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Sperm Cryopreservation in Live-Bearing *Xiphophorus* Fishes: Offspring Production from *Xiphophorus variatus* and Strategies for Establishment of Sperm Repositories

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Abstract

Cryopreservation of sperm from *Xiphophorus* fishes has produced live young in three species: *X. hellerii*, *X. couchianus*, and *X. maculatus*. In this study, the goal was to establish protocols for sperm cryopreservation and artificial insemination to produce live young in *X. variatus*, and to identify needs for repository development. The objectives were to: 1) collect basic biological characteristics of males; 2) cryopreserve sperm from *X. variatus*, 3) harvest live young from cryopreserved sperm, and 4) discuss the requirements for establishment of sperm repositories. The 35 males used in this study had a body weight of 0.298 ± 0.096 g (mean ± SD), body length of 2.5 ± 0.2 cm, and testis weight of 6.4 ± 3.4 mg. The sperm production per gram of testis was $2.33 \pm 1.32 \times 10^9$ cells. After freezing, the post-thaw motility decreased significantly to 37% ± 17% (ranging from 5% to 70%) ($p = 0.000$) from 57% ± 14% (40%–80%) of fresh sperm (N = 20). Artificial insemination of post-thaw sperm produced confirmed offspring from females of *X. hellerii* and *X. variatus*. This research, taken together with previous studies, provides a foundation for development of strategies for sperm repositories of *Xiphophorus* fishes. This includes: 1) the need for breeding strategies for regeneration of target populations, 2) identification of minimum fertilization capacity of frozen samples, 3) identification of fish numbers necessary for sampling and their genetic relationships, 4) selection of packaging containers for labeling and biosecurity, 5) assurance of quality control and standardization of procedures, 6) information systems that can manage the data associated with cryopreserved samples, including the genetic data, 7) biological data of sampled fish, 8) inventory data associated with frozen samples, and 9) data linking germplasm samples with other related materials such as body tissues or cells saved for DNA and RNA analyses.

Introduction

Cryopreservation is an established approach for preservation of unique genetic resources, and can be applied for the following fields: 1) germplasm banking of species or strains with valuable phenotypic traits; 2) conservation of endangered species; 3) assistance of artificial fertilization or insemination, including hybridization; 4) preservation of cell lines or strains, including stem cells, and 5) preservation of cells, tissues, or organs as genetic resources for DNA. At present, gamete cryopreservation has been used extensively in mammals and is rapidly expanding in application with aquatic species,[1] birds,[2] reptiles,[3] and invertebrates.[4]

Xiphophorus is a genus of fishes in the Family Poeciliidae of the Order Cyprinodontiformes, and is characterized by small body sizes (less than 10 cm) and internal fertilization as the mode of reproduction. Most *Xiphophorus* species are native to Mexico, Belize, Guatemala, and Honduras.[5] The use of these fishes as a research model began in the mid-1920's, and lead to the establishment of the *Xiphophorus* Genetic Stock Center (XGSC) in 1939.[6] After more than 70 years of effort, the XGSC currently maintains 58 pedigreed lines among 24 of the 27 known *Xiphophorus* species. *Xiphophorus* fishes are composed of platyfish and swordtail and have become valuable in the hobbyist trade and as experimental research models. Due to the capability of producing fertile interspecies hybrids and their species divergence, *Xiphophorus* fishes have become useful in several fields of scientific research, including spontaneous and induced tumorigenesis,[7–9] evolutionary genetics,[10] behavioral ecology,[11] and sexual selection and sex determination.[12] In addition, several species such as *X. hellerii*,

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X. maculatus, and *X. variatus* are highly prized by aquarium hobbyists, along with other live-bearing fishes such as the mollies and guppies.[13] In the wild, due to human activities impacting their native habitats, many wild populations of *Xiphophorus* fishes are listed on the Red List of the International Union for Conservation of Nature (www.iucn.org) as being rare (*X. clemenciae*), endangered (*X. gordoni* and *X. meyeri*), or critically endangered and perhaps extinct in nature (*X. couchianus*). Because of this, cryopreservation may be needed to assist the preservation of these valuable fishes.

Sustained research on sperm cryopreservation of *Xiphophorus* fishes has been performed for the past 6 years.[14–21] To date, cryopreserved sperm have been used to produce live young in three species: *X. hellerii,*[18] *X. couchianus,*[19] and *X. maculatus,*[20] with confirmation of paternity by use of both genotypic and phenotypic markers. In this study, we report fertilization and offspring production by cryopreserved sperm from an important fourth species, *X. variatus.* The natural distribution of this species is from southern Tamaulipas to northern Veracruz in Mexico, and it has become established in a number of locations in United States due to releases related to the aquarium trade. It is the third most common *Xiphophorus* species in the aquarium trade besides the Southern platy (*X. maculatus*) and swordtail (*X. hellerii*). The original protocol developed for *X. hellerii*[18] has been adapted for sperm cryopreservation and artificial insemination in *X. variatus* including use of a hypertonic (500 mOsmol/kg) extender. Females of *X. variatus and X. hellerii* were artificially inseminated with cryopreserved sperm for fertility estimation. The goal of this study was to establish protocols for sperm cryopreservation and artificial insemination to produce live young in *X. variatus*, and identify needs to enable repository development. The objectives were to: 1) collect basic biological characteristics of males; 2) cryopreserve sperm from *X. variatus*, 3) harvest live young from cryopreserved sperm, and 4) discuss the requirements for establishment of sperm repositories. This research, when viewed together with the three previous studies,[18–20] has established the protocols for sperm cryopreservation and artificial insemination, and laid a foundation for development of sperm repositories (and gene banking) for *Xiphophorus* fishes and other live-bearing species.

Materials and Methods

Animals

The male *Xiphophorus variatus* (Encino) used in this study were sexually mature (beyond 5 months of age) (XGSC pedigree 11129 at 6–7 months old, and pedigree 11136 at 10–11 months old). They were cultured in 20-L tanks with static water at a photoperiod of 14 h light: 10 h dark, fed twice daily, once with live *Artemia* larvae and once with beef liver paste (see http://www.xiphophorus.txstate.edu/research.html).[22] The fish were shipped overnight from the XGSC to the Aquaculture Research Station of the Louisiana State University Agricultural Center (Baton Rouge, LA). Upon arrival, the fishes were maintained in 5-L tanks at 26°C with 2–3 fish/L in an freshwater aquarium system (Aquatic Habitats™, Aquatic Eco-systems, Inc. Apopka, FL), and were fed twice daily with commercial flakes (Flake Fish Food, Aquatic Ecosystem) and live *Artemia* larvae grown from cysts (INVE group; Grantsville, UT). The photoperiod was set at 14 h light: 10 h dark. The water quality conditions for this system were: dissolved oxygen > 6.5 mg/L; total ammonia-N < 3.0 mg/L, nitrite-N < 0.1 mg/L, and hardness (as Ca_2CO_3) > 100 mg/L. Guidelines from the Institutional Animal Care and Use Committees (IACUC) of the Louisiana State University Agricultural Center and Texas State University were followed for animal care in this study.

Sperm collection

Before collection, males were anesthetized by covering with ice for 1 min until movement stopped, and were blotted with a paper towel to dry the body. Prior to removal of the testes, the fish were measured for standard length (from snout tip to the base of the tail) and body weight. Testes were separated from the surrounding lipid tissues by dissection at 10-x magnification, and were transferred to a tared 1.5-mL centrifuge tube for weighing. Gonadosomatic index (GSI) was calculated as the percentage of testis weight to body weight, and body condition factor was calculated as the percentage of body weight (g) to the cube of standard body length (cm). Sperm were collected by crushing of the testis in 100–150 μL of Hanks' balanced salt solution (based on gross assessment of testis size) at an osmolality of 500 mOsmol/kg (HBSS500), and sperm concentration was estimated by measuring the absorbance of the sample at 400 nm and calculating with an equation generated from standard curves between absorbance and sperm concentration.[23] HBSS500 was prepared by adjusting the water volume from 1 L to $\sim$ 600 mL for the standard recipe of HBSS (0.137 M NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose, pH = 7.2).[24] The osmolality was measured with a vapor pressure osmometer (Model 5520, Wescor Inc., Logan, UT), and pH was determined (AB15, Fisher Scientific). Sperm samples were held on ice before and during use in experiments.

Motility estimation

Sperm motility was estimated by placing 1 μL of sperm suspension on a glass slide, addition of 19 μL of HBSS at an osmolality of 300 mOsmol/kg, and observation of the sperm by naked eye[18] at 200-x magnification using dark-phase microscopy (Optiphot 2, Nikon Inc., Garden City, NY). The percentage of sperm that moved actively in a forward direction was recorded as sperm motility. Sperm vibrating in place were not considered to be motile.

Sperm cryopreservation, thawing, washing, and concentrating

The protocol used in this study for sperm cryopreservation and artificial insemination was adapted from the protocol used to produce live young from *X. hellerii* cryopreserved sperm.[18] An equal volume of pre-made glycerol-HBSS500 mixture (28%, v/v) was mixed with the sperm suspension to yield a final concentration of 14% glycerol (as cryoprotectant). Then, 200 μL of the sperm suspension with glycerol were loaded into labeled 250-μL straws for freezing. The equilibration time from the addition of glycerol to initiation of the freezing program at 5°C was 20 min. Samples packaged in straws were frozen at a cooling rate of 20°C/min (based on sample probe temperature) from 5°C to −80°C in a computer-

controlled programmable freezer (Kryo 10 series II; Planer Products, Sunbury-on-Thames, UK), and transferred into liquid nitrogen for long-term storage.

After storage for 1–3 weeks in liquid nitrogen, the straws were thawed by immersion in a water bath at 40°C for 5 sec, the sperm suspensions were transferred to 1.5-mL centrifuge tubes on ice, and motility of the thawed sperm was estimated within 30 sec. To concentrate the samples for artificial insemination, the thawed sperm samples were centrifuged (1000 *g*) for 5 min at 4°C; the supernatant was decanted, the sperm pellet was re-suspended in 10 μL of fresh HBSS500 by gentle pipetting.

Artificial insemination

Artificial insemination was performed at the XGSC. Virgin females of *X. variatus* and *X. hellerii* were used for insemination in this study because *Xiphophorus* females can store sperm within the reproductive tract sufficient for production of 3 to 5 broods without further mating.[25] Virgin females were identified and separated from mixed-sex broods at around 6 weeks of age when they are sexually dimorphic but not yet sexually mature.[22] Before insemination, the females were anesthetized in 0.01% MS-222 (w/v), and transferred to a Petri dish with the belly facing up. To inject sperm, a specially constructed insemination device was used (see http://www.xiphophorus.txstate.edu/research.html). The tip end of the device was loaded with sperm sample, and gently pushed into the genital duct (viewed at 10X magnification), and a 5-μL sperm volume from individual males was injected into the genital duct. Sperm samples from each male were aliquoted into two parts, and each was used to inseminate females of *X. variatus* and one *X. hellerii*. After insemination, the females were grouped (5 fish per aquarium) and returned to fresh aquaria, and maintained for collection of live young at the XGSC. Generally, *Xiphophorus* fishes can give birth at a fairly regular interval of 28 days, but the intervals of between the first few broods may be irregular and vary between 26 and 90 days. Therefore, the inseminated females were maintained with routine care and checked daily for offspring production for at least 90 days after artificial insemination. Then, all of the females were dissected for examination of the reproductive tract to identify developing embryos for determination of fertilization.

Phenotypic identification of parents and live young

Live young harvested from this study were cultured, and photographed at April 14, 2010 (about 6–7 months of age). The phenotypes of *X. variatus* and its hybrid with *X. hellerii* (Doce or Bel stocks) were photographed at the same time for paternity confirmation.

Data collection

Data in this study were analyzed using SYSTAT 13 (Systat Software, Inc., Chicago, IL). Correlation analysis was made among standard length, body weight, testis weight, GSI, and condition factor of the males used in this study. The variance equality test (F test) was used for analysis of variation of post-thaw motility. The effects of treatments were analyzed by T-test.

Results

Basic biological factors of male X. variatus

A total of 35 males were used in this study with a body weight of 0.298 ± 0.096 g (mean $\pm$ SD), body length of 2.5 ± 0.2 cm, and testis weight of 6.4 ± 3.4 mg. On average, the sperm production was $2.33 \pm 1.32 \times 10^9$ cells per gram testis (Table 1). Correlation analysis (Fig. 1) showed that body weight was significantly correlated with standard length ($p = 0.000$); testis weight was correlated with body weight ($p = 0.010$) and body condition factor ($p = 0.020$), but not with standard length ($p = 0.080$), the gonadosomatic index was not correlated with standard length ($p = 1.000$) or body condition factor ($p = 1.000$) (Fig. 1).

Sperm cryopreservation

Before freezing, the motility of fresh sperm from 20 males ranged from 40% to 80% (57 ± 14%). After freezing, the post-thaw motility was significantly decreased to 37 ± 17% ranging from 5% to 70% ($p = 0.000$) (Table 1). The variance for fresh motility did not show a difference with that of post-thaw motility ($p = 0.387$). Correlation analysis showed that post-thaw motility was correlated with fresh motility ($p = 0.000$), but not with testis weight ($p = 0.122$), body condition factor ($p = 1.000$), or gonadosomatic index ($p = 0.845$). Fresh sperm motility was correlated with testis weight ($p = 0.003$).

Live young collection

For fresh sperm samples, live young were collected from female *X. hellerii* in one tank (insemination by males #1–5) at 32 days, and a second brood was collected from the same tank on day 134 (Table 2). However, there were no live young collected from females of *X. variatus* that were inseminated with fresh sperm from the same males. For cryopreserved sperm, live young were collected from females of *X. hellerii* (at day 32) and *X. variatus* (at day 72) that had been inseminated with sperm from the same males (males #30–35) (Table 2). No embryos were observed within the females in the aquaria from which live young were not harvested.

Paternity confirmation of live young

The paternity of live young collected in this study was verified based on the phenotypic characteristics of each cross. Males of *X. variatus* (Encino) exhibit yellow on the dorsal and tail surfaces (DyTy) (Fig. 2). Females of *X. variatus* Encino do not show scoreable traits. Offspring from females of *X. hellerii* (Doce) crossed with male *X. variatus* Encino display an elongated body type and the vertical barring typical of *X. hellerii*, and the male offspring also display the (Ty) trait found in *X. variatus* (Encino). Offspring from female *X. hellerii* (Bel) crossed with male *X. variatus* (Encino) display an elongated body type compared to *X. hellerii* from the Sarabia line.

Discussion

Sperm cryopreservation and artificial insemination for Xiphophorus *fishes*

With the completion of this study, sperm cryopreservation in *Xiphophorus* fishes has been studied in four species that have yielded offspring production,[18–20] confirming the

Table 1. Biological Data for Males, Sperm Production, Sperm Concentration, and Sperm Motility Before Freezing, and Post-Thaw Motility for *Xiphophorus variatus* Used in This Study

Male	Weight (g)	Length (cm)	Testis weight (mg)	Sperm number per g of testis	Sperm density at freezing (sperm/mL)	Sperm density for injection (sperm/mL)	Sperm motility (%)	
							Fresh	Post-thaw
Fresh samples (controls)								
1	0.210	2.2	7.3	3.87E+09	–	1.29E+09	70	–
2	0.244	2.3	9.5	1.35E+09	–	4.49E+08	80	–
3	0.248	2.3	12.1	3.16E+09	–	1.05E+09	70	–
4	0.209	2.3	5.4	1.73E+09	–	5.76E+08	70	–
5	0.240	2.4	6.0	5.24E+08	–	1.75E+08	60	–
6	0.215	2.3	4.4	1.38E+09	–	4.59E+08	70	–
7	0.400	2.7	14.3	3.57E+09	–	1.19E+09	80	–
8	0.368	2.6	14.1	1.97E+09	–	6.56E+08	80	–
9	0.229	2.4	6.1	1.44E+09	–	4.81E+08	70	–
10	0.218	2.3	5.0	6.20E+08	–	2.07E+08	60	–
11	0.284	2.4	9.2	9.36E+08	–	3.12E+08	70	–
12	0.263	2.4	8.2	7.72E+08	–	2.57E+08	70	–
13	0.519	2.8	13.2	1.51E+09	–	5.04E+08	70	–
14	0.257	2.4	5.1	1.46E+09	–	4.87E+08	70	–
15	0.236	2.4	8.2	1.52E+09	–	5.08E+08	70	–
Cryopreserved samples								
16	0.307	2.5	5.8	2.95E+09	5.70E+07	1.14E+09	60	40
17	0.485	2.8	6.3	2.83E+09	5.95E+07	1.19E+09	70	40
18	0.511	2.9	10.0	5.80E+09	1.93E+08	3.87E+09	80	60
19	0.334	2.5	5.8	4.11E+09	7.95E+07	1.59E+09	70	40
20	0.130	1.9	2.6	1.43E+09	1.86E+07	3.72E+08	50	30
21	0.223	2.3	2.9	2.64E+09	3.83E+07	7.66E+08	50	40
22	0.267	2.4	3.2	1.60E+09	2.57E+07	5.13E+08	40	20
23	0.268	2.5	4.2	6.57E+08	1.38E+07	2.76E+08	50	30
24	0.281	2.5	3.3	1.63E+09	2.69E+07	5.38E+08	50	40
25	0.274	2.4	3.4	4.85E+09	8.24E+07	1.65E+09	60	50
26	0.284	2.5	3.8	1.78E+09	3.37E+07	6.75E+08	80	70
27	0.301	2.5	5.2	3.67E+09	6.37E+07	1.27E+09	60	30
28	0.278	2.5	3.8	4.57E+09	8.68E+07	1.74E+09	60	40
29	0.293	2.5	3.8	2.54E+09	4.82E+07	9.64E+08	50	40
30	0.379	2.7	4.8	2.66E+09	6.39E+07	1.28E+09	40	10
31	0.240	2.3	3.0	3.55E+09	5.32E+07	1.06E+09	40	20
32	0.337	2.6	3.4	3.16E+09	5.37E+07	1.07E+09	40	5
33	0.270	2.4	3.0	1.97E+09	2.95E+07	5.91E+08	40	30
34	0.563	3.1	11.1	3.06E+09	1.13E+08	2.27E+09	80	70
35	0.275	2.3	6.4	2.07E+09	4.42E+07	8.84E+08	60	40

capability of frozen sperm to produce fertilization in live bearing fishes after artificial insemination. The efficiency of offspring production was usually low (10%–30%), but this was not different from control groups inseminated with fresh sperm and is typical for artificial insemination in this genus. In this study, live young were collected in one (of three) aquaria for fresh sperm, and one (of four) aquaria for cryopreserved sperm. Based on data accumulated over more than 50 years at the XGSC, offspring production via artificial insemination for an experienced technician using freshly isolated sperm is about 20% (R. Walter, unpublished data). Thus, the low efficiency of thawed sperm was likely due to reproductive characteristics of *Xiphophorus* fishes in response to artificial insemination rather than technical problems related to cryopreservation. However, artificial insemination is an essential step to produce offspring from cryopreserved sperm for *Xiphophorus* fishes due to the internal fertilization and viviparity. Also *Xiphophorus* fishes are characterized by small body sizes (2–5 cm), and the sperm volume available from a single male is typically insufficient for insemination of more than 3 or 4 females. Therefore, increasing the efficiency of artificial insemination would be a critical step for application of germplasm preservation in *Xiphophorus* fishes.

Possible approaches for increasing offspring production efficiency with cryopreserved sperm

Studies on reproduction of *Xiphophorus* fishes started in the early 1920's by addressing sex differentiation and sex reversal,[26,27] followed by study of ovarian cycles,[28] embryonic development,[25] genetic control of the pituitary–gonadal axis,[29] and sex determination.[12] Most of these studies were performed with *X. hellerii* and *X. maculatus,* and the major findings were: 1) females can store sperm in folds of the oviduct for months and produce 3–5 broods from a single mating even in the absence of a male;[25,30] 2) females may reach sexual maturity within 4–6 months; however, maturity is governed by specific genes,[29] and may be influenced by other factors such as social behavior[31] and body size;[32] 3) embryonic development from fertilization to birth is about 22 days;[25]

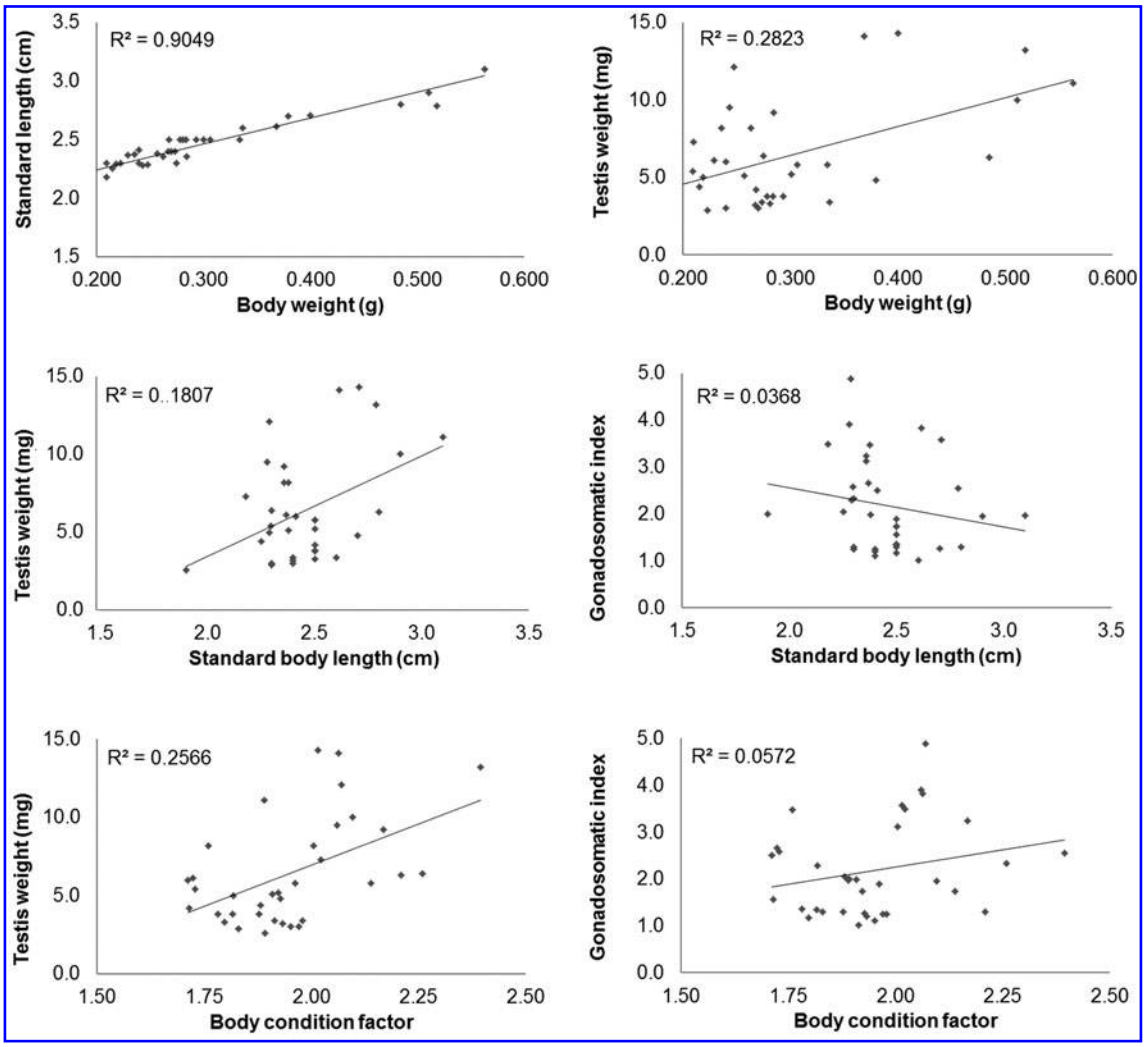

FIG. 1. Correlation analysis between biological factors from the 35 males used in this study: body weight (g), body standard length (cm), testis weight (mg), gonadosomatic index, and body condition factor. Gonadosomatic index was calculated as the percentage of testis weight to body weight, and body condition factor was calculated as the percentage of body weight (g) to the cube of standard body length (cm).

4) the time intervals between the first few broods in females can be irregular and vary between 26 and 90 days, although the tendency is to cycle at about 28 days with mature females, consistent maintenance, and a conducive environment.

Based on these observations, appropriate approaches for increasing artificial insemination success rates of *Xiphophorus* females may include:

1. Conditioning of males before sperm collection. Sperm collected from males in good health is usually of good quality and quantity. It has been shown that sperm of *X. hellerii* with higher swimming velocity can have higher fertilization success,[33] and in natural environments females prefer to mate with larger-sized males.[34,35] In this study, post-thaw motility was found to be correlated with fresh motility, and fresh motility was correlated with testis weight. Therefore, proper conditioning of males to reach maturity for spawning or mating could be an important approach to ensure sample quality and quantity for increasing the fertilization success of cryopreserved sperm.
2. One may determine how to increase female fecundity prior to artificial insemination by treatment with sex hormones or environmental manipulations. In *X. maculatus* fishes, sexual maturity can be determined by the genetic-controlled pituitary–gonadal axis,[29,36] and also can be controlled[37,38] or influenced[31] by social behavior and body size.[32] Therefore, to increase fertilization success with artificial insemination, it is critical to choose females that are mature and have high fecundity. In the present study, fresh sperm from the same males produced offspring after insemination of *X. hellerii* females but did not produce offspring when used to inseminate females of *X. variatus* (Table 2). Possible approaches to increase female fecundity include treatment with gonadotropic hormones, research into social effects on females grouped in tanks in terms of numbers, the presence or absence of males that may produce pheromones, enhanced conditioning of females (e.g., through nutrition or environmental factors) for maximum reproductive potential, and use of older, larger non-virgin females in which fertilization becomes more regular, and fecundity more certain.

Table 2. Artificial Insemination of Fresh (July 20, 2009) and Cryopreserved Sperm (July 21, 2009) from *Xiphophorus variatus*

			Live young collection	
Male identification number	*Female species*	*Strain*	*Date*	*Number and sex*
Fresh sperm				
1–5	*X. hellerii*	Bel	8/21/2009	1 female
			12/2/2009[a]	1 female and 1 male
	X. variatus	Encino	None	–
6–10	*X. hellerii*	Doce	None	–
	X. variatus	Encino	None	–
11–15	*X. hellerii*	Doce	None	–
	X. variatus	Encino	None	–
Cryopreserved sperm				
16–20	*X. hellerii*	Bel	None	–
	X. variatus	Encino	None	–
20–25	*X. hellerii*	Bel	None	–
	X. variatus	Encino	None	–
26–30	*X. hellerii*	Doce	None	–
	X. variatus	Encino	None	–
31–35	*X. hellerii*	Doce	8/21/2009	4 females and 3 males
	X. variatus	Encino	10/1/2009	1 male

[a]Second brood collected from inseminated females in this tank.

Sperm samples from each male were divided into two parts (5 μL each) for insemination of female *Xiphophorus hellerii* and *Xiphophorus variatus*. Five inseminated females were grouped in each tank (considered as a single replicate) for monitoring of live young production.

This latter approach would require safeguards to ensure that paternity could be verified.

3. Development of tools to assess female condition for ovulation. Sufficient *Xiphophorus* sperm can be stored for months in folds of the oviduct to fertilize as many as four or more broods.[25,30,39] Given the viability of stored sperm, fertilization could occur whenever oocytes are mature and ovulated. It is currently difficult to estimate the stage of ova development within females. Ultrasound is a noninvasive technique used for years in monitoring of fetal development in human medicine, and in recent years it has been used with large-bodied aquaculture fishes to identify ovarian developmental stages.[40] Thus far, the smallest fish for which conventional ultrasound has been used was the Neosho madtom *Noturus placidus* with 10 to 12 cm body length.[41] It is unknown if ultrasound techniques can be used with smaller fishes. Based on reproductive studies on *Xiphophorus* fishes, the ova begin to accumulate yolk at 450 μm, and are ready for fertilization at about 1.6 mm.[25,28,42] More investigation is needed to evaluate if ultrasound techniques can recognize developmental

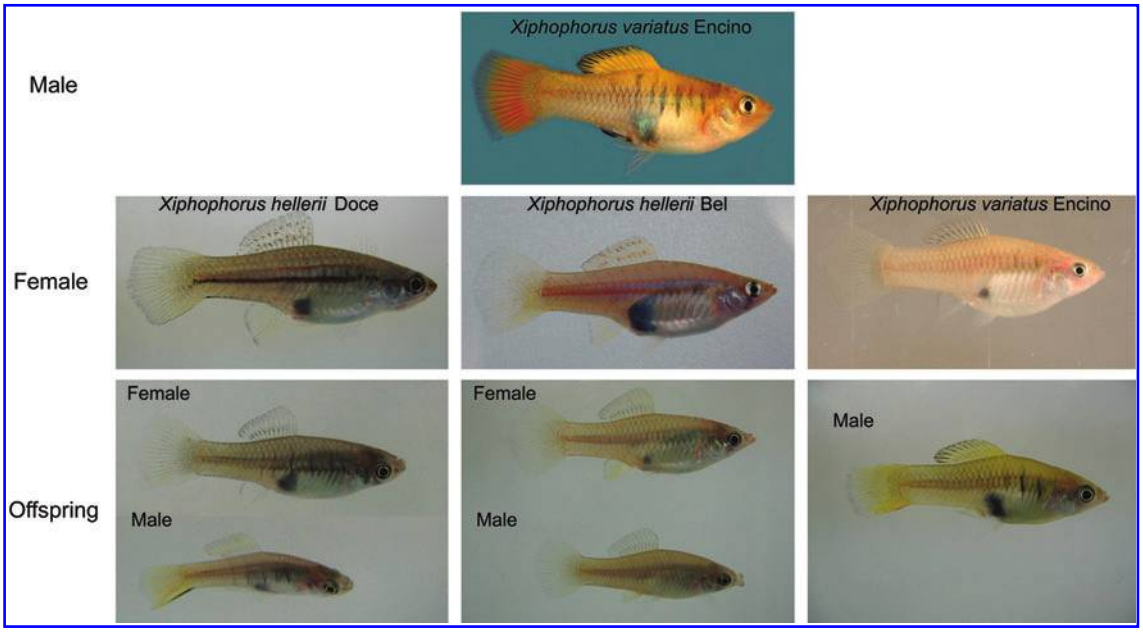

FIG. 2. Phenotypes of the parents and offspring that were collected in this study. Cryopreserved sperm from *Xiphophorus variatus* Encino were artificially inseminated into virgin females of *Xiphophorus variatus* Encino and *Xiphophorus hellerii* Doce or Bel. Color figure is available online at www.liebertpub.com/zeb

events in this size range, and if ultrasound can also be used to monitor embryonic development.
4. The culture conditions of inseminated females might be improved. Social interactions and courting behavior between males and females are normally employed by *Xiphophorus* fishes during mating.[43] However, it is unknown if fertilization always occurs when oocytes are mature and stored sperm are available, or if specific social interactions are also involved. Therefore, it would be useful to investigate culture conditions for females after artificial insemination, such as the effect of different numbers and densities of grouped females, and the presence or absence of males. Currently, most behavioral research on *Xiphophorus* fishes has addressed mating preferences, and we have not found reports that address the occurrence of fertilization.

Establishment of a sperm repository for Xiphophorus *fishes*

After development of protocols for sperm cryopreservation and artificial insemination, the final target is to establish a sperm repository for germplasm preservation. Currently, germplasm resource programs have been established mostly for domestic livestock species.[44–48] Few reports address establishment of fish germplasm repositories. Based on previous established germplasm repositories for livestock and the reproductive characteristics of *Xiphophorus* fishes, we propose basic approaches for establishment of sperm repositories:

Sampling and processing. At a minimum, the major purpose of a sperm repository would be to enable reconstitution of species, strains, or breeds, including specific mutated genes or chromosomes, or whole genomes, or populations. Depending on the type of reproduction and breeding efficiency, a strategy involving the following aspects should be developed before establishing a repository.

1. Development of breeding strategies for regeneration of target broods with cryopreserved sperm. A specific breeding plan should be in place, and females necessary for the breeding plan should to be identified. For example, a large number of specific strains, especially single-gene mutants, can be recovered through the use of a single population of wild-type females. Thus, maintenance of live females can be eliminated for most strains that have been cryopreserved.
2. Identification of the minimum capacity of frozen samples necessary for regeneration of viable strains or populations. This can be calculated based on breeding outputs, fecundity of females, and the fertility of cryopreserved sperm, especially when calculated on a per-sperm basis.
3. Identification of the number of individuals and their genetic relationship necessary for accession. For domestic livestock, a target of 50 unrelated animals (i.e., effective population size) has been recommended for reconstitution of particular populations to control the occurrence of inbreeding.[49] Knowledge of the genetic background of collected animals is essential for a germplasm repository, especially when the target is to regenerate populations. Genetic distancing is an approach for estimating genetic diversity, and genetic markers (especially microsatellite markers) have been recommended for such analyses.[50] For *Xiphophorus* fishes, the populations are mostly lines inbred for many generations. Therefore, consideration of the number of individuals necessary for a sperm repository should provide sufficient germplasm for recovery of live young based on the fertilization success of each individual, especially given the limited availability of sperm sample from these fishes. Future work should address cryopreservation for non-lethal collection of the stripped sperm to enable multiple collections for individual males.
4. Thoughtful selection of packaging containers and labeling formats for frozen samples to ensure integrity of biosecurity protocols, efficient storage and use, and easy inventory. All aspects of processing, storage, and use should be considered in this decision.
5. Assurance of quality control of sperm samples during collection, before freezing during storage and transport (if needed), and after thawing. Motility, sperm cell structure (such as plasma membrane integrity), and fertility are the most important quality control indices. A comprehensive quality control program would include screening of individual animals before entering the repository, and would assure the quality of cryopreserved sperm.
6. Standardization of procedures including sample labeling, packaging, sealing, sorting for storage, thawing, and use can assure the uniformity and reliability of the repository.

Information systems. A sperm repository should plan to preserve and store genetic materials for decades. Therefore, a flexible and comprehensive database is necessary to manage the cryopreserved samples. The database should include the following components:

1. Genetic data associated with the animals providing germplasm, such as species, line or strain, pedigree, phenotypes, and genotypes.
2. Biological data including body length, body weight, testis weight, and phenotypes.
3. Inventory data associated with frozen samples in the repository, such as the date of processing, sperm concentration and volume, sperm quality, straw numbers, labeling codes, storage location, and dispositions.
4. Data linkage among germplasm samples with other related materials such as body tissues or cells saved for DNA and RNA analysis.[1]
5. Data linkage to public research databases and interfaces of the stock centers for researchers to identify and request specific mutations from the repository.

Considerations should also be given to integrating comprehensive germplasm repository facilities with other comprehensive facilities and specific end users (such as research laboratories) to constitute a network-based repository system. This will place greater emphasis on standardization across all aspects (or at least a harmonization of different approaches). A linkage of databases and repositories as described above is being produced at the USDA National Animal Germplasm Program (www.nifa.usda.gov).

Conclusions

This study reports the production of offspring after artificial insemination using cryopreserved sperm of *X. variatus*. With this study, sperm cryopreservation has been accomplished in four *Xiphophorus* species that represent platyfish and swordtails. Future research on sperm cryopreservation in *Xiphophorus* fishes can move from protocol development and become more focused on procedural standardization and on efforts to improve the efficiency of artificial insemination. Based on the framework strategies above, a detailed working plan is now needed for future repository development.

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Disclosure Statement

No competing financial interests exist.

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