Cryobiology 82 (2018) 49–56

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

Cryopreservation of sperm bundles (spermatozeugmata) from endangered livebearing goodeids

Yue Liu, Leticia Torres, Terrence R. Tiersch*

Aquatic Germplasm and Genetic Resources Center, School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, LA, USA

ARTICLE INFO

Keywords:
Cryopreservation
Sperm
Sperm bundles
Spermatozeugmata
Livebearing fish
Viviparous
Goodeidae
Goodeids
Endangered species
Motility activation

ABSTRACT

More than half of fishes in the family Goodeidae are considered to be endangered, threatened, or vulnerable. Sperm cryopreservation is an effective tool for conserving genetic resources of imperiled populations, but development of protocols with livebearing fishes faces numerous challenges including the natural packaging of sperm into bundles. In this study the cryopreservation of sperm bundles (spermatozeugmata) of three goodeids species was evaluated. Sperm quality was evaluated by activation with NaCl-NaOH solution (at 300 mOsmol/kg and pH 11.8), and analysis of dissociable bundles and dissociation duration. Using Redtail Splitfin (*Xenotoca eiseni*) as a model, the effects of cryoprotectants (dimethyl sulfoxide, methanol, and glycerol) with different concentrations (5–15% v/v %), equilibration exposure times (1–60 min), cooling rates (5–40 °C/min), concentrations (4×10^4–4×10^6 bundles/ml), buffers (HBSS, PBS and NaCl), and buffer osmolalities (200–400 mOsmol/kg) were investigated. After cooling and thawing, sperm bundles maintained their packed form. A specific protocol was developed (10% dimethyl sulfoxide, 20-min equilibration, 10 °C/min cooling rate, 4×10^6 bundles/ml, and 300 mOsmol/kg HBSS). This protocol yielded 89 ± 5% of post-thaw dissociable bundles with 209 ± 10 s of dissociation duration for *X. eiseni*, 96 ± 9% with 814 ± 14 s for Blackfin Goodea (*Goodea atripinni*), and 66 ± 2% with 726 ± 25 s for Striped Goodeid (*Ataeniobius toweri*). This is the first study of cryopreservation of sperm within bundles for livebearing fishes and provides a basis for establishment of germplasm repositories for goodeids and other livebearers.

1. Introduction

Fishes of the family Goodeidae are considered to be among the most at-risk taxa in the world [10]. Goodeidae includes two subfamilies: Empetrichthyinae and Goodeinae. Empetrichthyinae comprises 3 extant oviparous (egg-laying) species in 2 genera that inhabit small-volume springs of the southwestern Great Basin of the United States [33]. The subfamily Goodeinae includes about 45 viviparous (livebearing) species (referred to as 'goodeids') in 18 genera inhabiting shallow freshwaters within the Central Mexican Plateau [33]. Of these, 12 are critically endangered, endangered, or vulnerable, and 3 are extinct among 17 that have been assessed by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species [21]. Another assessment (referred as 'the 2005 report') based on 18 years of field research in combination with a detailed scientific literature survey, suggested that about 35 species of goodeids were endangered, threatened, and vulnerable as of 2005 [6]. The rapid decline of goodeid populations has been attributed to the heavily disturbed aquatic ecosystem in central Mexico resulting from water pollution, reductions in levels of ground and surface waters, basin deforestation, habitat destruction, and introduction of exotic species [8]. Additionally, the resulting small population sizes potentially lead to losses of local populations via demographic stochasticity, increasing the risk of extinction due to inbreeding, decreases in genetic variation, and fixation of deleterious alleles [28]. Therefore, urgent action for conservation of goodeids is needed.

Current efforts toward goodeid conservation include protection and restoration of natural habitats and captive breeding [22]. The goal of habitat restoration is to maintain healthy populations and genetic diversity in historic habitats, however this goal is usually difficult to accomplish and can take decades, with population sizes and genetic diversity of concerned species continuing to decline in the wild before full restoration can be realized [1]. To address the difficulties of reaching long-term goals, short-term efforts, such as captive breeding, have also been initiated to ensure survival and to slow down losses of genetic diversity [25]. However, these approaches are costly, and may cause inbreeding depression, disease outbreaks, and long-term loss of genetic diversity [29]. Repositories of cryopreserved germplasm can become a

* Corresponding author. Aquatic Germplasm and Genetic Resources Center, Louisiana State University Agricultural Center, School of Renewable and Natural Resources, 2288 Gourrier Avenue, Baton Rouge, LA 70820, USA.
E-mail address: ttiersch@agcenter.lsu.edu (T.R. Tiersch).

https://doi.org/10.1016/j.cryobiol.2018.04.009
Received 9 January 2018; Received in revised form 22 March 2018; Accepted 13 April 2018
Available online 14 April 2018
0011-2240/

viable tool to address these challenges by preserving germplasm for future use at a relatively low cost, ensuring integrity of genetic diversity, enabling genetic assessment and management, and enhancing captive breeding [26,30]. This tool has been used to improve diversity in recovery of endangered mammals, such as black-footed ferrets (*Mustela nigripes*) [15], giant panda (*Ailuropoda melanoleuca*) [20], and Namibian cheetahs (*Acinonyx jubatus*) [5]. However, it has been neglected in conservation programs of imperiled fishes. For example, in our review of about 90 recovery plans for endangered or threatened fishes developed by the United States Fish and Wildlife Service since 1980s, only 2 mention cryopreservation of germplasm [32].

Development of cryopreservation protocols is the basis for the establishment of germplasm repositories [2,3]. Protocols for sperm cryopreservation of livebearing fishes have been developed for two genera within Poeciliidae. For example, *Xiphophorus* species have been the most studied due to their importance as cancer research models [35]. Fertilization success was less than 20% when females were inseminated with thawed or fresh sperm from *X. helleri* [41], *X. couchianus* [38], *X. maculatus* [39], and *X. variatus* [40]. Species from the genus *Poecilia*, such as the Guppy (*Poecilia reticulata*) and the Sailfin Molly (*P. latipinna*) are popular ornamental fishes and research models. In these species, fertilization success was ~50% when females were inseminated with thawed sperm [19]. To date, there are no reports of cryopreservation of sperm from goodeids.

Viviparous fishes employ unique reproductive features such as internal fertilization and the bearing of live young [13,34]. Freshwater livebearing fishes usually package sperm in bundles (spermatozeugmata), which are believed to facilitate the transfer of sperm from male to female [12]. The viviparity mechanisms of poeciliids and goodeids evolved independently, and thus there are differences in sperm bundles between the two groups. For example, bundles from *P. reticulata* and *Xiphophorus* species can be dissociated into free sperm by gentle crushing of testes, thus, sperm can be frozen as separate cells [19,39], and their quality could be evaluated by commonly used parameters, such as sperm motility, duration, and velocity. However, bundles from goodeids pose difficulties for sperm cryopreservation. For example, after testes were crushed, bundle dissociation occurred rarely (in less than 10% of males) [24]. To date, there are no reports on cryopreservation of fish sperm in the form of bundles, although methods to evaluate quality of sperm within bundles have been developed previously [23]. The goal of the present study was to develop protocols for cryopreservation of sperm bundles from endangered livebearing goodeids using three species as models, Redtail Splitfin (*Xenotoca eiseni*, listed as endangered in the 2005 report, but not assessed by the Red List), Blackfin Goodea (*Goodea atripinnis*, of least concern in the 2005 report and the Red List), and Striped Goodeid (*Ataeniobius toweri,* critically endangered in the 2005 report, and endangered in the Red List) [6,21]. The specific objectives were to: (1) investigate the effect of cryoprotectant, cooling rate, bundle concentration, and extender on post-thaw quality of bundles from *X. eiseni*, and (2) apply the protocol established with *X. eiseni* to *G. atripinnis* and *A. toweri*.

2. Materials and methods

2.1. Fish husbandry

Protocols for the use of animals in this study were reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee (Baton Rouge, LA). The *X. eiseni*, *G. atripinnis*, and *A. toweri* used in this study were 2-y old and maintained in the Aquatic Germplasm and Genetic Resources Center (AGGRC) at the Louisiana State University Agricultural Center (Baton Rouge, Louisiana, USA). These fishes were bred from aquarium-trade populations for research purposes and were not removed from wild populations. The *X. eiseni* and *G. atripinns* were bred by H. Grier [7] and transferred to the AGGRC at < 1-y old. The *A. toweri* were obtained from the Goodeid Working Group (http://www.goodeidworkinggroup.com). At the AGGRC, fishes were cultured indoors at 22–26 °C with a 14 h:10 h (light:dark) photoperiod in 80-l recirculating aquarium tanks (about 30 fish per tank) and fed twice daily with tropical flakes (Pentair Aquatic Eco-systems, FL, USA) and twice weekly with frozen brine shrimp (Sally's Frozen Brine Shrimp™, San Francisco Bay Brand, CA, USA). Males were mixed at a 2:1 ratio with females in each tank. Additional water quality parameters were monitored every 2–4 weeks and held within acceptable ranges including: pH (7–8), ammonia (0–0.1 mg/l), and nitrites (0–0.8 mg/l). The *X. eiseni* were used in *Experiments 1–3* as a model for protocol development, and a generalized protocol was applied to *X. eiseni*, *G. atripinns* and *A. toweri* in *Experiment 4*.

2.2. Collection of sperm bundles

Previous studies showed sperm from *X. eiseni* could not be collected by stripping [23]. As such, fish were euthanized by exposure to 300 mg/l Tricaine methanesulfonate (MS-222, Western Chemical, Inc. WA, USA) followed by severing of the spinal column behind the head. To eliminate MS-222 residues, the surface of fish was wiped with a paper towel and rinsed with an extender solution (NaCl at 300 mOsmol/kg buffered by 5 mM Tris-HCl at pH 7.0). The body wet weight and standard length were measured. Osmolalities of solutions were measured with a freezing point osmometer (Model 5010 OSMETTE III ™, Precision Systems Inc., MA, USA) and pH was measured with a meter (EcoSense® pH100A, YSI Inc., OH, USA). Testes were dissected, rinsed, weighed, placed in 50 µl of the extender onto a glass slide, and crushed with angled spade-tip forceps. Sperm bundles were released into extender and collected with a pipette into 1.5-ml centrifuge tubes (Fig. 1A). The concentration of sperm bundles was estimated using a hemocytometer (Hausser Scientific, Horsham, PA, USA). Body wet weight, standard length, and testes weight of males used in this study was 2.6 ± 0.49 g, 40.5 ± 5.0 mm, and 20.4 ± 1.9 mg for *X. eiseni*; 2.5 ± 0.26 g, 49.6 ± 1.5 mm, and 11.7 ± 1.1 mg for *G. atripinnis*, and 1.5 ± 0.3 g, 43.9 ± 1.3 mm, and 18.2 ± 0.8 mg for *A. toweri*.

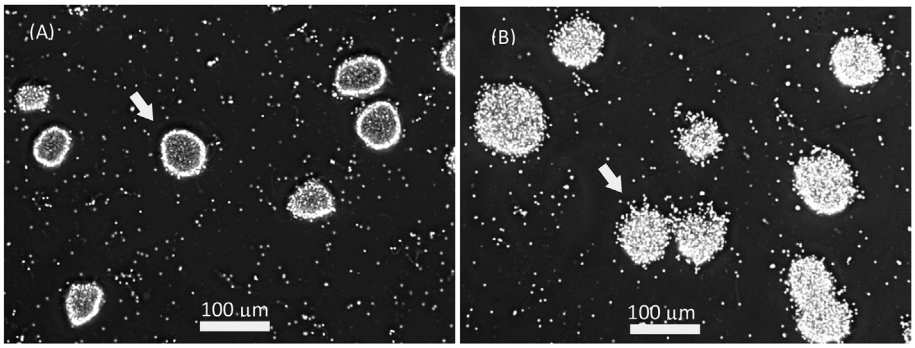

Fig. 1. Sperm bundles (indicated by white arrows) from *X. eiseni* before freezing (A) and after thawing (B) without activation (200-× magnification with darkfield). The white dots outside of the bundles were debris or free sperm (motility < 1%). After thawing, bundles were less compact, but packed sperm were still associated with the bundles. The samples were observed at room temperature (24–26 °C).

2.3. Bundle dissociation and evaluation

The capability of sperm to swim away from bundles is important in livebearing fishes, because to achieve internal fertilization, sperm need to dissociate from bundles and traverse the female reproductive tract. Preliminary studies demonstrated that NaCl solution at pH 11.8 activated sperm within bundles from goodeids, and this activation method could be used to evaluate the quality of sperm within bundles [24]. In the present study, bundle suspensions were mixed with an activation solution (NaCl at 300 mOsmol/kg at pH 11.8 adjusted by NaOH) on a Makler® counting chamber (Sefi-Medical Instruments, Haifa, Israel) at a ratio at 1: 9 (suspension: activation solution) followed by placing a cover slip on top. Before activation, sperm within bundles were quiescent. After activation, sperm first vibrated in place, then swam away from bundles, and finally stopped movement from bundles at the end of activation. In each observation, 5–15 bundles within a viewing area of a microscope (200-× magnification with darkfield, CX41, Olympus Corporation, Tokyo, Japan) were classified into dissociated and non-dissociated phases according to the degree of sperm dissociation. At the dissociated phase, at least 3 sperm were observed swimming away from bundles whereas no sperm were swimming at the un-dissociated phase. The percentage of dissociable bundles were calculated as number at dissociated phases/(dissociated phase + the un-dissociated phase) in a viewing area. This percentage can indicate the survival of sperm within bundles and the capability of these sperm for dissociating from bundles. The times when the first dissociated bundle was observed (T_1), and when all bundles stopped dissociating (T_2) were recorded. The percentage of dissociable bundles and the dissociation duration (T_2-T_1) of 2–4 observations for each replicate within each experimental treatment were averaged to evaluate the quality of sperm bundles.

2.4. Freezing and thawing procedures

Bundle suspensions with cryoprotectant (detailed below) were drawn into 0.25-ml French straws (IMV International, MN, USA) and held at room temperature (24–26 °C) for equilibration, and at 4 °C for 2 min before cooling in a controlled-rate freezer (IceCube 14M, SY-LAB, Neupurkersdorf, Austria) from 4 to −80 °C (cooling rates described as below) [18]. After freezing, the straws were transferred to a liquid nitrogen storage dewar. After a minimum of 3 d, the straws were thawed for 7 s in a 40 °C water bath. After thawing, bundle suspensions were transferred into 1.5-ml centrifuge tubes and centrifuged at 700 revolutions per min (about 770 × *g*) (Marathon 13 K/M, Fisher Scientific, PA, USA) for 10 s, followed by removal of supernatant, and resuspension of bundles in the extender for further tests.

2.5. Experiment 1: the effect of cryoprotectant before and after freezing

There were three trials in this experiment using *X. eiseni*. The first trial was to evaluate the acute toxicity of cryoprotectants. Bundle suspensions were mixed with an equal volume (80 μl) of NaCl solution at 300 mOsmol/kg (control), and three different cryoprotectants, dimethyl sulfoxide (DMSO), methanol, and glycerol to yield final concentrations of each cryoprotectant of 5, 10, and 15% (v/v %). Percent dissociable bundles and dissociation duration were estimated at 1, 10, 20, and 40 min after mixing at room temperature.

The second and third trials were used to evaluate post-thaw bundle quality. Based on the results of the first trial, DMSO and methanol at final concentrations of 5, 10, and, 15%, and glycerol at 5% were selected for testing in the second trial. The cryoprotectants and NaCl solution were mixed with an equal volume (80 μl) of bundle suspensions, and equilibrated for 10 min at room temperature before freezing. In the third trial, bundle suspensions were mixed with DMSO at final concentrations of 5, 10, and 15%, and glycerol at 5%, and equilibrated for 10, 20, 40, and 60 min at room temperature before initiation of cooling. In the second and third trials, the bundle-cryoprotectant suspensions were cooled at 20 °C/min from 4 to −80 °C before plunging into liquid nitrogen, and percent dissociable bundles and dissociation duration were estimated immediately after thawing. Because of the miniscule volumes of sperm available per male (< 10 μl) [24] and the multiple variables tested, in each of the three trials four separate pools of samples (two to three different males in each pool for a total of 14 males) were used to provide four separate replicates. The concentration of bundles was adjusted to 1–3 × 10^6/ml prior to mixing with cryoprotectants.

2.6. Experiment 2: the effect of cooling rate and bundle concentration

Based on the results of the previous experiments, DMSO at 10% (in NaCl solution at 300 mOsmol/kg) with a 20-min equilibration were used for subsequent studies. There were two trials in this experiment using *X. eiseni*. In the first trial, the equilibrated suspensions were cooled at 5, 10, 20, 30, and 40 °C/min from 4 to −80 °C before plunging into liquid nitrogen. Based on the results of the first trial, the cooling rate of 10 °C/min was used in the subsequent trials. In the second trial, the final concentrations of 4.0×10^6, 2.0×10^6, 4.0×10^5, and 4.0×10^4 bundles/ml were tested. In each trial, the percent dissociable bundles and dissociation duration were estimated immediately after thawing. In each trial, four different males were used as four replicates.

2.7. Experiment 3: the effect of extenders

In the first trial of this experiment, bundles from *X. eiseni* were suspended in NaCl solutions (buffered by 5 mM Tris-HCl at pH 7.2) at 200, 250, 300, 350, and 400 mOsmol/kg. The percent dissociable bundles and dissociation duration were estimated immediately after thawing. In the second trial, calcium-free Hanks' balanced salt solution (Ca^{2+}-free HBSS: 0.137 M NaCl, 5.4 mM KCl, 1.0 mM MgSO4, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM NaHCO3, and 5.55 mM glucose), phosphate-buffered saline (PBS: 0.01 M phosphate, 0.138 M NaCl, 0.0027 M KCl, prepared from pre-mixed powder, Sigma-Aldrich, MO, USA), and the NaCl solution described above were used as extenders. Based on the results from the first trial, the osmolalities of each extender in the second trial were adjusted to 300 mOsmol/kg. After thawing and resuspending with each extender, the samples were held at 4 °C. Percent dissociable bundles and the dissociation duration were estimated immediately, and at 2, 6, 12, 24, 48, 72, 96, 120, 144, 168, and 192 h after thawing. Internal fertilization place greater demands on sperm survival than does external fertilization, and therefore we tested motility duration for as long as 9 d. Four different males were used as four replicates in this experiment.

2.8. Experiment 4: Generalization

Based on results from the previous experiments, sperm bundles from *X. eiseni*, *G. atripinnis*, and *A. toweri* was suspended with HBSS at 300 mOsmol/kg with a concentration of 4 ± 1 × 10^6 bundles/ml, and mixed with DMSO at a final concentration of 10%. After a 20-min equilibration, the suspensions were cooled at 10 °C/min from 4 to −80 °C. After thawing and resuspending, the samples were held at 4 °C, and percent dissociable bundles and dissociation duration were estimated immediately, and at 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 h. Fresh sperm of livebearing fishes can swim > 2 d [19,24], therefore intervals ≥ 24 h were used for observation in this experiment. Four different males of each species were used as four replicates in this experiment.

2.9. Data analysis

One-way ANOVA with Tukey's multiple comparisons test were used to identify significant differences in the percent of dissociable bundles

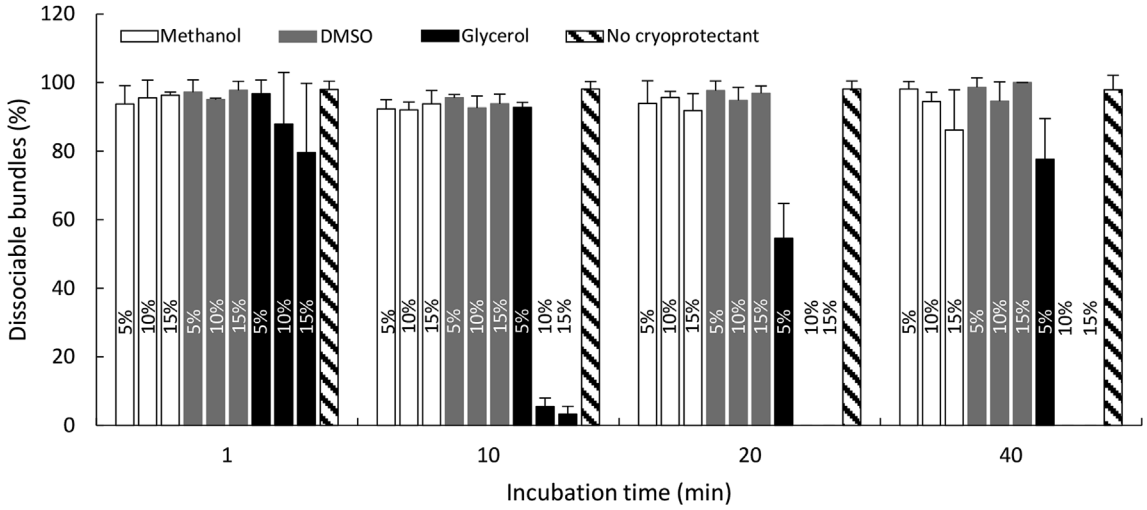

Fig. 2. The effects on bundle dissociation of the cryoprotectants methanol, dimethyl sulfoxide (DMSO), and glycerol at final concentrations of 5, 10, and 15% (in NaCl solution at 300 mOsmol/kg), were evaluated with *X. eiseni* for 1, 10, 20, and 40 min at room temperature. Bars represent the mean ± SD of four replicates (each replicate was a different pool of two to three males).

and the dissociation duration among different treatments. When data did not follow normal distribution or homogeneity of variance, Kruskal-Wallis (K-W test) with multiple comparisons were used. The results were considered statistically significant at $P < 0.05$. The software used was SAS 9.4 (SAS Institute, Cary, North Carolina, USA).

3. Results

3.1. Effect of cryoprotectant before and after freezing

The control group (without addition of cryoprotectant) had > 97% dissociated bundles at 1, 10, 20, and 40 min after mixing of bundle suspensions with extender solutions (NaCl solution at 300 mOsmol/kg) (Fig. 2). One min after mixing with cryoprotectants, there were no significant differences ($P = 0.1840$, K-W test) in percent dissociable bundles among treatments. After 10 min incubation, the percentages of dissociable bundles were significantly different among treatments ($P < 0.0001$, ANOVA), with the 10 and 15% glycerol treatments lower than the DMSO, methanol, and 5% glycerol treatments. At 20 and 40 min after mixing, there were significant differences among cryoprotectant groups ($P \leq 0.0006$, K-W test). The percent dissociable bundles ranged 86–100% for samples with DMSO and methanol, 55–78% for 5% glycerol, and 0% for 10 and 15% glycerol.

In the second trial before addition of cryoprotectant, there were 95 ± 6% the dissociable bundles and dissociation duration was 186 ± 18 s. After thawing, the bundles seemed less compact (Fig. 1B) compared with observations before freezing (Fig. 1A), but they remained at the un-dissociated phase before activation. After activation, sperm from peripheral layers of bundles vibrated in place or swam away, but the inner layers remained quiescent and un-dissociated. No dissociable bundles were observed in the control and methanol treatments (Fig. 3). There were significant differences in percent dissociable bundles ($P < 0.0001$, ANOVA) and dissociation duration ($P = 0.0026$, ANOVA) among samples with DMSO and 5% glycerol. Samples with 15% DMSO had the highest percent dissociable bundles (82 ± 10%) and duration (126 ± 19 s), and 5% glycerol had the lowest percent dissociable bundles (24 ± 9%) and duration (65 ± 7 s).

In the third trial after thawing (Fig. 4), percent dissociable bundles and dissociation duration were each lowest in 5% glycerol for 10, 20, 40, and 60 min equilibration ($P < 0.0001$). There were no significant differences among 5, 10, and 15% DMSO in percent dissociable bundles after 20 ($P = 0.2189$), 40 ($P = 0.4481$), and 60 ($P = 0.3433$) min equilibration. The highest percent dissociable bundles (93 ± 2%) was found with 10% DMSO and 20 min equilibration.

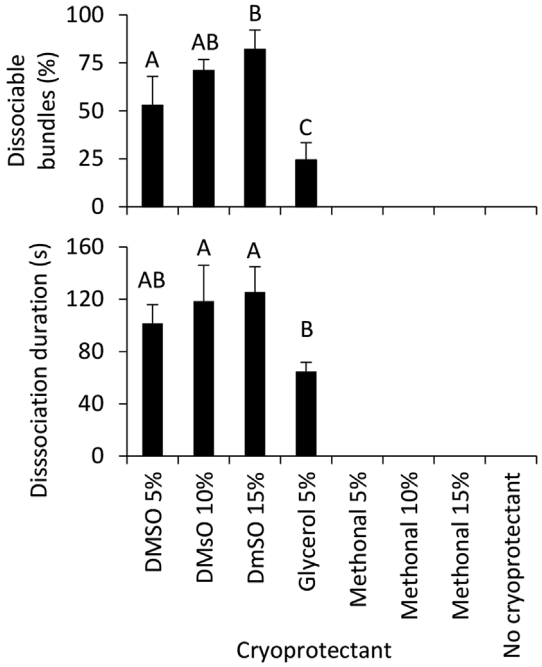

Fig. 3. The percent dissociable bundles and dissociation duration of thawed bundles from *X. eiseni* were evaluated after cryopreservation with methanol (5, 10, and 15%), dimethyl sulfoxide (DMSO, 5, 10, and 15%), and glycerol (5%) in NaCl solution at 300 mOsmol/kg. Bars represent the mean ± SD of four replicates (each replicate was a different pool of two to three males). Treatments sharing the same letter were not significantly different.

3.2. Effect of cooling rate and bundle concentration

After thawing, there were no significant differences in percent dissociable bundles ($P = 0.1829$, ANOVA) and dissociation duration ($P = 0.1554$, ANOVA) among samples with cooling rates of 5, 10, 20, 30, and 40 °C/min with 10% DMSO (in NaCl solution at 300 mOsmol/kg) with 20-min equilibration. There were no significant differences in percent dissociable bundles ($P = 0.2947$, ANOVA) and dissociation duration ($P = 0.5948$, ANOVA) among sample concentrations at 4.0×10^6, 2.0×10^6, 4.0×10^5, and 4.0×10^4 bundles/ml.

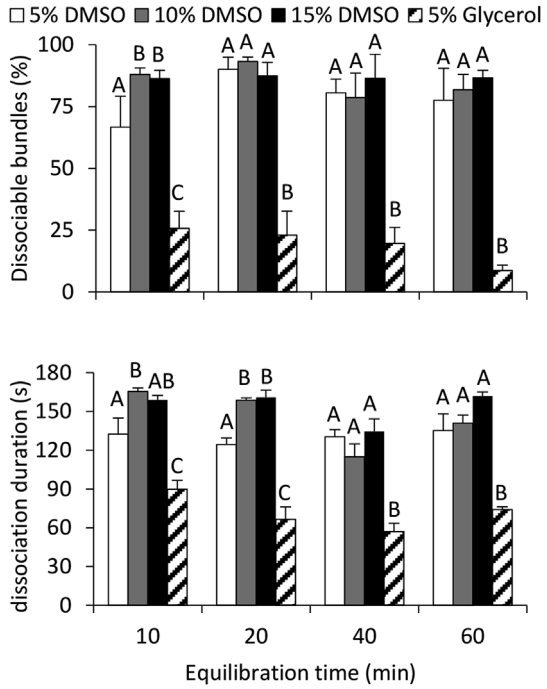

Fig. 4. The percent dissociable bundles and dissociation duration of thawed bundles from *X. eiseni* were evaluated after cryopreservation with dimethyl sulfoxide (DMSO, 5, 10, and 15%), and glycerol (5%) in NaCl solution at 300 mOsmol/kg, and equilibration for 10, 20, 40, and 60 min at room temperature before freezing. Bars represent the mean ± SD of four replicates (each replicate was a different pool of two to three males). Treatments sharing the same letter were not significantly different.

3.3. Effect of extenders

After thawing (Fig. 5), the percent dissociable bundles of samples suspended with 300 mOsmol/kg NaCl solutions (87 ± 7%) was higher ($P < 0.0001$) than 200, 250, 350, and 400 mOsmol/kg. The dissociation duration with 300 mOsmol/kg (151 ± 6 s) was higher ($P < 0.0001$) than 200, 350, and 400 mOsmol/kg, but not significant

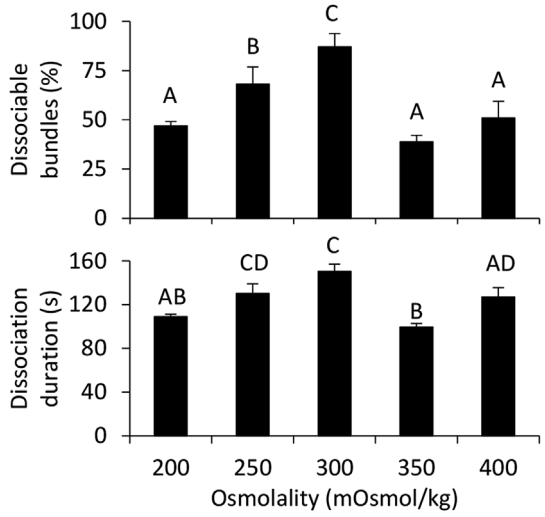

Fig. 5. The percent dissociable bundles and dissociation duration of thawed bundles from *X. eiseni* were evaluated after cryopreservation with NaCl solutions at 200, 250, 300, 350, and 400 mOsmol/kg as extenders. The samples were observed at room temperature. Bars represent the mean ± SD of four replicates (four different males). Treatments sharing the same letter were not significantly different.

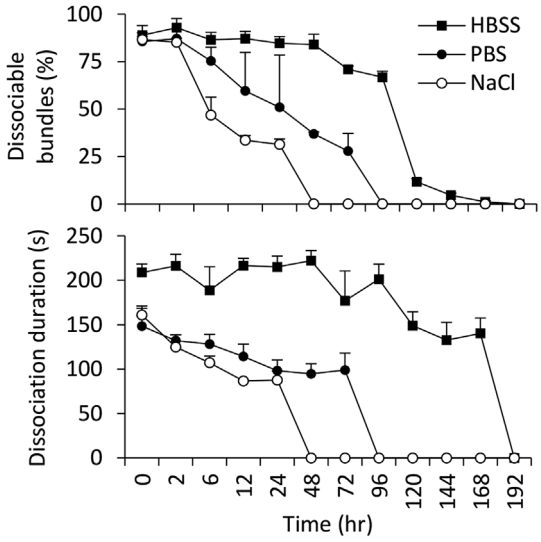

Fig. 6. The percent dissociable bundles and dissociation duration of thawed bundles from *X. eiseni* evaluated after cryopreservation with HBSS, PBS, and NaCl at 300 mOsmol/kg as extenders. The samples were held at 4 °C after thawing. Bars represent the mean ± SD of four replicates (four different males).

different with 250 mOsmol/kg.

Immediately and at 2 h after thawing (Fig. 6), the percent dissociable bundles of samples suspended with Ca^{2+}-free HBSS, PBS, and NaCl were above 85%, and declined thereafter beyond 2 h. The percent dissociable bundles of samples declined the most rapidly in NaCl (0% at 48 h), whereas in Ca^{2+}-free HBSS the decline was slowest (above 65% at 96 h). Samples in Ca^{2+}-free HBSS had the highest dissociable duration among the 3 buffers tested from immediate observation (209 ± 10 s) to 168 h (140 ± 18 s) after thawing.

3.4. Generalization

Immediately after thawing (Fig. 7), percent dissociable bundles from *X. eiseni*, *G. atripinnis*, and *A. toweri* were all > 60%. The percentages declined to about 20% after 24 h for samples from *G. atripinnis* and *A. toweri*; decreased to 0% at 48 h for *G. atripinnis*, and declined from 17 ± 5% at 48 h to 3 ± 2% at 192 h for *A. toweri*. Percent dissociable bundles of samples from *X. eiseni* declined the least with 67 ± 3% at 96 h. Although *A. toweri* had a rapid decline in percent dissociable bundles, the dissociation duration remained the highest from immediate observation (814 ± 14 s) to 192 h (550 ± 24 s). The dissociation duration remained > 500 s until 24 h for bundles from *G. atripinnis*, and 108 s until 192 h for *X. eiseni*.

4. Discussion

This is the first study on sperm cryopreservation for goodeids (the second family of livebearing fish that has been reported). Sperm bundles were cryopreserved in this study instead of free sperm for two reasons: (1) the majority of bundles from goodeids cannot be dissociated into free sperm in extender solutions; and (2) bundles may facilitate functioning of sperm in the female reproductive tract after insemination. Livebearing fishes from the family Poeciliidae and Goodeidae each produce sperm bundles, however the independent evolution of the viviparity for these two groups resulted in different reproductive characteristics. For example, milt can be collected by stripping from poeciliids but not from goodeids [23]. As such, males were euthanized in the present study. The fishes used were bred for research purposes beginning from a few founders in aquarium-trade populations without known genetic management, and thus, no valuable

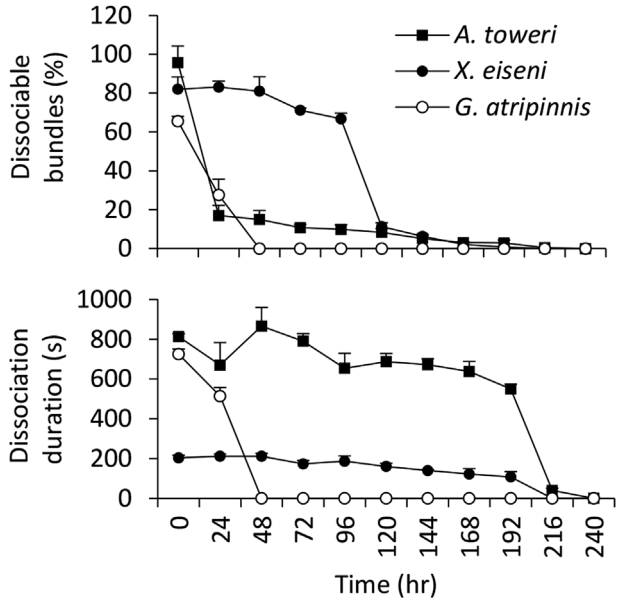

Fig. 7. The dissociable bundles and dissociation duration of thawed bundles from *A. toweri*, *X. eiseni*, and *G. atripinnis* evaluated after cryopreservation in 0.25-ml French straws with a generalized protocol: suspension of sperm bundles in Ca^{2+}-free HBSS at 300 mOsml/kg, use of 10% DMSO as cryoprotectant with a 20-min equilibration time, cooling at 10 °C/min at 4×10^6 bundles/mL, and thawing at 40 °C in a water bath for 7 s. The samples were held at 4 °C after thawing. Bars represent the mean ± SD of four replicates (four different males).

fishes or genetic resources were directly removed from the wild for this study. In addition, fish testes are usually dissected to collect sperm samples in research and applications for preservation of their genetic resources [35], in which cases small numbers of males are killed to collect a maximum volume of sperm rather than many males being stressed with lower mortalities but small volumes of sperm [35].

Because the typical methods for quality evaluation of free sperm were not suitable for bundles, percent dissociable bundles and dissociation duration after activation were used to assess bundle quality. A NaCl solution at pH 11.8 was used to activate bundles, based on previous studies that showed with this pH level, intact bundles could be activated in a short time (within 1 min) and be evaluated with a standardized method [23]. It has not been confirmed whether this pH level is present in the actual fluidic environment of the reproductive tract of female goodeids, but it is evident that the sperm activated in the present study were alive. It is possible that sperm which remained quiescent within bundles were viable, but their motility was not initiated by the activation methods. Because a bundle is a group of cells and the sizes of bundles are usually > 30 μm [23], assays to evaluate sperm viability using flow cytometry [31] may not be suitable. Imaging tools such as fluorescence microscopy can be more feasible to evaluate cell quality with fluorescent staining assays, however, such assays have not been established for sperm bundles. As a pilot study to investigate the feasibility of cryopreservation of sperm in bundle form, the present study did not include evaluation methods such as sperm viability and fertilization, which can be further studied.

Three permeable cryoprotectants were chosen in the present study because of their potential to interact with the densely packed sperm within the bundles. Glycerol has been suggested as being superior to DMSO for livebearing fishes, because of the unique morphology and physiology of sperm from internally fertilized species [19]. However, in the present study, compared with DMSO, glycerol showed significantly higher toxic effects before freezing, and lower post-thaw percentages of dissociable bundles and dissociation duration in all incubation times. Compared with free sperm, the highly compacted cells within bundles have a reduced surface area of exposure to the extracellular environment, thus decreasing the rate at which cryoprotectants can permeate the cells. Therefore, the higher post-thaw percent dissociable bundles using DMSO could have resulted from a higher permeability of the sperm plasma membrane to DMSO than to glycerol [4]. No post-thaw dissociable bundles were observed for the control group without incubation with DMSO, indicating the protective effects of DMSO of sperm within bundles. The limited access of cryoprotectant to cells can also explain the phenomenon that only the outer layers of sperm within bundles were observed to dissociate during activation. In the present study, the percentage of post-thaw dissociating bundles varied with the different concentrations of DMSO at a 10-min equilibration time, but this relationship was not observed for equilibration times of ≥ 20 min. This could indicate that 20 min was sufficient for full equilibration of water and DMSO. For externally fertilized fish, methanol has been often chosen as optimal cryoprotectant because it is less toxic to sperm than DMSO and glycerol prior to cooling and after thawing [14,16,37]. In the present study, however, the toxic effect of methanol was similar with DMSO, but less than glycerol prior to cooling, however, methanol yielded no post-thaw dissociation of bundles. This might be due to a low permeability of methanol to goodeid sperm or some other cellular features of the sperm.

Sperm from livebearing fishes can tolerate a wide range of cooling rates spanning from 5 to 45 °C/min, but highest post-thaw motility has usually been observed at between 15 and 35 °C/min [17–19]. In the present study, no significant differences in percent dissociating bundles and dissociation duration were found for cooling rates between 5 and 40 °C/min. Using faster cooling rates can reduce the time costs of the freezing process. But in practice, longer cooling durations can provide personnel with more time to prepare for sorting and storage procedures. As such, a cooling rate of 10 °C/min (~8 min of cooling duration) was chosen for the cryopreservation protocol.

Another factor affecting post-thaw quality is sperm concentration. For example, it directly determined the level of agglutination of thawed sperm from Pacific Oysters (*Crassostrea gigas*) [9], and significantly affected motility of thawed sperm from Rainbow Trout (*Oncorhynchus mykiss*) [27]. In the present study, no significant difference in post-thaw quality was found among bundle concentrations of 4.0×10^4 to 4.0×10^6. It is possible that the concentrations chosen in the present study were within an acceptable range, and were not high enough to compress inter-bundle space to sufficiently affect equilibration of cryoprotectant. As such, the packed space between cells could be the limiting factor for flow of water and cryoprotectant across plasma membranes during equilibration and freezing.

In cryopreservation, extender solutions are important for the dilution of milt, adjustment of sperm concentration, and for prevention of sperm activation. For externally fertilized fishes, sperm are typically immotile in extenders that are isotonic to the testis environment, and can usually be activated by hypotonic conditions for freshwater species, or by hypertonic conditions for saltwater species [11]. Thus, isotonic solutions are usually chosen as extenders for cryopreservation of externally fertilized species. However, sperm from internally fertilized fishes are motile in isotonic solutions, and immotile in hypotonic or hypertonic solutions [35]. For sperm from *X. helleri*, no significant difference in post-thaw motility was found between isotonic (HBSS at 310 mOsmol/kg) and hypertonic (HBSS at 500 mOsmol/kg) extenders; therefore, it was suggested that sperm from internally fertilized fish can be cryopreserved in hypertonic extenders in an unactivated state to minimize reduction of the energetic capacities necessary for insemination, traversal, and residence within the female reproductive tract prior to fertilization [36]. In the present study, sperm bundles suspended at 300 mOsmol/kg showed significantly higher post-thaw quality than did extenders at lower or higher osmolalities. This indicates that isotonic extenders can be effective for cryopreservation of goodeid sperm, although a wider range of osmotic pressures should be tested. In addition to their immobilization function, extenders can also

provide a stable physiochemical environment for cells during refrigerated storage and freezing. In the present study, sperm bundles suspended in HBSS had the longest survival and highest dissociation duration until about 144 h after thawing. After thawing and artificial insemination, a suitable extender can prolong the survival duration of sperm from livebearing fish for travel through the female reproductive tract.

Using the protocol developed for *X. eiseni* resulted in > 60% dissociable bundles immediately after thawing for *A. toweri* and *G. atripinnis* with about 20% remaining at 24 h. Although the percent survival of bundles declined rapidly after 24 h, the dissociation duration of bundles from *A. toweri* and *G. atripinnis* was about 2–3-fold longer than for those from *X. eiseni*, which could compensate for the loss of percent survival. The development of cryopreservation protocols can be costly, and time consuming, but by process generalization, a protocol developed with one species can often be applied as a starting point to other species within the same group to avoid cost of *de novo* protocol development [17,18,35,36,38–41]. For example, a generalized protocol developed for 3 *Xiphophorus* species at the AGGRC has been used successfully to freeze sperm from > 20 other species within the same genus. In addition, a generalized protocol for cryopreservation of sperm from Poeciliidae was also proposed [19]. There are more than 20,000 fish species in the world. In the future, protocol development of sperm cryopreservation for a large number of species will be needed because of the growing demand of germplasm repositories for aquaculture (food and ornamental fish), biomedical research, stock enhancement, and conservation programs. Process generalization of protocol development can be an efficient and reliable approach for future expansion of germplasm repositories for aquatic species. Generalization would be greatly enhanced if standardization or harmonization efforts could enable direct comparison of protocols and results [30].

In summary, a generalized protocol development process was tested with *X. eiseni*, *G. atripinnis*, and *A. toweri*: sperm bundles suspended in Ca^{2+}-free HBSS at 300 mOsmol/kg were mixed with 10% DMSO as cryoprotectant for 20 min of equilibration, loaded into 0.25-ml French straws, and cooled at a rate of 10 °C/min at 4.0×10^6 bundles/ml. Thawing was done at 40 °C in a water bath for 7 s, and bundles were resuspended in Ca^{2+}-free HBSS extender after centrifuging at 770 × *g* for 10 s. The protocol established with sperm bundles in the present study can be further tested for use with free sperm and could progress to study of fertilization. The evaluation of sperm bundles can be further enhanced by evaluation of sperm that are dissociated from bundles using assays such as viability, motility and morphology. The method of freezing sperm in bundle form can also be further studied on other fish groups that produce spermatozeugmata. This initial study on cryopreservation of goodeid sperm provides a foundation for further research and establishment of germplasm repositories for imperiled livebearing fishes.

Acknowledgements

This work was supported in part by funding from the National Institutes of Health, Office of Research Infrastructure Programs (R24-RR023998 and R24-OD011120), with additional support provided by the National Institute of Food and Agriculture, United States Department of Agriculture (Hatch project LAB94231), and the LSU-ACRES (Audubon Center for Research of Endangered Species) collaborative project (1617R0434). We thank H. Grier for suggestions and providing fish, and J. Schaff and J. Cane for maintaining fish and assisting with experiments. This manuscript was approved for publication by Louisiana State University Agricultural Center as number 2018-241-31609.

References

[1] N. Bailey, C.M. Garcia, M. Ritchie, Beyond the point of no return? A comparison of genetic diversity in captive and wild populations of two nearly extinct species of Goodeid fish reveals that one is inbred in the wild, Heredity 98 (2007) 360.
[2] H. Blackburn, Genebank development for the conservation of livestock genetic resources in the United States of America, Livest. Sci. 120 (2009) 196–203.
[3] H.D. Blackburn, The national animal germplasm program: challenges and opportunities for poultry genetic resources, Poult. Sci. 85 (2006) 210–215.
[4] A. Chaveiro, J. Liu, S. Mullen, H. Woelders, J.K. Critser, Determination of bull sperm membrane permeability to water and cryoprotectants using a concentration-dependent self-quenching fluorophore, Cryobiology 48 (2004) 72–80.
[5] A.E. Crosier, B.S. Pukazhenthi, J.N. Henghali, J. Howard, A.J. Dickman, L. Marker, D.E. Wildt, Cryopreservation of spermatozoa from wild-born Namibian cheetahs (*Acinonyx jubatus*) and influence of glycerol on cryosurvival, Cryobiology 52 (2006) 169–181.
[6] O. Domínguez-Domínguez, N. Mercado-Silva, J. Lyons, Conservation status of Mexican goodeids: problems, perspectives, and solutions, in: M.C. Uribe, H.J. Grier (Eds.), Viviparous Fishes, New Life Publications, Florida, 2005, pp. 515–524.
[7] O. Domínguez-Domínguez, N. Mercado-Silva, J. Lyons, H.J. Grier, The viviparous goodeid species, in: M.C. Uribe, H.J. Grier (Eds.), Viviparous Fishes, New Life Publications, Florida, 2005, pp. 525–569.
[8] O. Domínguez-Domínguez, E. Martínez-Meyer, L. Zambrano, L. De, G. Pérez Ponce, Using ecological-niche modeling as a conservation tool for freshwater species: livebearing fishes in central Mexico, Conserv. Biol. 20 (2006) 1730–1739.
[9] Q. Dong, C. Huang, T.R. Tiersch, Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters, Cryobiology 54 (2007) 87–98.
[10] J.R. Duncan, J.L. Lockwood, Extinction in a field of bullets: a search for causes in the decline of the world's freshwater fishes, Biol. Conserv. 102 (2001) 97–105.
[11] V. Dzyuba, J. Cosson, Motility of fish spermatozoa: from external signaling to flagella response, Reprod. Biol. 14 (2014) 165–175.
[12] H.J. Grier, Cellular organization of the testis and spermatogenesis in fishes, Am. Zool. 21 (1981) 345–357.
[13] A.J. Helmstetter, A.S. Papadopulos, J. Igea, T.J. Van Dooren, A.M. Leroi, V. Savolainen, Viviparity stimulates diversification in an order of fish, Nat. Commun. 7 (2016) 11271.
[14] Á. Horváth, W.R. Wayman, B. Urbányi, K.M. Ware, J.C. Dean, T.R. Tiersch, The relationship of the cryoprotectants methanol and dimethyl sulfoxide and hyperosmotic extenders on sperm cryopreservation of two North-American sturgeon species, Aquaculture 247 (2005) 243–251.
[15] J. Howard, C. Lynch, R. Santymire, P. Marinari, D. Wildt, Recovery of gene diversity using long-term cryopreserved spermatozoa and artificial insemination in the endangered black-footed ferret, Anim. Conserv. 19 (2015) 102–111.
[16] E. Hu, H. Yang, T.R. Tiersch, High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): establishment of an approach for commercial-scale processing, Cryobiology 62 (2011) 74–82.
[17] C. Huang, Q. Dong, T.R. Tiersch, Sperm cryopreservation of a live-bearing fish, the platyfish *Xiphophorus couchianus*, Theriogenology 62 (2004) 971–989.
[18] C. Huang, Q. Dong, R.B. Walter, T.R. Tiersch, Sperm cryopreservation of green swordtail *Xiphophorus helleri*, a fish with internal fertilization, Cryobiology 48 (2004) 295–308.
[19] C. Huang, C. Sun, X. Su, X. Zhao, M. Miao, Y. Liu, Q. Dong, Sperm cryopreservation in guppies and black mollies — a generalized freezing protocol for livebearers in poeciliidae, Cryobiology 59 (2009) 351–356.
[20] Y. Huang, D. Li, Y. Zhou, Q. Zhou, R. Li, C. Wang, Z. Huang, V. Hull, H. Zhang, Factors affecting the outcome of artificial insemination using cryopreserved spermatozoa in the giant panda (*Ailuropoda melanoleuca*), Zoo Biol. 31 (2012) 561–573.
[21] IUCN, The IUCN Red List of Threatened Species (Version 2017-3), the International Union for Conservation of Nature, (2017) accessed February 2018 http://www.iucnredlist.org.
[22] J. Kelley, A. Magurran, C.M. García, Captive breeding promotes aggression in an endangered Mexican fish, Biol. Conserv. 133 (2006) 169–177.
[23] Y. Liu, L. Torres, T.R. Tiersch, Quality evaluation of sperm from livebearing fishes: standardized assessment of sperm bundles (spermatozeugmata) from *Xenotoca eiseni* (Goodeidae), Theriogenology 107 (2018) 50–56.
[24] Y. Liu, H. Yang, L. Torres, T.R. Tiersch, Activation of free sperm and dissociation of sperm bundles (spermatozeugmata) of an endangered viviparous fish, *Xenotoca eiseni*, Comp. Biochem. Physiol., A: Mol. Integr. Physiol. 218 (2018) 35–45.
[25] A. Maceda-Veiga, O. Domínguez-Domínguez, J. Escribano-Alacid, J. Lyons, The aquarium hobby: can sinners become saints in freshwater fish conservation? Fish Fish. 17 (2016) 860–874.
[26] P. Mazur, S.P. Leibo, G.E. Seidel Jr., Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions, Biol. Reprod. 78 (2008) 2–12.
[27] J. Nynca, S. Judycka, E. Liszewska, S. Dobosz, A. Ciereszko, Standardization of spermatozoa concentration for cryopreservation of rainbow trout semen using a glucose-methanol extender, Aquaculture 477 (2017) 23–27.
[28] K. Piller, C. Kenway-Lynch, D. Camak, O. Domínguez-Domínguez, Phylogeography and population structure of the imperiled Redtail Splitfin (Goodeidae: *Xenotoca eiseni*): implications for conservation, Copeia 103 (2015) 440–454.
[29] N.F. Snyder, S.R. Derrickson, S.R. Beissinger, J.W. Wiley, T.B. Smith, W.D. Toone, B. Miller, Limitations of captive breeding in endangered species recovery, Conserv. Biol. 10 (1996) 338–348.
[30] L. Torres, E. Hu, T.R. Tiersch, Cryopreservation in fish: current status and pathways to quality assurance and quality control in repository development, Reprod. Fertil. Dev. 28 (2016) 1105–1115.
[31] L. Torres, Y. Liu, A. Guitreau, H. Yang, T.R. Tiersch, Challenges in development of sperm repositories for biomedical fishes: Quality control in small-bodied species,

Zebrafish 14 (2017) 552–560.
[32] USFWS, Listed FWS/Joint FWS and NMFS Species and Populations with Recovery Plans, the United States Fish and Wildlife Service, (2017) accessed February 2018 https://ecos.fws.gov/ecp0/pub/speciesRecovery.jsp?sort=1.
[33] S.A. Webb, J.A. Graves, C. Macias-Garcia, A.E. Magurran, D.O. Foighil, M.G. Ritchie, Molecular phylogeny of the livebearing Goodeidae (cyprinodontiformes), Mol. Phylogen. Evol. 30 (2004) 527–544.
[34] J.P. Wourms, Viviparity: the maternal-fetal relationship in fishes, Am. Zool. 21 (1981) 473–515.
[35] H. Yang, T.R. Tiersch, Current status of sperm cryopreservation in biomedical research fish models: zebrafish, medaka, and *Xiphophorus*, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 149 (2009) 224–232.
[36] H. Yang, L. Hazlewood, R.B. Walter, T.R. Tiersch, Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail *Xiphophorus helleri*, Cryobiology 52 (2006) 209–218.
[37] H. Yang, C. Carmichael, Z.M. Varga, T.R. Tiersch, Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in zebrafish *Danio rerio*, Theriogenology 68 (2007) 128–136.
[38] H. Yang, L. Hazlewood, R.B. Walter, T.R. Tiersch, Sperm cryopreservation of a live-bearing fish, *Xiphophorus couchianus*: male-to-male variation in post-thaw motility and production of F 1 hybrid offspring, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 149 (2009) 233–239.
[39] H. Yang, M.G. Savage, L. Hazlewood, R.B. Walter, T.R. Tiersch, Offspring production with cryopreserved sperm from a live-bearing fish *Xiphophorus maculatus* and implications for female fecundity, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155 (2012) 55–63.
[40] H. Yang, R. Cuevas-Uribe, M.G. Savage, R.B. Walter, T.R. Tiersch, Sperm cryopreservation in live-bearing *Xiphophorus* fishes: offspring production from *Xiphophorus variatus* and strategies for establishment of sperm repositories, Zebrafish 9 (2012) 126–134.
[41] H. Yang, L. Hazlewood, S.J. Heater, P.A. Guerrero, R.B. Walter, T.R. Tiersch, Production of F_1 interspecies hybrid offspring with cryopreserved sperm from a live-bearing fish, the Swordtail *Xiphophorus helleri*, Biol. Reprod. 76 (2007) 401–406.