several males release their sperm in an attempt to fertilize a single clutch of eggs (Beausoleil et al. 2012).

The Redside Dace was once an abundant native member of the fish community in many Great Lakes tributaries and played a key ecological role as a bio-indicator of ecosystem health in these important freshwater ecosystems (COSEWIC 2007). However, Redside Dace populations have declined in many areas across the species' North American range. For instance, the Redside Dace is listed as endangered in Canada (COSEWIC 2007) and in the states of Indiana (IDNR 2002) and Michigan (Goforth 2000), and it is a species of special concern in Wisconsin (Lyons et al. 2000). Thus, there is a need for protocols that can preserve unique genetic diversity in Redside Dace populations that are in imminent danger of being extirpated. Currently, protocols are not available for the cryogenic storage of fish eggs; therefore, cryopreservation of fish germplasm relies solely on paternal DNA. Ultimately, these protocols could enable fisheries managers to create germplasm repositories (i.e., sperm banks) and perform "genetic rescue," wherein genetically unrelated individuals from a larger population are infused into small, isolated populations that have inevitably lost genetic variability through inbreeding depression (Pimm et al. 2006; Fickel et al. 2007).

Sperm cryopreservation involves a series of chronological steps, each of which may cause cellular damage (Tiersch 2011). To successfully cryopreserve sperm for a given species, milt is typically diluted in a solution composed of salts and organic materials that inhibit motility (Suquet et al. 2000). For freezing the sperm of cyprinids, the best results thus far have been obtained when sperm were diluted in (1) Hanks' balanced salt solution (HBSS; Yang et al. 2007; Robles et al. 2009), (2) buffered sperm motility-inhibiting saline solution (BSMIS) + glycine (Lahnsteiner et al. 2000), or (3) sucrose (Babiak et al. 1998). In addition to a diluent, the freezing solutions (hereafter, "extenders") must also contain a liquid cryoprotective agent that reduces cell damage during the freezing and thawing process. Dimethyl sulfoxide (DMSO; Lahnsteiner et al. 2000) and methanol (Urbányi et al. 2006; Yang et al. 2007) are two of the most commonly used cryoprotectants for freezing the sperm of cyprinids. Propylene glycol (PG) and N,N-dimethylacetamide (DMA) are cryoprotectants that have been effective for freezing sperm from other fishes (Butts et al. 2010, 2011b; Boryshpolets et al. 2011).

Development of a successful cryopreservation procedure not only depends on the correct extender composition but also on the freezing protocol. For freezing sperm, a two-step procedure is generally applied: (1) spermatozoa are loaded into cryogenic straws and floated above liquid nitrogen on a styrofoam raft (Rideout et al. 2004) or placed into a programmable freezing unit and (2) the straws are then plunged directly into liquid nitrogen (DeGraaf and Berlinsky 2004; Butts et al. 2010, 2011a, 2011b). The use of a programmable freezing unit is advantageous because it ensures the same freezing rates and provides consistent results (Conget et al. 1996). Three-step procedures can also be programmed into freezing units, allowing specific freezing rates to be controlled at critical intervals (i.e., initial cooling plus supercooling, warming by the release of heat from fusion; Jamieson 1991). Finally, rapid thawing is required to avoid recrystallization (Suquet et al. 2000). Thawing rates used for fish have ranged from 1°C/min to 40°C/min (Bolla et al. 1987; Yao et al. 1995).

The goal of the present study was to develop a cryopreservation protocol for Redside Dace sperm. Specific objectives were to test the effects of extender toxicity, extender composition, freezing rate, and male-to-male variation on postthaw sperm quality (i.e., motility and velocity). The information gained from this work will assist in the creation of germplasm repositories and will aid in conserving biodiversity of Redside Dace populations that are in imminent danger of extirpation.

METHODS

Fish and gamete collection.—Redside Dace were caught during May 2011 and May 2012 by using a nylon seine (1.22 m × 3.05 m × 6.35 mm; Mid-Lakes Corp., Knoxville, Tennessee). The fish were collected from Rathburn Run in Wayne County, Ohio (40°48′39.4914″N, 82°1′23.988″W), and Paint Creek in Big Prairie, Ohio (40°36′2.6994″N, 82°3′34.0194″W). Mean TL ± SE of the fish ($n = 30$ males) at the time of capture was 8.3 ± 0.1 cm. Water temperature was 15–18°C during capture efforts. Upon capture, Redside Dace were immediately placed into submerged minnow traps (25.40-cm diameter × 35.56-cm length). Within 5 min, fish were removed from the traps and wiped dry with Kimwipes. Slight pressure was applied to the abdomen of each male, and milt was collected with a micropipette (set at 15 μL) as it was released from the vent. Extra care was taken to ensure that milt was not contaminated with blood, urine, or feces. Milt samples were then micropipetted into 1,000-μL microcentrifuge tubes and were held in a cooler filled with crushed ice and frozen ice packs. Temperature in the cooler was

approximately 8°C. Fish were measured for TL (±0.1 cm) and then released.

Sperm quality assessment.—Milt (<0.2 μL) was micropipetted into a chamber of a 2X-CEL glass slide (Hamilton Thorne Biosciences, Beverly, Massachusetts) and was covered with a coverslip (22 × 22 mm). Sperm were then activated with 15 μL of sperm activation medium consisting of 150-mOsmol/kg NaCl + 20-mM tris buffer adjusted to pH 8 and kept at 8°C. Sperm were video-recorded by using a black-and-white video camera (charge-coupled device [CCD], Model XC-ST50; Sony, Japan) module at 50-Hz vertical frequency; the camera was mounted on an external phase contrast microscope (Model CX41; Olympus, Melville, New York) with a 10× negative-phase magnification objective (Butts et al. 2012). Once recorded, sperm traits were analyzed by using the HTM-CEROS version 12 sperm analysis system (Hamilton Thorne Biosciences) set at the following parameters: number of frames = 60; minimum contrast = 11; minimum cell size = 3 pixels; and photometer = 55–65. To remove the potential effect of drift, sperm cells that had an average path velocity less than 20 μm/s and a straight-line velocity less than 10 μm/s were considered to be static and were excluded from the analyses (Rudolfsen et al. 2008). Postthaw sperm motility has been related to fertilization success in cyprinids (Urbányi et al. 2006); in Redside Dace competition trials, males with faster-swimming sperm fertilized more eggs (Beausoleil et al. 2012). Therefore, curvilinear velocity (hereafter, "sperm velocity") and the percentage of motile sperm were analyzed at 10 s postactivation. Each video recording was manually checked for quality control. Sperm tracks were removed from analyses if the software incorrectly combined the crossing tracks of multiple sperm, if the software split the track of a single sperm, or if a sperm swam out of the field of view before it could be adequately assessed.

Experiment 1: toxicity of extenders.—Each of three replicates in experiment 1 consisted of a pooled milt sample from four Redside Dace males. The toxicity of diluents and cryoprotectants that have been used successfully for other cyprinids (Babiak et al. 1998; Lahnsteiner et al. 2000; Urbányi et al. 2006; Yang et al. 2007; Robles et al. 2009) were tested in a full factorial design. Four diluents were evaluated: (1) BSMIS, which consisted of 75-mM NaCl (Product S-2830; ACP, Montreal, Quebec), 70-mM KCl (P-2940; ACP), 2-mM $CaCl_2$ (Product 1-1310; J. T. Baker, Phillipsburg, New Jersey), 1-mM $MgSO_4$ (M-0900; ACP), and 20-mM tris buffer (Product BP152-1; Fisher Scientific, Fair Lawn, New Jersey); (2) BSMIS + 0.5% glycine (weight/volume [w/v]; A-3637; ACP); (3) 300-mM sucrose (Product S-9378; Sigma-Aldrich, St. Louis, Missouri) + 20-mM tris buffer (hereafter, "sucrose"); and (4) HBSS (H8264; Sigma-Aldrich) + 20-mM tris buffer (hereafter, "HBSS"). All diluents were adjusted to pH 8.0, which is the pH of Redside Dace seminal plasma (osmolality = 276.5 mOsmol/kg; data not shown). Four cryoprotectants were evaluated: (1) DMSO (D-6400; ACP), (2) PG (P355-1; Fisher Scientific), (3) DMA (D137510; Sigma-Aldrich), and (4) methanol (9093-03; J. T. Baker). Diluents (3,600 μL) and cryoprotectants (400 μL) were mixed in disposable, 12- × 75-mm culture tubes (Fisher Scientific) and were kept at 8°C. Once homogeneous, these extender solutions (20 μL) were slowly micropipetted into 1,000-μL microcentrifuge tubes containing milt (2 μL) and then were mixed by flicking the tubes for 10–15 s. Sperm were allowed to incubate with each extender for 5 min at 8°C. Mean sperm activity for each of the three experimental replicates was used for statistical analyses; fresh sperm served as the control.

Experiment 2: effects of extender composition on postthaw sperm quality.—For experiment 2, milt was collected from nine Redside Dace. Males were not pooled; thus, each fish served as an experimental replicate. Diluents (3,600 μL) and cryoprotectants (400 μL) were mixed in disposable, 12- × 75-mm culture tubes and were held at 8°C. Using a full factorial design, milt samples (2.0 μL) were micropipetted into 1,000-μL microcentrifuge tubes and were diluted with each possible combination of diluent (BSMIS, BSMIS + glycine, HBSS, and sucrose) and cryoprotectant (DMSO, PG, and methanol) at a sperm-to-extender ratio of 1:10, resulting in a total of 12 different treatment combinations. The DMA had toxic effects on sperm quality and thus was excluded from further experimentation (refer to experiment 1 results). Diluted milt samples were drawn up into 2,500-μL cryogenic straws (Minitube Canada, Ingersoll, Ontario) by using a Microclassic micropipettor (Minitube Canada), and the straws were sealed with metal sealing beads (Minitube Canada). An equilibration time of approximately 3 min was allotted, as this was the time required for two researchers to fill nine straws and load them into the cryogenic freezer (Model CL-8800i; Biogenics, Inc., Napa, California) equipped with a 23-slot cryochamber (Model CC23F; Biogenics) maintained inside a cryobath (Biogenics). Cryogenesis version 5 (Biogenics) was used to freeze samples at a rate of 10°C/min from 10°C to −20°C and then at a rate of 5°C/min from −20°C to −120°C. Once samples reached −120°C, they were plunged directly into a liquid nitrogen Dewar and were kept frozen for 24 h. To evaluate postthaw sperm quality traits, straws were transferred from the liquid nitrogen into a temperature-controlled water bath (40.64- × 20.32- × 25.40-cm aquarium with heater) and were thawed for 7 s at 30°C. Straw tips were cut off and the contents were released into disposable, 1,000-μL microcentrifuge tubes. Postthaw activity of each sample was recorded within 1 min after thawing. For each straw, one activation trial was conducted to evaluate sperm motility and velocity as described above. Fresh sperm served as the control.

Experiment 3: effects of freezing rate on postthaw sperm quality.—Milt was collected from four Redside Dace for use in experiment 3. Sperm from each male was diluted (see methods above) into the top-three postthaw extender combinations (refer to experiment 2 results; BSMIS + glycine + DMSO; sucrose + DMSO; and HBSS + DMSO) at a sperm-to-extender ratio of 1:10. Each extender combination was frozen at a rate of 1, 5, or 10°C/min from 10°C to −20°C and then was frozen at 5°C/min from −20°C to −120°C. Once samples reached

−120°C, they were plunged directly into a liquid nitrogen Dewar and were kept frozen for 24 h. The thawing of samples and the evaluation of postthaw sperm activity were conducted according to the methods described for experiment 2.

Experiment 4: male-to-male variation in postthaw sperm quality.—Milt used in experiment 4 was collected from 22 Redside Dace. Sperm from each male was diluted in an extender composed of sucrose + DMSO at a 1:10 sperm-to-extender ratio and was frozen at a rate of 10°C/min from 10°C to −20°C and then at a rate of 5°C/min from −20°C to −120°C (see methods above). Thawing of the samples and evaluations of postthaw sperm activity were again performed according to the methods used in experiment 2.

Statistical analyses.—All data were analyzed using Statistical Analysis System (SAS) software version 9.1 (SAS 2003). Residuals were tested for normality (Shapiro–Wilk test; UNIVARIATE procedure in SAS; SAS 2003) and homogeneity of variance (plot of residuals versus predicted values; GPLOT procedure in SAS). When necessary, the data were transformed to meet assumptions of normality and homoscedasticity. The Kenward–Roger procedure was used to approximate the denominator df for all *F*-tests (Spilke et al. 2005). Alpha was set at 0.05 for main effects and interactions.

One-way ANOVA models were first run to compare extender toxicity and postthaw sperm quality of the cryopreservation treatment combinations with those of the fresh sperm samples. Treatment means were contrasted using the least-squares means method (LSMEANS/CL adjust = DUNNETT, MIXED procedure in SAS).

To further our understanding of the biological and physiological interactions involved in the cryopreservation process, we examined the effects of cryopreservation treatment (i.e., diluent, cryoprotectant, and freezing rate) on postthaw sperm activity by using a series of two-way ANOVA models (MIXED procedure in SAS). For experiments 1 and 2, the ANOVA models contained the source variables of (1) diluent, (2) cryoprotectant, and (3) the diluent × cryoprotectant interaction term. For experiment 3, the ANOVA models contained the source variables of (1) extender, (2) freezing rate, and (3) the extender × freezing rate interaction term. If the first-order interaction term was significant, the two-way ANOVA models were decomposed into a series of one-way ANOVAs. For experiments 1 and 2, the decomposed ANOVA models were run to determine the effect of cryoprotectant at each level of diluent. For experiment 3, ANOVA models were run to determine (1) the effect of freezing rate at each level of extender and (2) the effect of extender at each level of freezing rate. If the first-order interaction term was not significant, the main effects were interpreted. Diluent, cryoprotectant, and freezing rate were considered fixed factors, whereas male was considered a random factor. Treatment means were contrasted using the least-squares means method (LSMEANS/CL adjust = TUKEY, MIXED procedure in SAS). For experiment 4, mean ± SE values for fresh and postthaw sperm quality are reported for the 22 males. Additionally, sperm motility and velocity values were expressed as motility recovery and velocity recovery indices, calculated as [(postthaw sperm motility or velocity)/(fresh sperm motility or velocity)] × 100.

RESULTS

Experiment 1: Toxicity of Extenders

Incubation of the sperm in the 16 different extender combinations had a significant effect on sperm motility (one-way ANOVA: $F_{16, 30} = 16.25$, $P < 0.0001$). Mean motility ± SE of fresh sperm (91.00 ± 6.28%) was not significantly different from the mean motility for the BSMIS + glycine + methanol (76.50 ± 7.69%), BSMIS + glycine + PG (75.00 ± 6.28%), and HBSS + methanol (66.33 ± 6.28%) treatments. The two-way ANOVA model revealed a significant diluent × cryoprotectant interaction effect for sperm motility ($F_{9, 28} = 3.03$, $P = 0.012$). The ANOVA was therefore decomposed to determine the effect of cryoprotectant at each level of diluent. A significant effect was detected for all decomposed ANOVA models (one-way ANOVAs: all $F \geq 7.65$, $P \leq 0.013$). The cryoprotectant DMA resulted in the lowest sperm motility for all four diluents (Table 1). No significant differences in motility were detected among the cryoprotectants DMSO, methanol, and PG for the diluents BSMIS, BSMIS + glycine, and sucrose (Table 1). Sperm incubated in methanol had the highest motility for the HBSS diluent (Table 1).

Incubation of sperm in the extenders had a significant effect on sperm velocity (one-way ANOVA: $F_{16, 30} = 12.92$, $P < 0.0001$). Hanks' balanced salt solution + methanol (mean velocity ± SE = 135.87 ± 11.60 μm/s), sucrose + methanol (125.70 ± 11.60 μm/s), and sucrose + PG (113.17 ± 11.60 μm/s) were the only extender combinations for which sperm velocity did not significantly differ from that of fresh sperm (163.10 ± 11.60 μm/s). The two-way ANOVA model revealed a significant diluent × cryoprotectant interaction effect for sperm velocity ($F_{9, 28} = 3.34$, $P = 0.007$). The ANOVA was therefore decomposed to determine the effect of cryoprotectant at each level of diluent. When the models were decomposed, a significant effect was detected for the diluents BSMIS (one-way ANOVA: $F_{3, 7} = 38.65$, $P < 0.0001$), sucrose (one-way ANOVA: $F_{3, 7} = 4.42$, $P = 0.048$), and HBSS (one-way ANOVA: $F_{3, 6} = 60.07$, $P < 0.0001$), while a nonsignificant effect was detected for BSMIS + glycine (one-way ANOVA: $F_{3, 6} = 2.69$, $P = 0.139$). For BSMIS, the cryoprotectant DMA produced the lowest sperm velocity, whereas no significant differences were detected among the cryoprotectants DMSO, methanol, and PG (Table 1). When sucrose was used as the diluent, DMSO, methanol, and PG were not significantly different and produced the highest sperm velocities (Table 1). For the HBSS diluent, DMA resulted in the lowest sperm velocity and methanol produced the highest sperm velocity (Table 1).

Experiment 2: Effects of Extender Composition on Postthaw Sperm Quality

Postthaw motility was zero for sperm in extenders that contained BSMIS or PG. Therefore, BSMIS and PG were excluded

TABLE 1. Effects of extender toxicity on the motility and velocity of Redside Dace sperm. The toxicity of diluents and cryoprotectants was tested in a full factorial design. Due to higher-order interactions, one-way ANOVA models were run to determine the effect of cryoprotectant at each level of diluent. Least-squares means (±SE) are reported. For a given diluent, cryoprotectant means without a letter in common are significantly different ($P < 0.05$). For fresh sperm, mean motility was 91.00% and mean velocity was 163.10 μm/s. Diluents are buffered sperm motility-inhibiting saline solution (BSMIS; Lahnsteiner et al. 2000); BSMIS + 0.5% glycine; Hanks' balanced salt solution (HBSS) + 20-mM tris buffer; and 300-mM sucrose + 20-mM tris buffer. Cryoprotectants are N,N-dimethylacetamide (DMA), 99%; dimethyl sulfoxide (DMSO); methanol; and propylene glycol (PG).

Diluent	Cryoprotectant	Motility	Velocity
BSMIS	DMA	0.00 ± 0.00 z	0.00 ± 0.00 z
	DMSO	56.00 ± 11.94 y	86.15 ± 9.47 y
	Methanol	48.67 ± 9.75 y	111.53 ± 7.74 y
	PG	58.33 ± 9.75 y	82.17 ± 7.74 y
BSMIS + glycine	DMA	27.50 ± 6.78 z	78.95 ± 9.62 z
	DMSO	58.33 ± 5.54 y	86.00 ± 7.86 z
	Methanol	76.50 ± 6.78 y	102.15 ± 9.62 z
	PG	75.00 ± 5.54 y	109.57 ± 7.86 z
HBSS	DMA	0.00 ± 0.00 z	0.00 ± 0.00 z
	DMSO	49.50 ± 3.85 y	89.75 ± 8.94 y
	Methanol	66.33 ± 3.15 x	135.87 ± 7.30 x
	PG	45.67 ± 3.15 y	86.87 ± 7.30 y
Sucrose	DMA	9.67 ± 5.72 z	82.35 ± 11.30 z
	DMSO	56.00 ± 5.72 y	87.83 ± 9.23 zy
	Methanol	36.33 ± 5.72 y	125.70 ± 9.23 y
	PG	46.00 ± 5.72 y	113.17 ± 9.23 y

from further statistical analyses. Mean motility ± SE of fresh sperm (73.28 ± 6.42%) was significantly higher than the postthaw motility of sperm from all treatment combinations (one-way ANOVA: $F_{6, 48.1} = 44.18$, $P < 0.0001$). Postthaw sperm motility ranged from 5.72 ± 6.94% for BSMIS + glycine + methanol to 40.67 ± 6.25% for sucrose + DMSO. For postthaw sperm motility, the diluent × cryoprotectant interaction term was not significant (two-way ANOVA: $F_{2, 35.1} = 1.84$, $P = 0.174$), but there was a significant effect of diluent (two-way ANOVA: $F_{2, 34.6} = 3.85$, $P = 0.031$) and cryoprotectant (two-way ANOVA: $F_{2, 35.3} = 80.04$, $P < 0.0001$). Sperm that were frozen with sucrose as the diluent had significantly higher motility than sperm frozen with BSMIS + glycine, and sperm that were frozen with DMSO as the cryoprotectant had significantly higher motility than sperm frozen with methanol (Figure 1A).

For postthaw sperm velocity, extenders that contained methanol were excluded from further statistical analysis due to low numbers of motile sperm (see motility results above); therefore, data were analyzed by using a one-way ANOVA. Mean velocity ± SE of fresh sperm (140.69 ± 5.32 μm/s) was significantly higher than the velocities observed for all postthaw treatment combinations (one-way ANOVA: $F_{3, 28} = 67.57$, $P < 0.0001$; Figure 1B).

Experiment 3: Effects of Freezing Rate on Postthaw Sperm Quality

Mean motility ± SE of fresh sperm (66.94 ± 9.07%) was significantly higher than the means from all freezing rate treatment combinations (one-way ANOVA: $F_{9, 27} = 8.94$, $P < 0.0001$). Postthaw motility ranged from 4.69 ± 9.07% for sperm frozen with sucrose at a freezing rate of 1°C/min to 41.69 ± 9.07% for sperm frozen with sucrose at a rate of 10°C/min. The two-way ANOVA model revealed a significant extender × freezing rate interaction effect for postthaw sperm motility ($F_{4, 22.1} = 3.76$, $P = 0.018$). The model was therefore decomposed to determine the effect of freezing rate at each level of extender and the effect of extender at each level of freezing rate. When decomposed at each level of extender, the effect of freezing rate was not significant for BSMIS + glycine (one-way ANOVA: $F_{2, 6} = 1.93$, $P = 0.226$) or for HBSS (one-way ANOVA: $F_{2, 8} = 2.61$, $P = 0.134$). However, a significant effect was detected when sperm were frozen with sucrose (one-way ANOVA: $F_{2, 5.17} = 13.73$, $P = 0.009$) such that freezing rates of 5°C/min and 10°C/min produced higher postthaw motility than a freezing rate of 1°C/min (Figure 2A). When decomposed at each level of freezing rate, the effect of extender was not significant at 1°C/min (one-way ANOVA: $F_{2, 5.37} = 1.62$, $P = 0.282$) or 5°C/min (one-way ANOVA: $F_{2, 4.74} = 1.34$, $P = 0.347$), but a significant effect was detected at 10°C/min, as sucrose yielded the highest postthaw sperm motility (Figure 2B).

Mean velocity ± SE of fresh sperm (126.09 ± 5.55 μm/s) was significantly higher than the mean postthaw velocities observed for all treatment combinations (one-way ANOVA: $F_{9, 26} = 17.20$, $P < 0.0001$). Postthaw velocity ranged from 45.38 ± 5.50 μm/s for sperm frozen with BSMIS + glycine at a rate of 1°C/min to 65.60 ± 6.41 μm/s for sperm frozen with HBSS at a rate of 5°C/min. For postthaw sperm velocity, the extender × freezing rate interaction was not significant

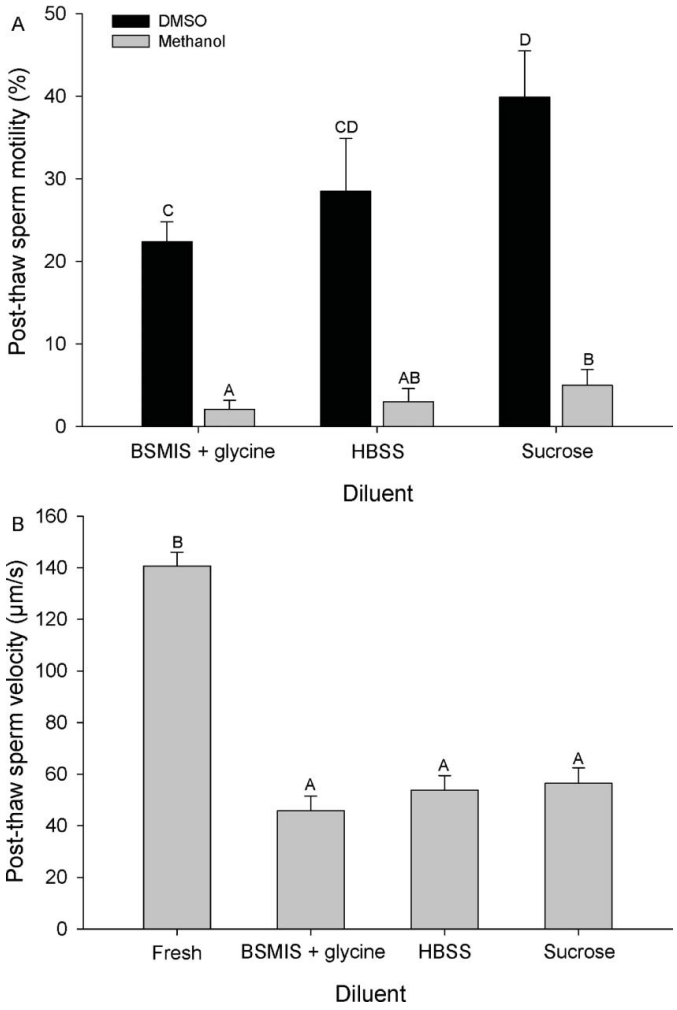

FIGURE 1. Effects of extender composition on postthaw sperm quality for Redside Dace ($n = 9$ males): **(A)** results of a two-way ANOVA for postthaw sperm motility; and **(B)** results of a one-way ANOVA for postthaw sperm velocity. Least-squares means (+SE) are reported. Treatment means without a letter in common were significantly different ($P < 0.05$). For fresh sperm, mean motility was 73.28% and mean velocity was 140.69 µm/s. Diluents are buffered sperm motility-inhibiting saline solution (BSMIS) + 0.5% glycine (weight/volume; Lahnsteiner et al. 2000); Hanks' balanced salt solution (HBSS) + 20-mM tris buffer; and 300-mM sucrose + 20-mM tris buffer. Cryoprotectants included dimethyl sulfoxide (DMSO) and methanol.

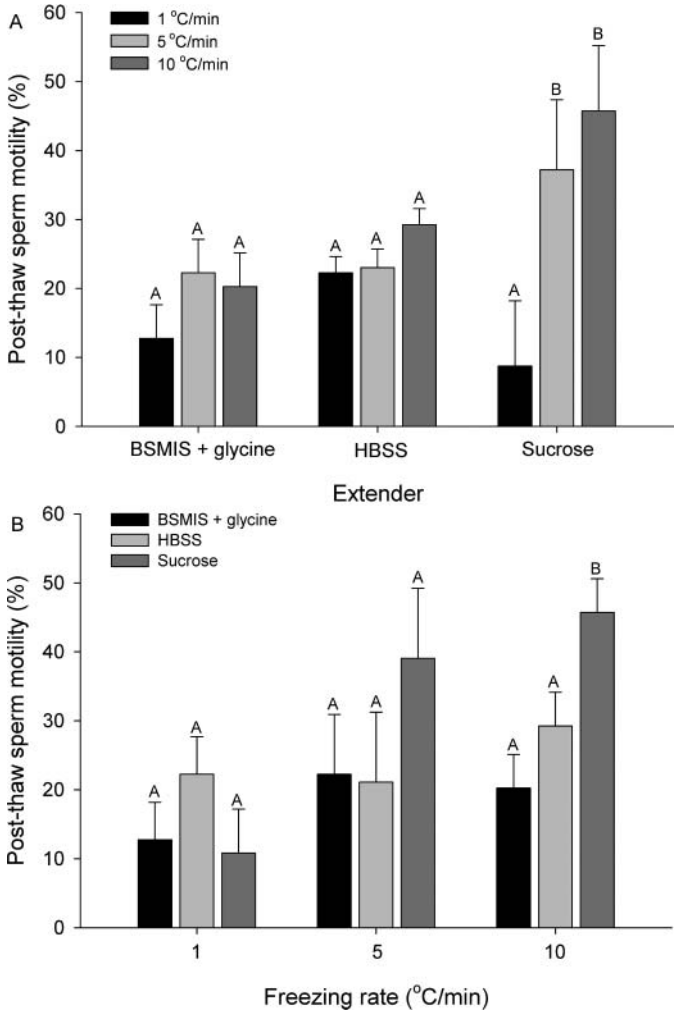

FIGURE 2. Results of one-way ANOVAs for postthaw motility of Redside Dace sperm ($n = 4$ males). One-way ANOVAs were run to determine **(A)** the effect of extender for each freezing rate (a rate of 1, 5, or 10°C/min from 10°C to −20°C; and then a rate of 5°C/min from −20°C to −120°C) and **(B)** the effect of freezing rate for each extender. These decomposed one-way ANOVAs were run because of a significant higher-order interaction. Least-squares means (+SE) are reported. Treatment means without a letter in common for the same extender (in A) or for the same freezing rate (in B) were significantly different ($P < 0.05$). Mean motility of fresh sperm was 66.94%. Extenders are defined in Figure 1.

(two-way ANOVA: $F_{4, 22} = 1.03$, $P = 0.414$), but there was a significant effect of diluent (two-way ANOVA: $F_{2, 22} = 8.42$, $P = 0.002$) such that sperm frozen with HBSS or sucrose had a significantly higher postthaw velocity than sperm frozen with BSMIS + glycine (Figure 3A). The main effect of freezing rate was not significant (two-way ANOVA: $F_{4, 22} = 0.06$, $P = 0.940$; Figure 3B).

Experiment 4: Male-to-Male Variation in Postthaw Sperm Quality

For the 22 Redside Dace males whose sperm were cryopreserved, the mean motility and velocity (±SE) of fresh sperm were 71.50 ± 3.33% and 140.21 ± 3.65 µm/s, respectively, whereas the mean motility and velocity of postthaw sperm were 35.18 ± 3.84% and 52.14 ± 2.87 µm/s, respectively (Figure 4). Motility of fresh sperm ranged from 37% to 97%, while that of postthaw sperm ranged from 6% to 65% (Figure 4A). The velocity of fresh sperm ranged from 105.5 to 170.1 µm/s, while the velocity of postthaw sperm ranged from 33.0 to 81.4 µm/s (Figure 4B). Furthermore, the sperm motility recovery index ranged from 6.67% to 79.27%, while the sperm velocity recovery index ranged from 21.37% to 57.33%.

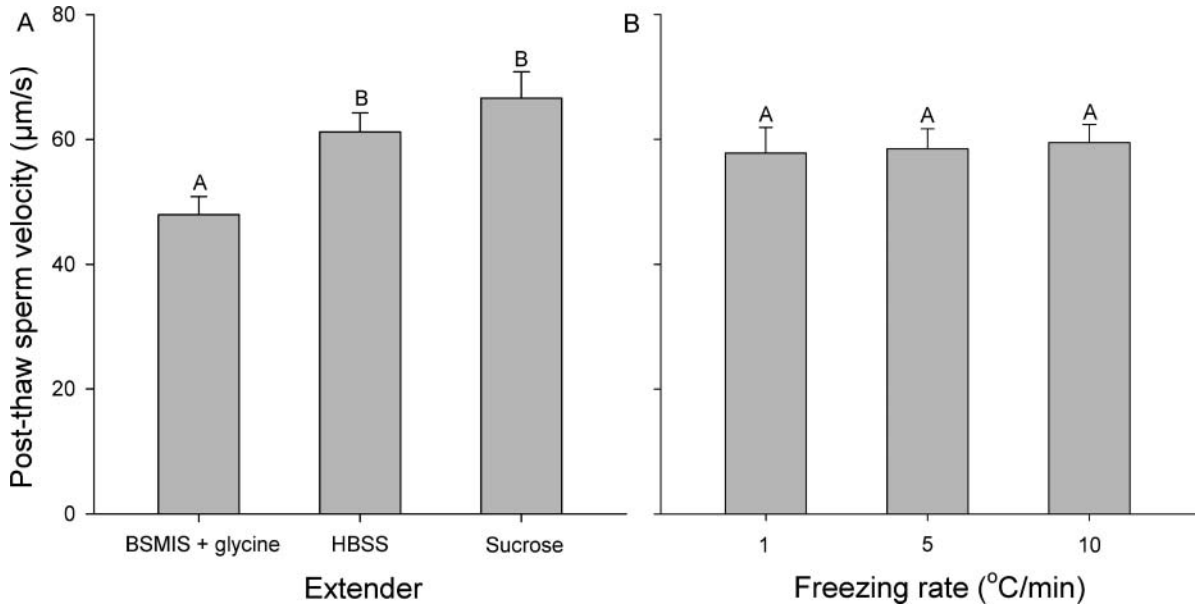

FIGURE 3. Results of a two-way ANOVA for postthaw velocity of Redside Dace sperm ($n = 4$ males). The extender × freezing rate interaction was not significant; thus, the main effects of **(A)** extender and **(B)** freezing rate were analyzed. Least-squares means (+SE) are reported. Treatment means without a letter in common were significantly different ($P < 0.05$). Mean velocity of fresh sperm was 126.09 μm/s. Extenders are defined in Figure 1; freezing rate treatments are defined in Figure 2.

DISCUSSION

Redside Dace are declining across the species' geographic range (Goforth 2000; Lyons et al. 2000; IDNR 2002; COSEWIC 2007). Despite ongoing efforts to restore native populations (RDRT 2010), Redside Dace receive little scientific attention, especially with regard to their reproductive biology. A literature search (Web of Science search [13 August 2012] with topic as "Redside Dace") yielded 14 publications, and only two of those were related to the species' reproductive biology. One study focused on spawning coloration, female choice, and sperm competition (Beausoleil et al. 2012), while another study examined sperm form and function (Pitcher et al. 2009). Therefore, it is apparent that the current work, which develops a functional sperm cryopreservation protocol, advances the knowledge base in this area and could have immediate ramifications for conserving disappearing biodiversity in populations facing imminent local extinction.

We undertook a multistep statistical approach that employed one-way and factorial ANOVA models. In the former, our goal was to determine which extender media or freezing rate generated sperm quality characteristics (after sperm were incubated in different extender media or were cryopreserved) that were most similar to those of fresh sperm. In the latter approach, we furthered our understanding of the biological and physiological interactions involved in the cryopreservation process. Overall, our results showed that (1) the incubation of sperm in various extender media generated results similar to those observed for fresh sperm; (2) the quality of sperm (either incubated or cryopreserved) was strongly influenced by the presence of higher-order interactions; (3) the cryopreservation process decreased sperm quality for all frozen–thawed treatment combinations; and (4) some males' sperm were highly active after the freezing–thawing process, whereas the sperm of other males performed poorly.

Toxicity of Extenders

Motility of Redside Dace sperm decreased from 91.0% to 27.5% or less after 5 min in extenders containing DMA. This clearly indicates that DMA is not a suitable cryoprotectant for Redside Dace sperm; similar results have been reported for Danube Bleak *Chalcalburnus chalcoides* (Lahnsteiner et al. 2000). Dimethyl sulfoxide also showed toxicity to sperm; however, during the freezing and thawing process, it ranked as the best cryoprotectant (discussed in detail below). Therefore, prefreezing and postthawing incubation times should be kept to a minimum when using this cryoprotectant. On the contrary, PG and methanol—depending on the diluent used—were nontoxic in some cases, thus further strengthening the importance of full factorial experimental designs when developing sperm cryopreservation protocols (Suquet et al. 2000; Babiak et al. 2001; Butts et al. 2010, 2011b). Previous studies have also indicated that methanol and PG provide opportunities for storage of sperm without a loss of viability (Christensen and Tiersch 1996; Tiersch et al. 1998; Rideout et al. 2004). For instance, Channel Catfish *Ictalurus punctatus* sperm that were suspended in a buffer containing methanol retained motility significantly longer than sperm that were suspended in a buffer without methanol (Christensen and Tiersch 1996). In Haddock *Melanogrammus aeglefinus* and Atlantic Cod *Gadus morhua*, sperm that were mixed with PG and kept at 5°C for 20 min

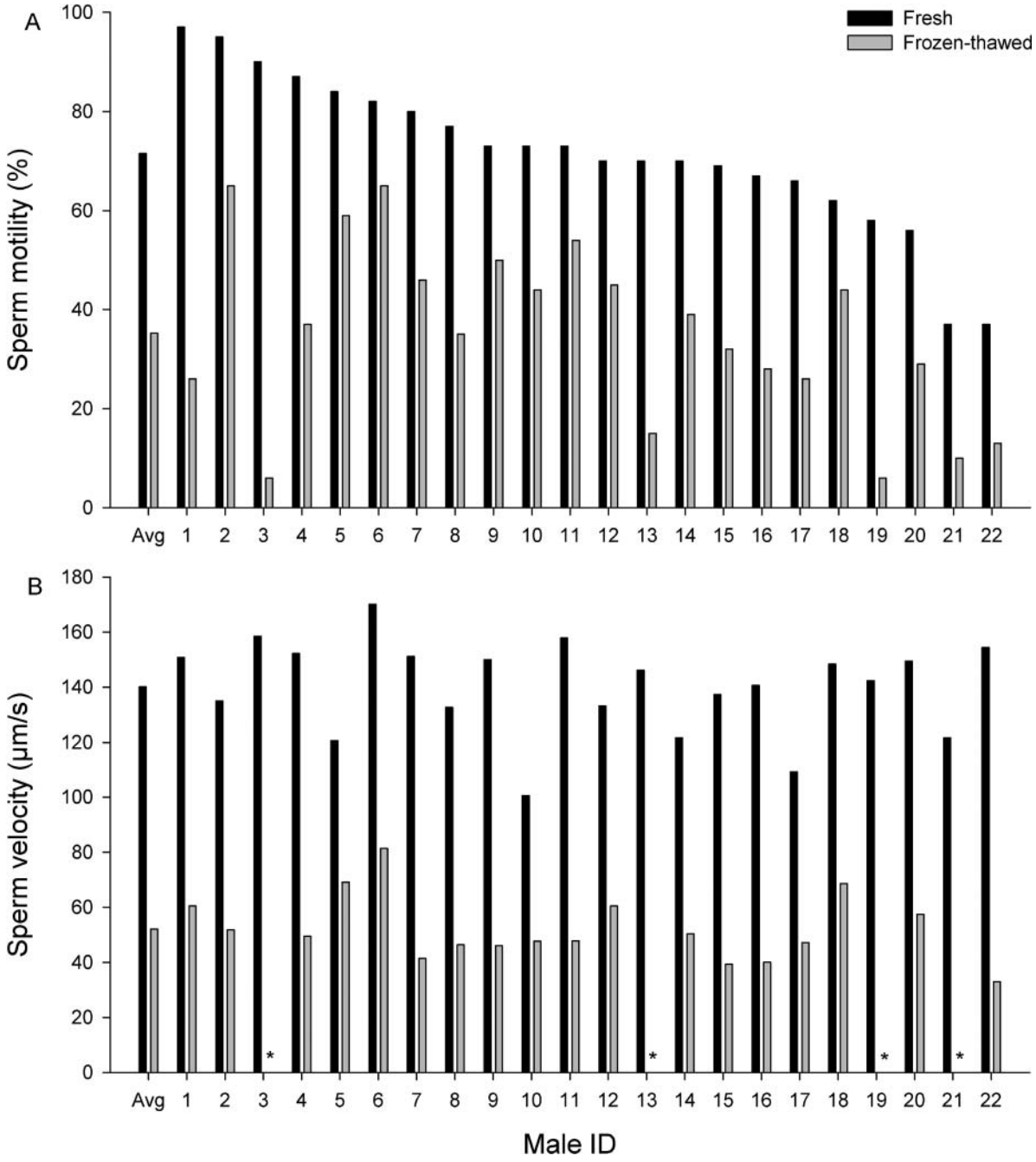

FIGURE 4. Effect of Redside Dace male-to-male variation in **(A)** motility and **(B)** velocity of fresh sperm and postthaw sperm ($n = 22$ males). Vertical bars represent the mean value for each male (* = velocity not measured due to an insufficient number of motile sperm).

experienced no reduction in percent motility or mean and maximum swimming speeds relative to those of fresh sperm (Rideout et al. 2004).

Effects of Extender Composition on Postthaw Sperm Quality

Although the cryopreservation process decreased sperm quality for all frozen–thawed treatment combinations, our postthaw sperm motility and velocity results are still more than adequate for preserving genetic diversity via sperm banks and for fertilizing eggs (Beausoleil et al. 2012). The sucrose-based diluent provided the best protection for sperm during the freezing–thawing process. In this regard, it has been shown that sucrose stabilizes liposomal membranes during freezing (Quinn 1985) and promotes cell dehydration (Gwo 2000), thus protecting the integrity of the cell.

As previously mentioned, methanol and PG were able to sustain sperm quality during incubation; however, they were both inferior to DMSO at protecting the sperm during cryopreservation. Dimethyl sulfoxide has also been proven successful for freezing the sperm of various cyprinids, such as the Common Carp *Cyprinus carpio* (Horváth et al. 2003), Danube Bleak, Barbel *Barbus barbus*, Nase *Chondrostoma nasus*, Grass Carp *Ctenopharyngodon idella*, Silver Carp *Hypophthalmichthys molitrix*, European Chub *Leuciscus cephalus*, Lake Chub *Rutilus meidingerii*, Vimba Bream *Vimba vimba* (Lahnsteiner et al. 2000), and Olive Barb *Puntius sarana* (Nahiduzzaman et al. 2011). The success of DMSO may be explained by its ability to quickly penetrate the sperm and by its interactions with the phospholipids of the sperm membrane (Suquet et al. 2000), therefore creating a rapid balance between the intracellular and extracellular media.

Effects of Freezing Rate on Postthaw Sperm Quality

One of the most critical steps during the cryopreservation process is the freezing protocol. If the freezing rate is too slow, then too much water leaves the cell and dehydration causes cell death; if the freezing rate is too fast, not enough water leaves the cell and large intracellular ice crystals form, causing the cell to rupture (Yang et al. 2007). In this study, we used a programmable freezing unit to test our desired freezing rates, as this unit ensures accurate freezing rates and provides consistent results (Conget et al. 1996; Babiak et al. 1999). In the present study, freezing rate had an effect on postthaw sperm motility via a significant interaction. Therefore, determining the response of various interacting factors is essential for proper development and assessment of the best freezing protocols, as the osmotic balance varies based on the extender used and the physiology of the sperm (Yang et al. 2007). Our results indicated that the sucrose + DMSO extender and a freezing rate of 10°C/min resulted in the highest postthaw quality of Redside Dace sperm.

Male-to-Male Variation in Postthaw Sperm Quality

In the present study, we noticed that variation in the ability of sperm to endure freezing was quite high. This male-to-male variation poses a problem in that the "bad" sperm donors might be of high genetic value. Furthermore, males with poor cryopreservation potential require higher sperm numbers to compensate for poor sperm quality, thus jeopardizing the enhancement of conservation breeding programs. Therefore, a suggestion for future research includes addressing the impact of milt quality on sperm cryopreservation success for Redside Dace by using a controlled experimental design that considers such factors as fish size, age, genetic heritability, and nutritional status. It is also essential to consider the biochemical characteristics (e.g., fatty acids, enzymes, protein, and antioxidants) of the milt so that factors with negative influences on postthaw sperm traits can be accurately pinpointed and properly evaluated (Pustowka et al. 2000; Butts et al. 2011a).

Conclusions and Implications

Overall, we found that the best results were obtained by diluting the Redside Dace sperm in an extender composed of sucrose + DMSO and freezing at a rate of 10°C/min from 10°C to −20°C and then at 5°C/min from −20°C to −120°C. Therefore, we have successfully developed the first functional sperm cryopreservation protocol for the Redside Dace. With this newly developed sperm cryopreservation protocol, we now need to preserve genetic resources by creating sperm banks. Males should be collected from all regions of the species' distribution, with the aim of maximizing the level of genetic diversity that is frozen in perpetuity. Ultimately, this will provide the greatest chance of successful reintroduction through the establishment of genetically viable Redside Dace populations that have a greater ability to adapt to present and future challenges via favorable gene complexes.

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