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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