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Production of live young with cryopreserved sperm from the endangered livebearing fish Redtail Splitfin (*Xenotoca eiseni*, Rutter, 1896)

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ABSTRACT

Previous studies of sperm cryopreservation of livebearing fish have been limited to two genera within the family Poeciliidae. The goal of the present study was to investigate the feasibility to produce live young of livebearing goodeids (family Goodeidae) with cryopreserved sperm, using aquarium-trade populations of the endangered species Redtail Splitfin (*Xenotoca eiseni*, Rutter, 1896). Reproductive condition of females was evaluated by histological categorization of ovarian development. A total of 117 females were inseminated with cryopreserved sperm, 81 were inseminated with fresh sperm, 27 were mixed with males for natural breeding, and 30 were maintained without males or insemination. Histological images of 34 mature females indicated 68% of ovaries had primary- or secondary-growth oocytes, and 32% had ovulated eggs. Ovarian development had no significant relationship ($P = 0.508$) with body wet weight, but had a relationship ($P < 0.001$) with ovary weight and gonadosomatic index. Sperm cells were observed within ovaries that were fixed at 12 h after insemination with fresh sperm. A total of 29 live young were produced from two females inseminated with thawed sperm (8% post-thaw motility with HBSS300 as extender, 20 min incubation in 15% DMSO, cooling rate at 10 °C/min, and thawing at 40 °C for 7 s), 12 were produced from two females with fresh sperm (1%–20% motility), 41 were produced from five naturally spawned females, and no live young were produced from the female-only group. This study provides a foundation for establishment of germplasm repositories for endangered goodeids to assist conservation programs.

1. Introduction

Sperm from more than 200 fish species have been cryopreserved (Torres et al., 2016), but among these only six are livebearing (viviparous) species (Huang et al., 2004a,c; Huang et al., 2009; Yang et al., 2012a,b). Compared to egg-laying (oviparous) fish, there are more challenges in developing procedures to study and apply sperm cryopreservation to livebearing species. For example, livebearing fish have internal fertilization (Meyer and Lydeard, 1993), and thus assessment of fertilization or hatching rates requires dissection of females or waiting periods of 30 to 80 d until females give birth (Wourms, 1981; Yang et al., 2007b). The fertilization or

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hatching rate of most of egg-laying fish, however, can simply be estimated by observation of embryonic development or hatching within hours or days after insemination (Linhart et al., 2000; Glogowski et al., 2002). As another example, the egg quality of egg-laying species can be evaluated upon spawning, but such evaluations would require destructive dissection of females for livebearing species.

Current studies of cryopreservation of livebearing fishes have been limited to two genera within the family Poeciliidae. *Xiphophorus* species have been the most studied due to their importance as cancer research models (Walter and Kazianis, 2001; Yang and Tiersch, 2009). Live young were harvested from about 20% of females inseminated with fresh or thawed sperm from *X. helleri* (Yang et al., 2007b), *X. couchianus* (Yang et al., 2009), *X. maculatus* (Yang et al., 2012b), and *X. variatus* (Yang et al., 2012a). Species from the genus *Poecilia*, such as the Guppy (*Poecilia reticulata*) and the Sailfin Molly (*P. latipinna*) are popular ornamental fish and research models. In these species, live young were harvested from about 50% of females inseminated with thawed sperm (Huang et al., 2009). There are no reports of production of live young with cryopreserved sperm of fish outside the family Poeciliidae. It is not known whether protocols developed for poeciliids can be successfully applied to fishes of the family Goodeidae (the second largest freshwater livebearing family), because the reproductive characteristics related to viviparity and internal fertilization of the two taxa evolved independently (Helmstetter et al., 2016), resulting in many differences. For example, freshwater livebearing fish usually package sperm in bundles, spermatozeugmata (naked bundles) or spermatophores (membrane enclosed bundles), which are believed to facilitate the transfer of sperm from male to female (Grier, 1981). Spermatozeugmata from poeciliids contain sperm with outwardly directed nuclei (Uribe et al., 2014) and can be thoroughly dissociated by crushing of the testes (Huang et al., 2004b), whereas bundles from goodeids hold sperm with inwardly directed nuclei (Uribe et al., 2014; Liu et al., 2018c) and can remain intact upon crushing of testes (Liu et al., 2018b). In addition, sperm cells of poeciliids have narrow-cylindrical heads and well-developed mitochondrial sheaths in elongated midpieces (Huang et al., 2004c), whereas heads of goodeid sperm are elliptical to spherical without elongated midpieces (Liu et al., 2018c). Such physical differences between sperm from poeciliids and goodeids could produce differential responses to physicochemical stressors that are inherent in the cryopreservation process (Watson, 2000).

The family Goodeidae is considered to be one of the most at-risk fish taxa in the world (Duncan and Lockwood, 2001). It is composed of four oviparous species inhabiting small springs of the southwestern Great Basin of the United States (Webb et al., 2004), and about 45 viviparous species in 18 genera inhabiting shallow freshwater streams within the Central Mexican Plateau (Webb et al., 2004). Among the viviparous goodeids, 12 are listed as critically endangered, endangered, or vulnerable, and three are extinct according to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (referred as 'the Red List') (IUCN, 2017). Moreover, another assessment (referred as 'the 2005 report') based on 18 years of field research in combination with a detailed scientific literature survey estimated that 35 species of livebearing goodeids were endangered, threatened, and vulnerable as of 2005 (Domínguez-Domínguez et al., 2005). Repositories based on sperm cryopreservation can be an important resource to goodeid conservation programs by preserving germplasm for future use at a relatively low cost, ensuring integrity of genetic diversity, assisting genetic management, and enhancing captive breeding (Mazur et al., 2008; Torres et al., 2016).

Previous studies have resulted in reports of sperm survival within bundles of goodeids after cryopreservation (Liu et al., 2018a), but no protocols have been developed for use with free sperm outside bundles and no live young have been produced with thawed sperm for goodeid fish. The goal of the present study was to investigate the feasibility to produce live young of viviparous goodeids with cryopreserved sperm, using an endangered species, the Redtail Splitfin (*Xenotoca eiseni*, Rutter, 1896) as a model. The specific objectives were to: (1) evaluate reproductive condition of males and females from a captive population, (2) develop sperm cryopreservation protocols based on the previous studies, and (3) evaluate production of live young. This study, therefore, was conducted for the evaluation of ovarian development in combination with sperm cryopreservation of livebearing fishes, and Goodeidae is the second family of freshwater livebearing fish documented for production of live young with use of cryopreserved sperm.

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, USA). The *X. eiseni* used in this study were an aquarium population of unknown genetic background maintained and bred for research purposes by H. Grier, and transported at about 2 months of age from Florida to the Aquatic Germplasm and Genetic Resources Center (AGGRC) of the Louisiana State University Agricultural Center (Baton Rouge, LA, USA) by overnight shipping. Fish were cultured at the AGGRC at 20–22 °C with 14 h:10 h (light:dark) photoperiod in two 800-l recirculating aquaculture systems with four tanks in each system. Fish were fed twice daily with tropical flakes (Pentair Aquatic Ecosystems, FL, USA) supplemented once to twice a week with thawed brine shrimp (Sally's Frozen Brine Shrimp™, San Francisco Bay Brand, CA, USA). Bubble-bead filters (Aquaculture Systems Technologies, LLC., LA, USA) on the recirculating systems were backflushed weekly. Additional water quality variables were monitored weekly and maintained within acceptable ranges including: pH (7.0–8.0), ammonia (0–1.0 mg/l), and nitrites (0–0.8 mg/l).

Males were identified by presence of orange-red coloration on posterior caudal peduncle, bluish coloration on the anterior caudal peduncle (adjacent to the orange-red area), and well-developed andropodia (split anal fins; Fig. 1A). Females were identified by the absence of orange-red and blue colorations on the caudal peduncle, black areas on the posterior abdomen, and absence of andropodia (Fig. 1B). Males and females were identified and separated at 4 months of age. At this age, there are some fish that are too small to be identified for gender. As such, to ensure no males were present in the female tanks, fish were individually evaluated again monthly from 4 to 8 months old. Two males were detected that had previously been placed in the female tanks at the 5th month and one male

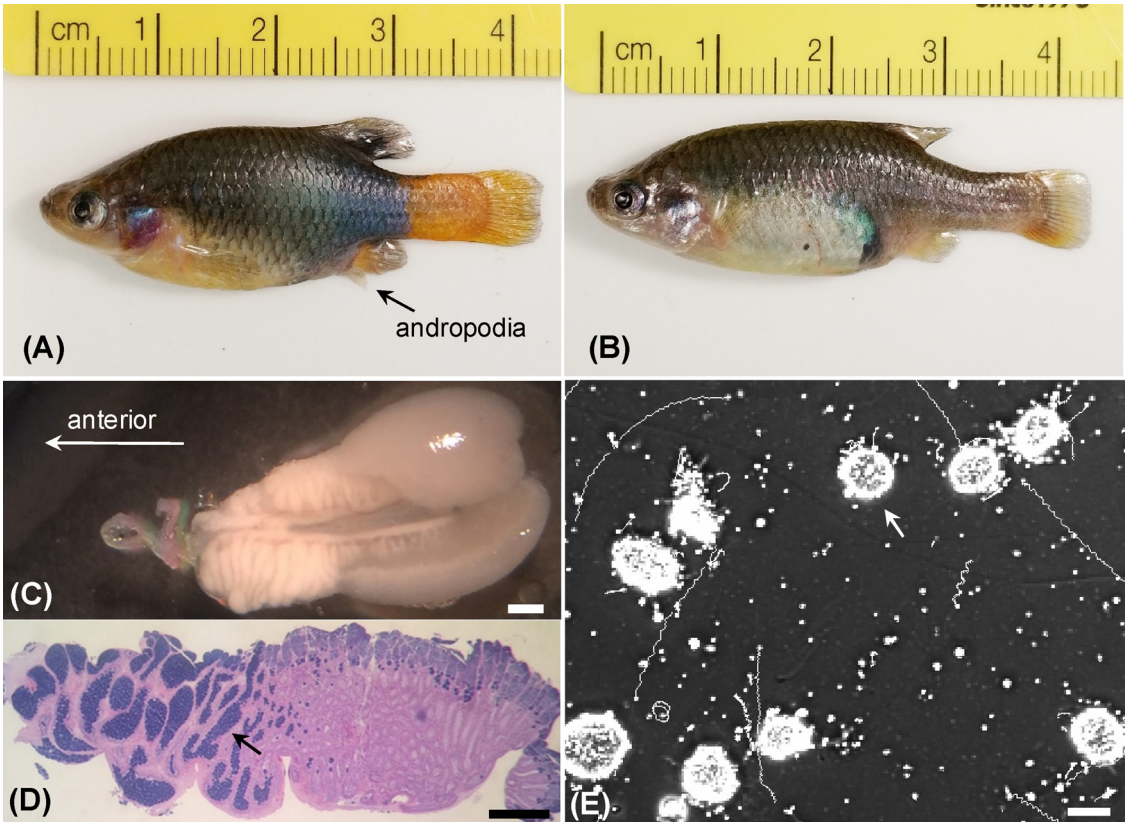

Fig. 1. Sperm collection from *X. eiseni*; (A) Mature males were identified by the presence of orange-red coloration on the posterior caudal peduncle, bluish coloration on the anterior caudal peduncle (adjacent to the orange-red area), and well-developed andropodia (split anal fins); (B) Female were identified by the absence of orange-red and blue colorations on the caudal peduncle, black areas on the posterior abdomen, and absence of andropodia; (C) Whitish coloration within the anterior half of a testis dissected from *X. eiseni*; Bar = 0.5 mm; (D) Sperm bundles (black arrow) were compactly distributed within anterior half of a testis observed by histological image (H-E staining); Bar = 0.5 mm; (E) Observation of free sperm and sperm bundles by use of a computer-assisted sperm analysis system (100 frames during 1.67 s) at 200-× magnification (the dots indicate quiescent sperm, tracks indicate sperm movement, and the arrow indicates a sperm bundle); Bar = 50 μm.

was found at the 6th month in the tanks containing the females. At the 7th and 8th months, only females were detected in the female tanks. Previous observations with the captivity conditions used for males and females indicated that the fish were sexually mature at 11 months of age, and the gestation duration was 55 to 65 d. Prior to artificial insemination, no females (at 12–14 months old) in the female tanks had given birth. There is no evidence that goodeids can store sperm for > 5 d (Macías-Garcia and Saborío, 2004) (this is different from poeciliids which can store sperm for more than a month). As such, the females used in this study were considered to have not been previously inseminated (virgin).

2.2. Histology

Histological samples were prepared to evaluate ovarian development, and to assist identification of sperm and sperm bundles within the female reproductive tract or ovary after artificial insemination. Fish were anesthetized with 0.03% tricaine methanesulfonate (MS-222, Western Chemical, Inc. WA, USA) and body wet weight and standard body length were measured. Ovaries and testes (Fig. 1C) were dissected, weighed, and fixed in 10% neutral buffered formalin for 12 h (MCC, Torrance, Canada) and subsequently stored in 70% ethanol prior to sample processing at the Histology Laboratory of the School of Veterinary Medicine, Louisiana State University. Samples were embedded with paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H-E). The oocyte development of 34 virgin females at 12 months of age (capable of reproduction based on our previous observations) was classified into six stages (from early to late) as oogonial proliferation (OP), chromatin-nucleolus (CN), primary growth (previtellogenesis, PG), secondary growth (vitellogenesis, SG), oocyte maturation (OM), and ovulation (OV) (Fig. 2A–D) (Grier et al., 2009). The gonadosomatic index (GSI) was calculated as: (testes or ovaries weight/body wet weight) × 100%.

2.3. Sperm collection

Mature males at 12–14 months old were anesthetized with 0.03% MS-222 and body wet weight and standard body length were

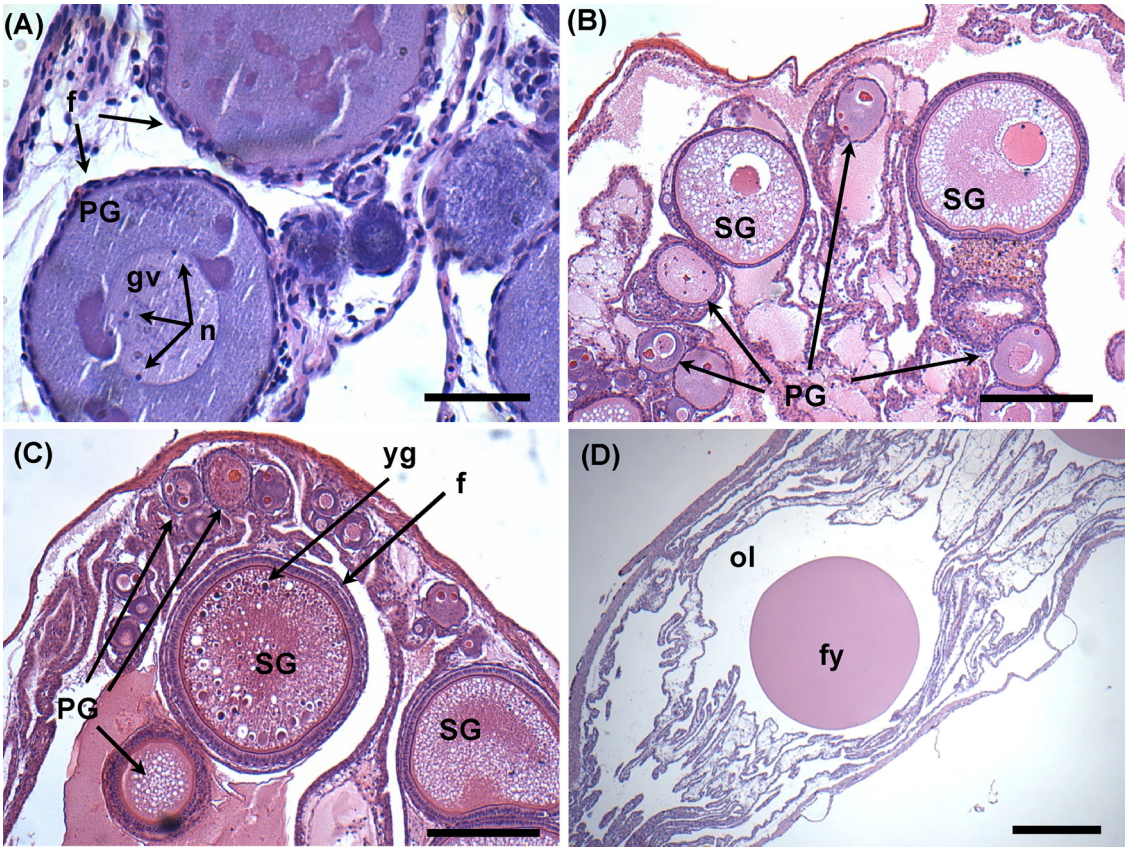

Fig. 2. Ovarian development of *X. eiseni* in 6 μm sections stained with H-E. (A–C) Immature oocytes; Primary-growth (PG) and secondary-growth (SG) oocytes were restricted to within follicle cells (f). In primary-growth oocytes, multiple nucleoli (n) were observed inside germinal vesicles (gv), and oil droplets (round globule shapes) were detected; Yolk globules (yg, dark stained) accumulated within secondary-growth oocytes but not in primary-growth oocytes. (D) Ovulated eggs were observed in the ovarian lumen (ol); Yolk globules fused to form fluid yolk (fy); Bar = 50 μm (A), 0.2 mm (B), 0.2 mm (C), and 0.4 mm (D).

measured. Histological images (Figs. 1D, 3 A and B) indicated sperm cells of *X. eiseni* were concentrated in the anterior section of the testes (Fig. 1C and D). This section was dissected, rinsed, weighed, placed in 50 μl of extender solution on a glass slide, and gently crushed with angled spade-tip forceps. The extender solution was Hanks' balanced salt solution at 300 mOsmol/kg (HBSS300) (Yang et al., 2007a). Sperm were released into the extender and collected with a pipette into a 1.5 ml centrifuge tube, and the volumes of suspensions (referred to as 'sperm suspensions') were adjusted to 50 μl by addition of the extender. Osmolality of the extenders were measured by use of a freezing point osmometer (Model 5010 OSMETTE III™, Precision Systems Inc., MA, USA).

2.4. Sperm evaluation

Sperm collected from crushed testes were found to be packed within spermatozeugmata (referred to as 'sperm bundles') or outside without packing (referred to as 'free sperm'). Bundles and free sperm were often mixed in suspensions (Fig. 1E). Free sperm were observed to be motile for more than 2 d in HBSS300 (Liu et al., 2018c), and thus no activation solutions were used. Sperm suspensions were mixed with extender at ratios ranging from 1:5 to 1:9 (sperm suspension:extender) on the base of a Makler® counting chamber (Sefi-Medical Instruments, Haifa, Israel), the cover slip was placed on top, and the concentration of free sperm was estimated. The motility and curvilinear velocity (VCL) of free sperm were measured at 10 s after mixing by use of a computer-assisted sperm analysis (CASA) system (HTM-CEROS, version 14 Build 013, Hamilton Thorne Biosciences, MA, USA) coupled with a dark-field microscope (CX41, Olympus Corporation, Tokyo, Japan) (200-× magnification) (Torres et al., 2017). Two to three measurements from different viewing areas with 100 to 200 free sperm in each observation were applied and averaged for each observation.

Activation of sperm within bundles was evaluated by classification of a total of 10–15 bundles distributed across a viewing area (with a total of three viewing areas in each observation) as 'quiescent bundles' or 'dissociating bundles'. In quiescent bundles, ≤ three sperm attached to the bundles were vibrating in place, and no sperm within the bundles were motile. In dissociating bundles > three sperm attached to the bundles were vibrating in place, or ≥ one sperm within the bundles swam away. The percentage of dissociating bundles (PDB) were used to evaluate activation levels of sperm within the bundles. Concentrations of bundles were measured by counting the number of bundles within three 1 × 1 mm squares of a standard hemocytometer (Hausser Scientific, Horsham, PA, USA)

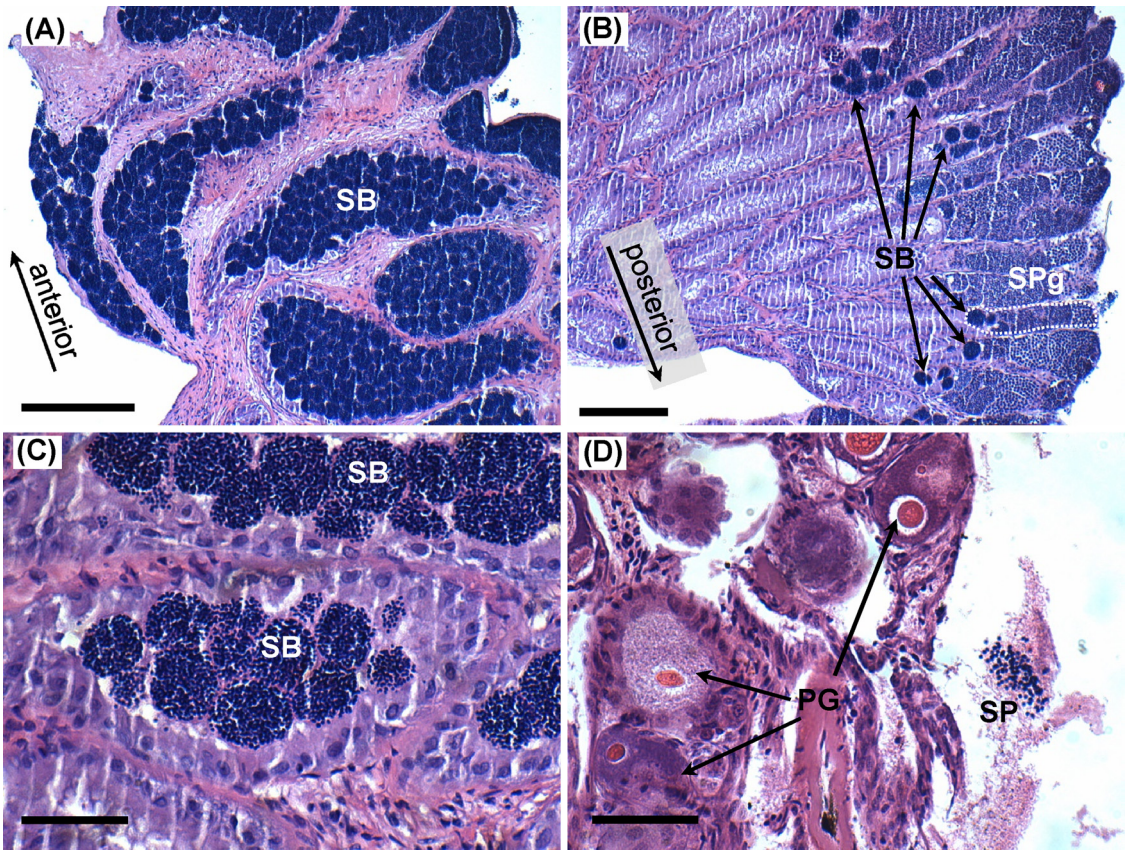

Fig. 3. Sperm cells within testis (A–C) and ovary (D) of *X. eiseni* after staining with H-E; Sperm cells were packed into sperm bundles (SB); (A) Sperm bundles were compact in the anterior section of the testis; (B) Spermatogenesis (SPg) processed within posterior sections of the testis, beginning with spermatogonia restricted to distal areas of lobules, with development progressing toward the proximal lobules, and ending with a few (< 5) packed sperm bundles within lobules; (C) Sperm cells within testes stained dark purple with a solid round shape; (D) Sperm cells (SP) in an ovary that was fixed at 12 h after insemination with fresh sperm; Primary-growth oocytes (PG) and sperm were present in the same section; Bars = 0.2 mm (A), 0.4 mm (B), 50 μm (C), and 50 μm (D).

(Liu et al., 2018b). A total of 24 mature males were used to evaluate testis development (within 30 min after dissection) by testing relationships among testis weight, GSI, PDB, motility, and concentrations of free sperm and bundles.

2.5. Freezing and thawing

Previous studies (Liu et al., 2018a) compared the effects of the cryoprotectants dimethyl sulfoxide (DMSO), methanol, and glycerol on quality of sperm bundles, showing that sperm within bundles had greatest post-thaw survival rates when mixed with 10% DMSO, equilibrated for 20 min, and cooled at 10 °C/min. Two trials were used in the present study to assess whether the protocol developed for bundles would be applicable to free sperm. In the first trial, sperm suspensions were mixed with equal volumes of the cryoprotectants (DMSO, methanol, and glycerol) at a final concentration of 10% diluted with HBSS300. Based on the results of this assessment, DMSO at final concentrations of 5%, 10%, and 15% were compared in a second assessment. Sperm suspensions mixed with cryoprotectant were drawn into 0.25 ml French straws (IMV International, Minnesota, USA), sealed with a handheld electronic straw sealer (Ultra Seal 21, Minitube USA, Inc., WI, USA), stored at room temperature (24–26 °C) for 20 min, and cooled in a controlled-rate freezer (IceCube 14M, SY-LAB, Neupurkersdorf, Austria) at a rate of 10 °C/min from 4 to −80 °C. 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 water bath at 40 °C. Motility and VCL of free sperm were measured immediately after equilibration (at the start of the freezing program), and at 2 min, 6 h, and 24 h after thawing. Four males were used in the first assessment as four separate replicates, and five males were used in the second assessment as five separate replicates.

2.6. Artificial insemination

Fresh (without cryopreservation) and thawed (cryopreserved with 5%, 10%, and 15% DMSO) sperm were used for artificial insemination. Free-sperm concentration was adjusted to 1–2 × 10^8 sperm/ml and bundle concentration was adjusted to 1 to 8 × 10^5

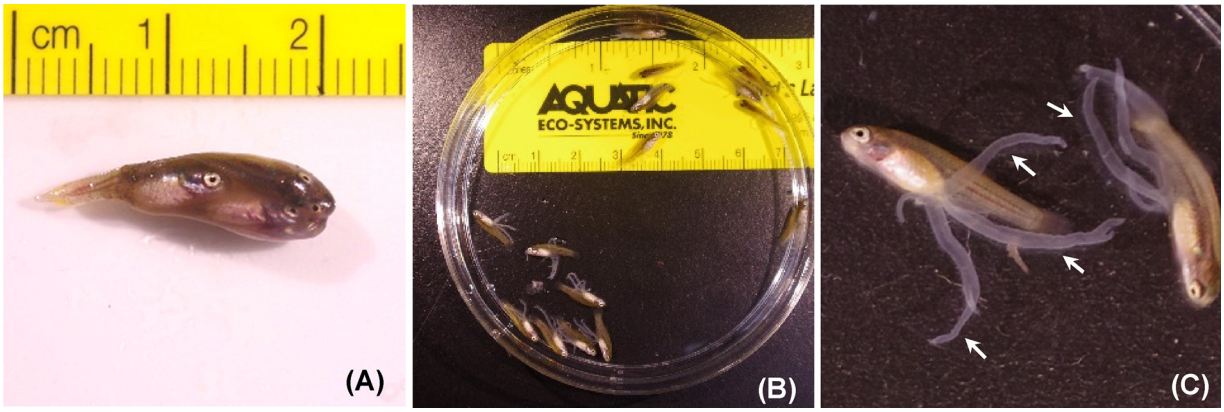

Fig. 4. Live young of *X. eiseni* produced by cryopreserved sperm; (A) Live young inside of a dissected ovary; (B) Live young in tank water after release from dissected ovaries; (C) Trophotaenia (ribbon-like structures indicated by white arrows contributing to maternal-fetal nutrient delivery) were observed connected to live young after collection.

bundles/ml. Females were anesthetized with 0.03% MS-222, and body wet weight and standard body length were measured. Specific growth rates of females were calculated as $((\ln_{\text{weight at insemination}} - \ln_{\text{weight at dissection}}) / \text{number of days}) \times 100\%$. Fish were rinsed with the extender (HBSS300), dried with kimwipes (Kimberly-Clark, TX, USA), placed on their back on a centrally hollowed sponge. A total of 117 females were inseminated with thawed sperm, and 81 females with fresh sperm. Sperm suspensions (3–5 μl) were injected into the genital opening of females with a 10-μl capillary (Drummond Scientific, PA, USA) by breath pressure through a rubber tube (Drummond Scientific) (Yang et al., 2007b). After artificial insemination, females were returned to tank water for recovery and held in a 1200-l recirculating water system with eight tanks until dissection. A tank containing16 males and 27 females without artificial insemination were used as a natural spawning group to serve as a positive control, and a tank with 30 virgin females without males was used as a negative control. In a separate experiment, at 30 min, 60 min, 120 min, and 12 h after artificial insemination with fresh sperm, ovaries from 3 to 7 were dissected, fixed, and processed for histology for observation of sperm presence and transport through the female reproductive tract.

The gestation duration of *X. eiseni* was 55–65 d in the water quality conditions in which the present study was conducted and based on previous observations (Schindler and de Vries, 1987). To avoid predation by adult fish, live young were collected by dissection of females at 52–60 d after artificial insemination. At this stage, embryo development at dissection was considered as near complete and females were considered to be at a reproductive stage that was near the time when birth would occur. Females were classified as being pregnant when live young were present in the ovaries at dissection (Fig. 4A). A term used in mammalian reproduction research (Hu, 2012), 'conception rate' (Grimard et al., 2006), was used in this study to characterize pregnancy, and was calculated as: (number of pregnant females / number of artificially inseminated females) × 100%. Fertilized females with embryos that did not develop live young (at dissection) were not able to be reliably identified with these techniques, and thus the number of females that were fertilized could be greater than those that were classified as pregnant at 52–60 d post-insemination.

No live young were released from females prior to dissection based on daily observation. Body wet weights of 233 females were measured, ovaries were dissected, and ovary weights were measured (ovaries of another 34 representative females were sampled for histology as described above). The live young were collected into tank water in petri dishes, and standard lengths were measured. After removal of live young, ovary tissues were weighed. The body wet weights of live young were estimated as: (total ovary weight – residual ovary tissue weight) / number of live young. Live young were transferred to 2.8-l tanks in a recirculating system (Aquaneering, CA, USA), and the mortality was calculated at 5 d after birth. Mortalities of female broodstock were calculated as ((number at artificial insemination – number at dissection) / number at artificial insemination) × 100%.

2.7. Data analysis

All statistical analyses were performed using SAS 9.4 (SAS Institute, NC, USA). Simple linear regressions (PROC REG) were performed to predict standard length and ovarian weight as associated with body weight of females, and to evaluate the paired relationships between variables in male: weight, standard length, testis weight, GSI, PDB, motility, free sperm concentration, and bundle concentration. A two-sample *t*-test (PROC TTEST) was used to compare the mortality of females used for insemination with thawed and fresh sperm. One-way analysis of variance (ANOVA) was used to identify significant differences in body weights and ovary weights, and GSI of females among different ovarian development stages (Bonferroni's test for unequally sized samples), and in sperm motility and VCL among different cryoprotectants (Tukey's multiple comparisons for equal-sized samples). Repeated-measures ANOVA was used to identify significant differences in post-thaw motility and VCL among different concentrations of DMSO over post-thaw duration. Data were square-root or reciprocal transformed (for evaluation of ovarian development) prior to statistical analyses when assumptions of one-way ANOVA of normal distribution or equal variances were not met. When the assumptions were not met after transformations, Kruskal-Wallis non-parametric ANOVA (PROC NPAR1WAY) was performed. The results were considered

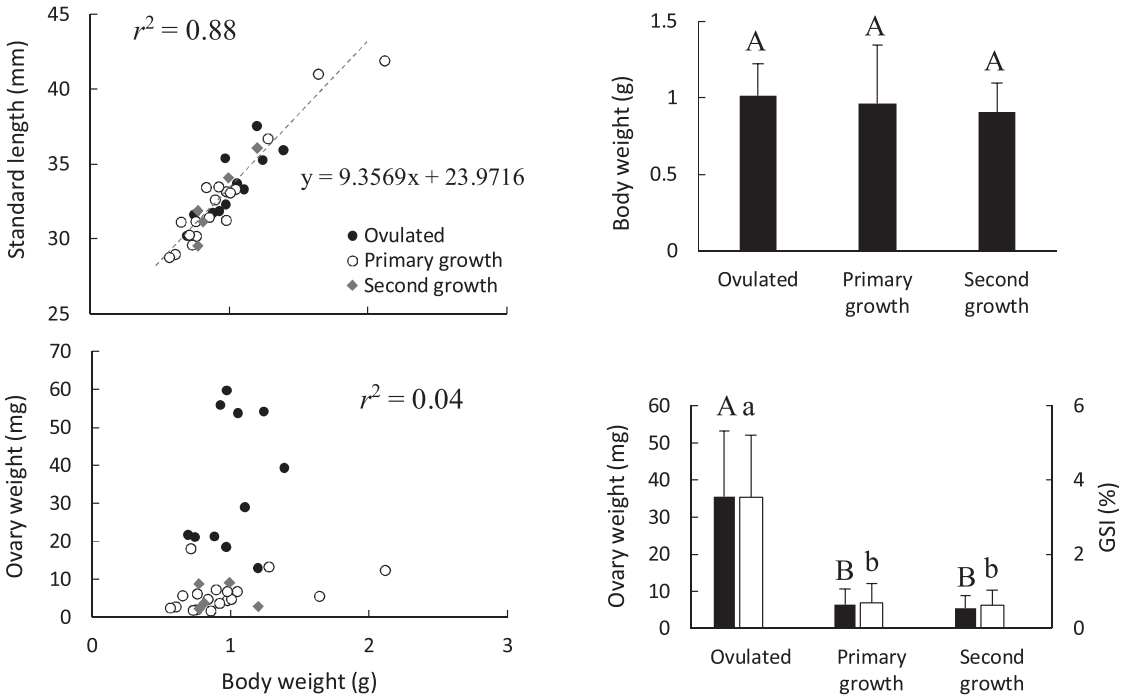

Fig. 5. Ovarian development of 34 female *X. eiseni* (12–14 months old) from a captive population; Relationships between body weight and standard length and ovary weight are depicted in the left panels; Among 34 ovaries, 15% were classified as primary-growth stages, 53% were as secondary-growth stages, and 32% had ovulated eggs; Ovarian development had no apparent relationship (top right) with body weight, but had a significant relationship (bottom right) with ovary weight (black bars with capitalized letters) and GSI (white bars with lower-cased letters).

statistically significant at $P < 0.05$.

3. Results

3.1. Ovarian development

The weights of ovaries (used in histological observations) were 15.6 ± 14.3 mg and the lengths were 7.8 ± 2.1 mm. The most advanced developmental stages detected in 34 ovaries were primary growth (53%), secondary growth (15%), and ovulated eggs (32%) (Figs. 2A–D, 4). Primary-growth and secondary-growth oocytes were only located within ovarian follicles. In primary-growth oocytes, one or multiple nucleoli could be observed oriented close to the inner membranes of the germinal vesicles (Fig. 2A and B). In later primary growth steps, oil droplets began to accumulate, but no yolk globules were observed. In secondary-growth ovaries scattered yolk globules were observed (Fig. 2C). Ovulated eggs were observed in the ovarian lumen without follicles and yolk globules were completely fused, forming fluid yolk (Fig. 2D). The diameter of oocytes increased from 80 to 150 μm for primary-growth stages, to 0.3 to 0.4 mm for secondary-growth stages, and ultimately to 0.5 to 0.8 mm for ovulated eggs.

Body weight had a significant linear relationship with standard length ($P < 0.001$, $r^2 = 0.88$) but not with ovary weight ($P = 0.263$, $r^2 = 0.04$) (Fig. 5). There were no differences in body weight among females at the primary-growth, secondary-growth, or ovulated oocyte stages ($P = 0.508$) (Fig. 5). Ovarian weights ($P < 0.001$) and GSI ($P < 0.001$) were greater in females with ovulated eggs than in females with primary-growth and secondary-growth oocytes (Fig. 5).

3.2. Testis development

Sperm nuclei were solid, round, and stained dark purple in histological images. Sperm were packed into sperm bundles within the testes (Fig. 3A–C). Within anterior testis sections (Fig. 1D, 3A) sperm bundles were grouped within sperm ducts. Within posterior sections (Fig. 1D, 3B) various stages of spermatogenesis were observed, beginning as spermatogonia restricted to the distal areas of lobules, developing toward proximal lobules, and ending with a few (< 5) packed sperm bundles within lobules.

Variables of 24 males at 12–14 months old were used to evaluate reproductive development (Table 1). The percentage of dissociating bundles (PDB) was 20 ± 13% (mean ± SD), and the motility of free sperm was 7 ± 17%. The motility varied from a minimum of 0% to a maximum of 79%. There were linear relationships between variables for body weight, standard length, testis weight, GSI, sperm concentration, and bundle concentration, but there were no significant linear relationships between these variables and PDB, or sperm motility. Also, there was no significant linear relationship between PDB and sperm motility.

Table 1
Reproductive condition of 24 male *X. eiseni* (12–14 months old) in a captive population.

	Body weight (g)	Standard length (mm)	Testis weight (mg)	GSI[a] (%)	PDB[b] (%)	Motility (%)	Concentration	
							Sperm ($\times 10^8$/ml)	Bundle ($\times 10^5$/ml)
Variables								
Mean ± SD	1.5 ± 0.9	36.6 ± 8.1	10.6 ± 8.3	0.64 ± 0.17	20 ± 13	7 ± 17	2.2 ± 1.0	8.0 ± 3.5
Maximum	3.3	49.4	27.6	1.01	47	79	4.2	14.6
Minimum	0.5	26.0	2.4	0.35	0	0	0.8	1
Relationships (*P* values[c])								
Body weight (g)		< 0.001	< 0.001	0.002	0.849	0.435	0.011	0.010
Standard length (mm)			< 0.001	0.012	0.739	0.418	0.026	0.024
Testis weight (mg)				< 0.001	0.573	0.373	0.002	0.001
GSI[a] (%)					0.359	0.364	0.002	0.002
PDB[b] (%)						0.166	0.574	0.621
Motility (%)							0.086	0.297
Sperm concentration ($\times 10^8$)								0.018

[a] GSI: Gonadosomatic index.
[b] PDB: Percentage of dissociating bundles.
[c] *P* values were generated from simple linear regression between each two variables.

3.3. Sperm cryopreservation

In the first experiment, three different cryoprotectants were compared at a final concentration of 10%. There were no significant differences ($P = 0.118$) in motility (16 ± 12% to 34 ± 14%) or VCL (70.8 ± 3.6 μm/s to 98.9 ± 6.2 μm/s) between fresh sperm and samples after equilibration with 10% DMSO, methanol, and glycerol (Fig. 6). Post-thaw motility of sperm incubated with DMSO (5 ± 2%) was greater ($P = 0.016$) than those with glycerol (1 ± 1%), but there were no significant differences ($P = 0.888$) of post-

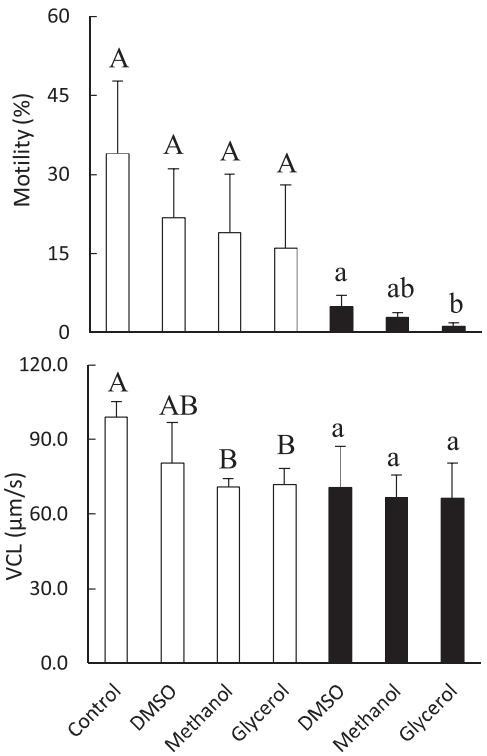

Fig. 6. Effects of 10% of dimethyl sulfoxide (DMSO), methanol, and glycerol as cryoprotectants on equilibration (white bars) and post-thaw (black bars) motility (upper panel) and VCL (lower panel) of sperm from *X. eiseni*; Shared capitalized letters above the bars represent no significant differences between control (fresh sperm) and equilibration, and shared lowercase letters represent no differences between post-thaw samples; $n = 4$ males.

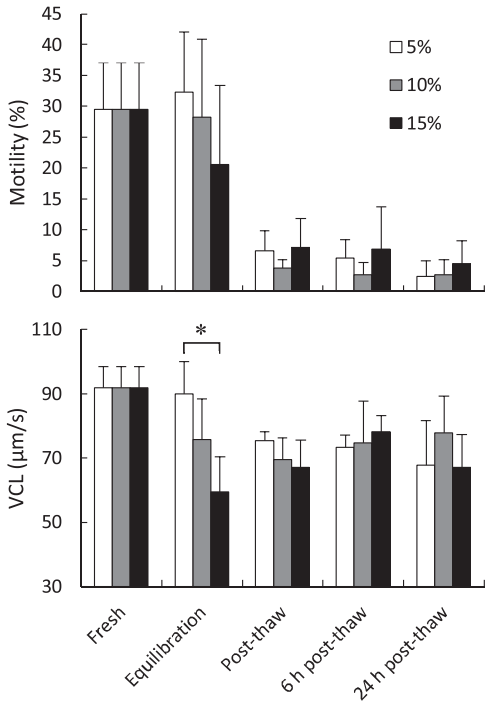

Fig. 7. Effects of 5, 10, and 15% of dimethyl sulfoxide (DMSO) as cryoprotectant on equilibration and post-thaw motility and VCL of sperm from *X. eiseni*. Only equilibration velocity were different (asterisk) among the three concentrations; $n = 5$ males.

thaw VCL (66.4 ± 14.2 to 70.6 ± 16.6 μm/s) among DMSO, methanol, and glycerol treatments (Fig. 6).

Based on the results of the first trial, DMSO was used as cryoprotectant in the second experiment. Motility of fresh sperm in this trial was 29 ± 8% and VCL was 92.0 ± 6.5 μm/s (Fig. 7). After 20-min equilibration, there were no significant differences ($P = 0.817$) in motility (21 ± 13% to 32 ± 10%) among the 5%, 10%, and 15% of DMSO treatments, but VCL of 5% DMSO (89.9 ± 10.1 μm/s) was greater ($P = 0.004$) than 15% (59.3 ± 11.0 μm/s; Fig. 7). Motility of sperm with 5%, 10%, and 15% of DMSO at 2 min, 6 h, and 24 h after thawing ranged from 2 ± 3% to 7 ± 7%. Post-thaw VCL with use of the three concentrations of DMSO at the three time points ranged from 67.1 ± 8.6 μm/s to 78.1 ± 5.1 μm/s (Fig. 7). There were no significant differences in post-thaw motility and VCL among the three concentrations ($P = 0.232$ for motility and $P = 0.845$ for VCL), or among the three time points ($P = 0.081$ for motility and $P = 0.377$ for VCL).

The post-thaw motility and VCL of cryopreserved sperm used for artificial insemination was 7%–9% and 48.4–76.8 μm/s (Table 2). The motility and VCL of fresh sperm (without cryopreservation) used for artificial insemination was 1%–79% and 89.6–97.5 μm/s (Table 2).

3.4. Harvest of live young

There were no differences ($P = 0.855$) in body weight of females at insemination among groups of thawed sperm, fresh sperm, natural spawning, and female only. At 52–60 d after artificial insemination, live young were harvested by dissection of ovaries. After release into tank water, live young began to swim within 10 s (Fig. 4B). Trophotaenia (ribbon-like structures contributing to maternal-fetal nutrient delivery) (Wourms, 1981; Iida et al., 2015) were observed connected to abdominal areas (Fig. 4C). At dissection, there were no significant differences ($P = 0.738$) in mortality of females between insemination with thawed (2%–21%) and fresh (0%–25%) sperm (Table 2). No females died within the natural-spawning and female-only groups.

The specific growth rate of female body weight varied from tank to tank, ranging from 0.20% to 1.50%/d (Table 2). No live young were harvested from females without artificial insemination or natural spawning (female-only negative control), or from females inseminated with sperm frozen with 5% or 10% DMSO. Live young were harvested from females of the fresh-sperm group with a conception rate of 6% to 7% per tank, and the 15%-DMSO group with a conception rate of 6%, and the natural spawning group with a conception rate of 19%. Brood size was 15 for the 15%-DMSO group, 5 to 7 for the fresh-sperm group, and 8 for the natural spawning group. Based on these brood sizes, the number of live young per female was 0.8 for the 15%-DMSO group, 0.3 to 0.4 for the fresh-sperm group, and 1.6 for the natural spawning group. At 5 d after harvest, one live young died from the 15%-DMSO group, but no live young died from the fresh-sperm group or the natural spawning group.

Histological images (Fig. 3D) indicated sperm cells were present in an ovary that was fixed at 12 h after artificial insemination with fresh sperm. Sperm were observed to be grouped within the ovarian lumen (about 3 mm measured from the genital opening end), and primary-growth oocytes were observed (Fig. 3D) in the same ovary. No sperm were observed from ovaries fixed at 30, 60,

Table 2
Production of live young and reproductive parameters of males and females used in experiments.

	Thawed (5% DMSO)	Thawed (10% DMSO)	Thawed (10% DMSO)	Thawed (15% DMSO)	Fresh	Fresh	Fresh	Natural spawning	Female only
Males									
Male number	2	2	1	2	2	1	1	16	0
GSI[a] (%)	0.93	0.93	0.82	0.93	0.51	0.56	0.82	N/A	N/A
PDB[b] (%)	22	22	0	22	45	12	0	N/A	N/A
Fresh motility (%)	43	43	79	43	1	20	79	N/A	N/A
Post-thaw motility (%)	9	8	7	8	N/A	N/A	N/A	N/A	N/A
Post-thaw VCL (μm/s)	76.8	62.8	48.4	69.1	92.3	89.6	97.5	N/A	N/A
Females									
Body weight before AI[c] (g)	0.96 ± 0.34	1.21 ± 0.60	0.86 ± 0.58	0.72 ± 0.38	0.90 ± 0.22	0.90 ± 0.27	0.80 ± 0.34	0.84 ± 0.38	0.83 ± 0.30
Body weight at dissection (g)	1.08 ± 0.36	1.43 ± 0.58	2.17 ± 1.20	1.00 ± 0.59	1.44 ± 0.56	1.54 ± 0.40	1.16 ± 0.35	1.05 ± 0.49	1.45 ± 0.51
Number at dissection	40	22	11	34	15	17	37	27	30
Mortality (%)	2	15	21	6	0	0	25	0	0
SGR[d] (% BW/Day)	0.2	0.28	1.50	0.59	0.91	0.90	0.68	0.46	1.03
Conception number	0	0	0	2	1	1	0	5	0
Conception rate (%)	0	0	0	6	7	6	0	19	0
Offspring									
Number of offspring	0	0	0	29	5	7	0	41	0
Number per brood	0	0	0	15	5	7	0	8	0
Number per female used	0	0	0	0.8	0.3	0.4	0	1.6	0
Body weight (mg)	N/A	N/A	N/A	38	28	34	N/A	28	N/A
Standard length (mm)	N/A	N/A	N/A	10.7 ± 0.9	11.3 ± 0.4	11.0 ± 0.9	N/A	11.5 ± 1.1	N/A
Mortality after 14 h	N/A	N/A	N/A	3%	0	0	N/A	0	N/A

[a] GSI: gonadosomatic index.
[b] PDB: Percentage of dissociating bundles.
[c] AI: artificial insemination.
[d] SGR: specific growth rate.

or 120 min after artificial insemination.

4. Discussion

Previous production of live young in livebearing fish by use of cryopreserved sperm has been limited to six species in the family Poeciliidae. The goal of the present study was to investigate the feasibility of production of live young with thawed sperm in the family Goodeidae using *X. eiseni* as a model. For the first time in the present study, there were evaluation of ovarian development in the study of sperm cryopreservation of livebearing fish. Because fertilization occurs within the ovaries, dissection and histology procedures were utilized. Ovulation is not required for fertilization in goodeids because intrafollicular fertilization can occur prior to ovulation (Wourms, 1981), but mature intra-follicular oocytes were not observed in the present study. Thus, it appeared that mature oocytes in the present study had already undergone ovulation prior to dissection. As such, 32% of the females in this group were presumably capable of producing fertilized oocytes. The ovarian development of *X. eiseni* in natural conditions is unknown, but the 32% maturity rate observed was within the range of 5% to 50% that has been observed in another goodeid (*Ameca splendens*) sampled monthly from their natural habitats (Ortiz-Ordonez et al., 2007). In the present study, females with mature oocytes had greater ovarian weights and GIS values than did those with immature oocytes. Body weights, however, did not reflect these differences. In addition, there was a linear relationship between body weight and standard length, but not with ovarian weight, indicating that reproductive status of females could not predicted by body weight or standard length without dissection of the ovaries. Further studies could be done on the prediction of ovarian development using hormonal concentrations (Zohar and Mylonas, 2001) or physical examination techniques such as ultrasonography (Guitreau et al., 2012; Novelo and Tiersch, 2016).

In contrast to unpredictable female reproductive conditions, there was a linear relationship between concentrations of free sperm and bundles with body weight, standard length, testis weight and GSI. Thus, if a specific concentration of sperm or bundles is desired based on experimental or breeding designs, the number of males needed can be predicted to avoid waste. Different from concentration, however, the motility of free sperm and bundle dissociation were unpredictable using the variables that were previously described, suggesting that having males with more sperm did not indicate that there was greater sperm motility of free sperm or sperm within bundles. Interestingly, the motility of free sperm was markedly variable among individuals, ranging from a minimum of 0% to a maximum of 79%. It is unclear what factors affected sperm motility that resulted in this large amount of variation, but further studies could be conducted on the effects of feeding, water quality, or seasonality. The body weight (1.5 ± 0.9 g), standard length (36.6 ± 8.1 mm) and testis weight (10.6 ± 8.3) were generally greater than those reported for poecilids such as *P. reticulata* (Huang et al., 2009), *P. latipinna* (Huang et al., 2009), and *X. helleri* (Yang et al., 2007b) (0.29-0.8 g for body weight, 23.2 to 34.8 mm for standard length, and 6.9 to 10.3 mg for testis weight). In 50 µl of sperm suspension for each *X. eiseni*, the average concentration was 2.2×10^8/ml for free sperm and 8.0×10^5/ml for bundles. Based on 5000 sperm per bundle (Uribe et al., 2009), the total number of sperm cells (within bundles + outside) per testis can be calculated as 1.5×10^7, less than those reported for poeciliids which had 5.5 to 11.4×10^7 sperm per testis (Huang et al., 2009). Histological images indicated sperm cells were compact within bundles in the anterior testes. Thus, collection of sperm from the anterior half of testes could avoid having undue debris in the sample.

The use of glycerol as cryoprotectant resulted in greater post-thaw sperm motility in poeciliids than with use of other cryoprotectants such as DMSO, methanol, *N*-dimethyl formamide (DMF) (Huang et al., 2004a,c; Huang et al., 2009). Previous studies, however, indicated that use of DMSO resulted in a greater post-thaw viability of sperm within bundles in goodeids than with use of glycerol (Liu et al., 2018a). When free sperm were considered in the present study, the use of DMSO resulted in marginally greater post-thaw motility than with use of glycerol, consistent with previous findings using cryopreserved bundles. With cryopreservation of sperm from *Xiphophorus* species the removal of glycerol by washing resulted in longer post-thaw sperm motility (Dong et al., 2006), suggesting possible toxic effects of glycerol on sperm health after thawing. The post-thaw condition of sperm is especially important for livebearing species, because sperm must traverse the female reproductive tract to complete fertilization. As such, DMSO was chosen for cryopreservation, although the marginal superiority of DMSO was not sufficient to ascertain an advantage compared with use of glycerol from a fertilization perspective. There were no significant differences in post-equilibration sperm motility when DMSO, methanol, and glycerol were used, and for fresh sperm controls, indicating that differences in post-thaw sperm motility when using DMSO and glycerol were attributed to the differential protective effect during freezing and thawing, and not to toxic effects at room temperatures. There were no significant differences in post-thaw sperm motility with use of 5%, 10%, and 15% DMSO, or at 2 min, 6 h, and 24 h after thawing, suggesting that sperm from *X. eiseni* were not sensitive to the concentration of DMSO within this range, and once the sperm survived freezing and thawing sperm could survive the presence of DMSO for 24 h. Post-thaw sperm motilities were 50% to 70% for poeciliids (Huang et al., 2004c; Yang et al., 2007b; Huang et al., 2009; Yang et al., 2009, 2012a,b), which was greater than the 2% to 9% observed in the present study. This could be because of the relatively less quality of fresh sperm used in the present study with initial motilities of 29% to 34% compared to the 70% to 90% initial motilities used in the cryopreservation of poeciliids.

The post-thaw velocity of sperm from livebearing fish is important because sperm have to traverse the female reproductive tract for fertilization to occur within the ovaries. With the present study, it was the first time sperm velocities (VCL) were reported for thawed sperm of livebearing fish. Although different cryoprotectants affected the survival of sperm, there were no significant differences in VCL among different cryoprotectants when using different concentrations of DMSO, and at different times after thawing. The exact length of the reproductive tracts of females were not measured in the present study because the tracts were too short (< 0.8 mm) to be distinguishable from the ovaries. The average ovary length observed was about 7.8 mm, and the observed VCL was about 90 µm/s for fresh sperm, and 70 µm/s for thawed sperm cryopreserved with DMSO.

The precise paths of sperm transport within the female reproductive tract remain unknown (to fertilize eggs, sperm might traverse

the anterior end of ovary, stop at the entrance, or be located somewhere between), and thus the maximum distance that sperm might have traversed in a straight line was estimated (conservatively) as 7.8 + 0.8 = 8.6 mm (The combined-straight-line distance of the entire reproductive tract). Based on these values, it was calculated that it would require about 96 s for sperm to travel directly to the anterior end (8.6 mm) of the ovaries for fresh sperm, and about 123 s for the thawed sperm. Interestingly, sperm were not observed in ovaries fixed after 30, 60, or 120 min after insemination with fresh sperm, but were observed at 12 h after insemination. It is possible that sperm could enter the ovaries before 12 h but this was not observed, perhaps because the sample sizes were small (< 7 ovaries for each time point) and only 2–33 sections of each ovary were stained for histology. The observation of sperm within the ovaries, however, indicates that fresh sperm can enter the ovaries within 12 h. If it is assumed that resistant forces (viscosity) (Butts et al., 2017) of ovarian fluid and swimming paths (Wilson-Leedy and Ingermann, 2007) of sperm were consistent among individual females, thawed sperm could arrive at the anterior end of the ovaries within about 16 h based on calculations previously described. This estimate is consistent with the survival of cryopreserved sperm observed within 24 h after thawing as previously described.

In the present study, live offspring were harvested from females inseminated with cryopreserved and fresh sperm. Poeciliid females can store sperm, and sperm from a single spawning or insemination can result in fertilization of multiple broods (Holt and Lloyd, 2010). Thus, to ensure that the live young produced were fertilized by inseminated cryopreserved sperm, hybridization and paternity assessments were usually performed (Yang et al., 2007b; Yang and Tiersch, 2009). Unlike poeciliids, goodeid females do not store sperm for more than several d (Uribe et al., 2009) and usually have a 2-month gestation period. As such, females can be considered to be non-inseminated if they were held without males for more than 2 months prior to insemination without pregnancy or production of young. In the present study males and females were separated at 4 months of age, and at least 4 months prior to insemination no males were observed in the female tank. To ensure that there was not fertilization in females prior to insemination, a negative control tank was maintained with females only, and no pregnancies were observed, and no live young were produced in this group. Live young were harvested from thawed (15% DMSO) sperm with post-thaw motility of 8%, and from fresh sperm with motility of 1% and 20%, but none were produced with fresh sperm with 79% motility, suggesting that post-thaw motility *per se* was not a critical factor of live young production, or that sperm bundles contributed to fertilization.

The conception rates of poeciliids inseminated with cryopreserved sperm was similar to that of fresh sperm (< 20%) for the genus *Xiphophorus* for *X. helleri* (Yang et al., 2007b), *X. couchianus* (Yang et al., 2009), *X. maculatus* (Yang et al., 2012b), and *X. variatus* (Yang et al., 2012a), and about 50% for the genus *Poecilia*, such as *P. reticulata* and *P. latipinna* (Huang et al., 2009). Each of these were greater than the 0% to 6% conception rate with cryopreserved sperm in the present study. A relatively less oocyte maturity rate (32%), however, could account for the approximate 70% the conception failure. Conception rates of the natural spawning group (19%) and fresh sperm group (6%–7%) appeared to be greater than that of thawed sperm group (not tested statistically), suggesting that natural infertility, artificial insemination, and cryopreservation could also account for the conception rate of the cryopreserved group, and be factors in addition to female reproductive conditions that accounted for most of the low productivities. These calculations also suggested that assessment of ovarian development of livebearing fish is important for quality control of the cryopreservation procedures when selecting females to be used for insemination. This also appears to be true for Poeciliidae (Yang et al., 2012a,b). The present study, however, was designed to investigate feasibility of production of live young with cryopreserved goodeid sperm with relatively small sample sizes for females, and thus further studies are needed to statistically analyze the effects of each factor on production of live young. Future studies of sperm cryopreservation for livebearing fishes could utilize females which have previously produced live young as proven spawners to improve reproductive efficiency. For species that do not store sperm for more than a few days (such as goodeids), female proven spawners could be isolated from males for a safe time prior to artificial insemination, whereas for species that can store sperm for months (such as poeciliids) live young can be produced by interspecies hybridization to provide proven spawners for fertilization assessments.

In addition, it is important to predict the productivity (number of live young that potentially can be harvested) and costs (e.g., number of male and female broodstock needed) of conducting procedures. For example, in a hypothetical conservation program if 100 live young are needed to be produced each year to maintain specific amount of genetic diversity for the next 20 years, based on the estimation in the present study (0.8 offspring produced for each female used for insemination), it can be calculated that the female broodstock needed for this program per year would be = 100 / 0.8 = 125. Calculations such as these can be conducted for prediction of broodstock needs and sample costs, and with modifications based on improvements in procedures would be useful to direct future applied goals in addition to research.

5. Conclusions

Overall, the results from this pilot study provide insights as to the feasibility of producing live young goodeid (*X. eiseni* as a model) by use of cryopreserved sperm. This is the first report with this research focus for the family Goodeidae and the second family for freshwater livebearing fish. A total of 29 live young were produced by two of 34 females that were inseminated with cryopreserved sperm (8% post-thaw motility). Ovarian development was evaluated for the first time in the present study of sperm cryopreservation of livebearing fish, indicating mature oocytes were present in 32% of females from the captive population. Sperm (fresh) were observed in histological images of ovaries that were fixed at 12 h after insemination. Productivities of live young were evaluated and could be used to assist future planning of germplasm repositories. The present study provides a foundation for establishment of germplasm repositories for endangered goodeids, which can be integrated into comprehensive conservation programs.

Declarations of interest

The authors have no conflicts of interest to declare.

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