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Production of F_1 Interspecies Hybrid Offspring with Cryopreserved Sperm from a Live-Bearing Fish, the Swordtail *Xiphophorus helleri*[1]

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ABSTRACT

Despite study of sperm cryopreservation in more than 200 fish species, production of broods from cryopreserved sperm in live-bearing fish has not been demonstrated. This has not been due to a lack of effort, but instead is a result of the unique morphology, biology, and biochemistry of reproduction in viviparous fishes. For example, sperm of *Xiphophorus helleri* have a cylindrical nucleus, can swim for days after being activated, have glycolytic capabilities, and can reside in the female reproduction tract for months before fertilization. These traits are not found in fishes with external fertilization. The long-standing research use of the genus *Xiphophorus* has led to development of over 60 pedigreed lines among the 26 species maintained around the world. These species and lines serve as contemporary models in medical research, although they must be maintained as live populations. Previous attempts at establishing sperm cryopreservation protocols for *Xiphophorus* have not produced live young. To address this we have been studying the parameters surrounding cryobiology of *Xiphophorus* sperm and applying this information to an improved understanding of internal fertilization and reproduction. Here we report the first successful fertilization and offspring production by cryopreserved sperm in any live-bearing fish. This claim is supported by our use of artificial insemination between two species that yield distinct hybrid offspring to verify paternity via cryopreserved sperm. We provide a practical approach for preservation of valuable genetic resources from live-bearing fish species, a group that is rapidly being lost due to destruction of native habitats.

sperm, sperm motility, testis, transport

INTRODUCTION

Hybrid crosses between various species of live-bearing fishes of the genus *Xiphophorus* have provided well-studied spontaneous or induced tumor models for biomedical research [1–3]. Over the past 80 years, valuable pedigreed *Xiphophorus* research stocks and inbred lines have been created and are currently maintained as live animals in the *Xiphophorus* Genetic Stock Center (XGSC; http://www.xiphophorus.org) [4, 5]. Sperm cryopreservation is a useful technique that needs to be developed and employed to maintain these valuable strains or lines.

Sperm cryopreservation has been studied in more than 200 fish species with different protocols, but prior to 2004 there were no publications addressing live-bearing fishes [6]. Compared with the vast majority of fish species, live-bearing (i.e., viviparous) fishes reproduce by internal fertilization, and consequently their sperm possess specialized morphology and physiology. For fishes of the genus *Xiphophorus*, sperm are characterized by the following characteristics: 1) mature sperm have a cylindrical nucleus, a short thickened midpiece, and an elongated axoneme [7]; 2) after deposition of sperm in the female the spermatozoa remain in discrete head-head agglutination bundles, termed *spermatozeugmata*, within the female reproductive tract [8, 9]; 3) inseminated sperm can be stored live for months in the female reproductive tract before intrafollicular fertilization of mature oocytes, and may produce broods at approximately 30-day intervals over 4–5 months after a single insemination [10]; 4) sperm motility can be activated by suspension in buffer isotonic to *Xiphophorus* plasma [11]; and 5) sperm can remain continuously motile for 4–7 days of storage at 4°C [12]. These unique characteristics are very different from the sperm of fish with external fertilization, where motility is short lived (seconds to minutes) and is activated by hypotonic (for freshwater fish) or hypertonic (for marine fish) media [13]. Additionally, *Xiphophorus* fishes are further characterized by small body sizes (2–5 cm), and the availability of sperm is limited to microliter volumes. Accordingly, sperm cryopreservation and fertilization trials with cryopreserved sperm present significant challenges in *Xiphophorus* compared with oviparous fish species that use external fertilization.

Our recent studies have evaluated sperm cryopreservation in *X. helleri*, with sperm motility as a general indicator of gamete quality [12, 14], and by use of a shape-independent differential scanning calorimeter technique [15]. However, these attempts did not yield fertilization or offspring production. To address this, we examined in a previous study [11] the basic parameters surrounding *X. helleri* sperm cryobiology and concluded that: 1) immobilization of sperm by hypertonic osmolality can extend retention of motility during refrigerated (nonfrozen) storage compared with storage of nonimmobilized sperm in isotonic solutions, and it did not affect the motility of sperm after thawing; 2) washing of thawed sperm by centrifugation could remove the cryoprotectant and prolong motility from 24 to 72 h; and 3) centrifugation of thawed sperm during washing served to concentrate and increase the number of sperm injected for artificial insemination. This is especially important for *Xiphophorus* fishes, because the sperm volume injectable

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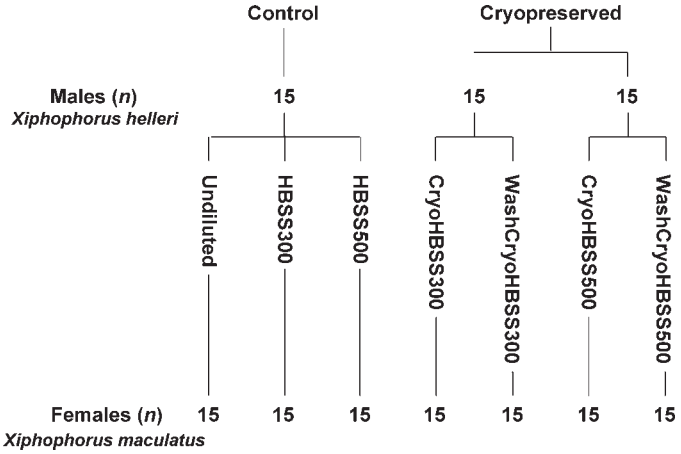

FIG. 1. The experimental design and numbers of fish used in each treatment in this study.

during artificial insemination (~5 μl) is limited by the small female body size.

In the present study we used information from previous reports to develop approaches for artificial insemination with cryopreserved sperm [11, 12, 14]. Here we report the first successful fertilization and offspring production by cryopreserved sperm in a viviparous fish. This claim is validated by the use of males and females of different species to yield interspecies hybrid offspring that verify the paternity of the sperm source. We developed a detailed practical protocol to assist protection of germplasm resources in domesticated and wild live-bearing fish species.

MATERIALS AND METHODS

Animals

Fish used in these experiments were obtained from the *Xiphophorus* Genetic Stock Center (Texas State University, San Marcos, TX). Males were mature (~1-year-old) *X. helleri* (strains of Sarabia, BXII, or Jalapa) with an average (mean ± SD) body length of 3.48 ± 0.30 cm, and body wet weight of 0.80 ± 0.18 g. Females used were *Xiphophorus maculatus* strain Jp 163 A and B, and virgin females were selected by separation from mixed-sex broods prior to maturation (at around 6 wk of age). Guidelines from the Institutional Animal Care and Use Committees (IACUC) of Louisiana State University and Texas State University were followed for animal care in this study, IACUC animal protocols and inspections are current (IACUC no. 05–05F7651F62), as is National Institutes of Health Protection from Research Risks approval.

Sperm Collection

Sperm were collected by crushing dissected testis. Males were anesthetized in 0.01% tricaine methane sulfonate (MS-222; Western Chemical Inc., Ferndale, WA), rinsed in fresh water, and blotted with a paper towel to dry the body. Before dissection of testes, the males were measured for standard length (from snout tip to the base of the tail) and body weight, and were dissected by use of 10× magnification. Testes were separated from the surrounding lipid tissues and transferred to a 1.5-ml centrifuge tube for weighing. Sperm were released by crushing the testis with or without Hanks balanced salt solution (HBSS) prepared at osmolalities of 310 mOsmol/kg (HBSS310: 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) or 500 mOsmol/kg (HBSS500) by adjusting the final volume of water (~600 ml). The sperm suspensions were held on ice before use in experiments.

Motility Estimation

To estimate sperm motility, 1 μl sperm suspension was placed on a glass slide, 20 μl of HBSS310 at room temperature (20°C–22°C) was added, and the motility of sperm was observed at 200× magnification using dark-phase microscopy (Optiphot 2; Nikon Inc., Garden City, NY). The motility of each sample was estimated two times, with three visual fields checked each time, and was expressed as the percentage of sperm that actively moved in a forward direction. Sperm vibrating in place were not considered to be motile.

Preparation of Control Sperm for Artificial Insemination

For comparison of control treatments with cryopreserved sperm (Fig. 1), testes from 15 males were dissected and individually processed into three parts: 1) the testis was crushed, and 1–2 μl sperm was removed (termed undiluted sperm); 2) half of the remaining crushed testis was suspended in 20 μl HBSS310 and stored at 4°C for 24 h (termed HBSS310 sperm); and 3) the other half of the remaining crushed testis was suspended in 20 μl HBSS500 and stored at 4°C for 24 h (termed HBSS500 sperm).

Preparation of Cryopreserved Sperm for Artificial Insemination

For cryopreservation, sperm suspensions were prepared by crushing an individual testis in HBSS310 or HBSS500 with a volume of approximately 40 times the testis weight, and mixing this with an equal volume of double-strength HBSS-glycerol to yield a final concentration of 14%. After about 15 min equilibration with glycerol, the sperm suspensions (220 μl) were loaded into 250-μl French straws (IMV International, Minneapolis, MN), and cooled from 5 to −80°C at 20°C per minute in a programmable freezer (Kryo 10 series II; Planer Products, Sunbury-on-Thames, UK). The straws were transferred to liquid nitrogen for storage. Dissected testes from 15 males were used to collect immobilized sperm by individually crushing in HBSS500 (termed CryoHBSS500 sperm), and another 15 males were used to collect nonimmobilized sperm by crushing of individual testis in HBSS310 (termed CryoHBSS310 sperm).

After storage for 1 wk in liquid nitrogen, frozen samples were thawed at 42°C for 5 sec for artificial insemination, and thawed sperm were transferred into 1.5-ml tubes for processing, centrifugation, washing, and use for injection within 30–60 min after thawing. A small aliquot of thawed sperm (20 μl) was used to estimate motility immediately and after 24 h of storage at 4°C, and the sperm density was measured using a hemocytometer.

Cryopreserved samples (CryoHBSS500 and CyoHBSS310 sperm) from each male were separated into two parts after thawing. One part was centrifuged once at 1000 × *g* to concentrate the sperm into a volume of approximately 10 μl without washing; the other part was washed once by addition 200 μl fresh HBSS500 or HBSS310 after the first centrifugation and centrifuged again to concentrate the sperm into a suspension of approximately 10 μl. In this study cryopreserved nonimmobilized sperm in HBSS310 with the additional wash is termed WashCryoHBSS310 sperm, and the cryopreserved immobilized sperm in HBSS500 with additional wash after thawing is termed WashCryoHBSS500 sperm.

Artificial Insemination

Artificial insemination was prepared by injecting sperm into the genital duct of females by use of a specially constructed injector [6]. A silicon tube was connected with a Daigger gel-loading pipette tip at one end that served as intromittent orifice for injecting sperm into females. A modified syringe handle at the other end was used for controlling the sperm flow pressure exerted from the mouth of the injector. A glass rod in the middle of the tube was used to hold the injector. Before sperm injection, females were anesthetized in 0.01% MS-222 and positioned on moist cotton in a Petri dish with the belly facing up for viewing by use of a dissecting scope at 10× magnification. The tip end of the injector was filled with sperm sample and gently pushed into the genital duct, and the sperm were ejected by breath pressure. After insemination, the females were returned to fresh water for recovery and were maintained in aquaria systems in the XGSC for harvest of live young. These inseminated females were cultured following routine maintenance protocols at the XGSC. When live young were harvested, or at 90 days after insemination (whichever came first), the inseminated females were dissected, preserved in 95% ethanol, and photographed.

Together with 3 control groups, a total of 7 groups of sperm were used for artificial insemination, with 15 samples from individual males in each group. For each group, the 15 virgin females of *X. maculatus* Jp 163 used for insemination were cultured in three tanks, with 5 females in each tank as a single replicate. The experimental design is outlined in Figure 1.

Phenotype and Genotype Confirmation of the Hybrid F_1 Offspring

Phenotypes were confirmed by observation of changes in pigment patterns that occur upon interspecies hybridization. Genotypes were determined by

analysis of microsatellite markers. Live or ethanol (95%)-preserved adult fish and live young (2–3 mo old) were used. Tailfin clips from each fish were collected and flash frozen in liquid nitrogen after the fish were briefly anesthetized in 0.01% MS-222.

Genomic DNA was extracted from the tailfin clips using the PureGene Cell and Tissue DNA Isolation Kit (Gentra Systems, Minneapolis, MN) following the manufacturer's protocol for DNA extraction from fish tissues. The concentration and A_{260}/A_{280} ratio of the extracted DNA samples were measured with a microvolume spectrophotometer (Nanodrop Technologies, Wilmington, DE). Stock DNA solutions were diluted to 50 ng/μl in 1× TE (10 mM Tris with 1 mM EDTA; pH 8.0) and stored at −20°C until use.

Prior to screening the experimental samples, several microsatellite primer sets were tested using DNA from *X. maculatus* strain Jp 163 A, *X. helleri*, and F_1 hybrids from *X. maculatus* Jp 163 A × *X. helleri* to ensure distinctive amplification using PCR. An optimized microsatellite primer set (Msc053; Integrated DNA Technologies, Coralville, IA) was used for analyzing the samples collected in this study. The sequence of the forward oligonucleotide was 5′-ATCAGTGCAATTCCAAATACTCATAATAT-3′, and the reverse oligonucleotide was 5′-GGAAACAACAAGAATGACACATCAGT-3′. Genomic DNA was amplified and analyzed by agarose gel electrophoresis. Each 20-μl reaction contained 25 ng genomic DNA, 0.2 μM each of forward and reverse oligonucleotide, 1.0 unit *Taq* polymerase (Invitrogen, Carlsbad, CA), containing 2 mM $MgCl_2$, 32 mM Tris-HCl (pH 8.4), and 80 mM KCl. PCR amplification consisted of initial denaturation at 94°C for 5 min, followed by 40 cycles of: denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. A final extension at 72°C for 7 min concluded the amplification. The PCR products were electrophoresed in 2% (w/v) agarose gel in 1× Tris-acetate EDTA containing 25 ng/ml ethidium bromide. For each PCR reaction, a set of controls consisting of genomic DNA from *X. maculatus* Jp 163 A, *X. helleri*, and a known hybrid of *X. maculatus* Jp 163 A × *X. helleri* were assayed alongside the experimental samples.

Data Analysis

Data were analyzed using SYSTAT version 11 (Systat Software Inc.). Treatment effects were tested by using a two-sample *t* test. Percentage data were arcsine transformed before analysis. The significance level was set at $P < 0.050$.

RESULTS

Motility of Fresh Sperm (Controls) and Cryopreserved Sperm Used for Artificial Insemination

After sperm were activated in HBSS with an osmolality equal to that of *Xiphophorus* plasma (310 mOsmol/kg), the motility of undiluted sperm ranged from 90%–95% before injection for artificial insemination (Table 1). For the HBSS310 sperm and HBSS500 sperm, motility was 80%–85% after storage at 4°C for 24 h prior to insemination (Table 1).

As for the cryopreserved sperm, the initial motility of sperm before cryopreservation processing in either CryoHBSS500 or CryoHBSS310 was 80%–90% (Table 1). After cryopreservation, the motility of thawed sperm varied from fish to fish, ranging from 20% to 70% (Table 1), which was significantly lower than the initial motility ($P < 0.012$). In addition, after storage at 4°C for 24 h, the motility of thawed sperm in CryoHBSS500 and CryoHBSS310 declined to 0%–5%.

Harvest of Live Young from the Females Inseminated with Fresh and Cryopreserved Sperm

The density of the fresh and cryopreserved sperm for insemination ranged from 0.5 to 11.0×10^9 cells/ml (Table 1), and the volume of sperm injected into each female for artificial insemination was 4–6 μl, except for fresh undiluted sperm, which was 1–2 μl.

At 27–56 days after artificial insemination, live young were harvested from all the experimental groups (Table 1). In females inseminated with the three control treatments (undiluted sperm, HBSS310 sperm, and HBSS500 sperm), live young were harvested in a single replicate (of three). In females inseminated with CryoHBSS310 sperm or CryoHBSS500 sperm, live young were harvested in a single replicate (of three). In females inseminated with WashCryoHBSS310 sperm, live young were harvested in a single replicate (of three), and a second, successive brood also was harvested from this replicate. In females inseminated with WashCryoHBSS500 sperm, live young were harvested from all three experimental replicates (Table 1).

The number of live young harvested was different from group to group, ranging from 1–8, and the sex phenotype distributions in these young were highly skewed; some broods had only males, and other broods had only females (Table 1). This was caused by the distinct sex determination mechanisms of the different strains of *X. helleri* males used in this study. For example, it is widely known that when the *X. helleri* Sarabia strain (collected in southern Mexico) is crossed with *X. maculatus,* only male offspring are produced. This is due to the Sarabia strain having a WY/YY sex determining mechanism and being crossed to *X. maculatus* with a XX/XY determination mechanism (i.e., XX females × YY sperm leads to all XY male offspring in this cross; Fig. 2). In contrast, crossing a strain of *X. helleri* (BXII) originally collected in Belize with *X. maculatus* can result in both sexes being represented the interspecies offspring (data not shown, XGSC records). The testes used for sperm collection, cryopreservation, and offspring production were derived from *X. helleri* Sarabia, *X. helleri* BXII, or *X. helleri* Jalapa stocks maintained at the XGSC [16].

Phenotype and Genotype Confirmation of Harvested Live Young

Phenotypes and genotypes were both used to confirm that the live young resulted from artificial insemination with the cryopreserved sperm and the control treatments. Phenotypic characters in adult hybrids (>6 mo) of *X. maculatus* × *X. helleri* include: 1) overexpression of the dorsal red (*Dr*) pigment pattern originating from *X. maculatus* (strain Jp 163 A). This is clearly observed (Fig. 2) in F_1 hybrids where the red pigment extends from just behind the pectoral fins to the caudal fin and covers the entire back half of the fish body. 2) The enhancement of the spotted dorsal (*Sd*) phenotype originating from *X. maculatus* Jp 163 A in the F_1 hybrid where small black macromelanophore spots restricted to the dorsal fin in the *X. maculatus* parent become enhanced to exhibit almost complete black pigmentation of the dorsal fin with spreading into the dorsal region of the body. The live young harvested from the cryopreserved sperm and the control animals showed all of the phenotypic characteristics expected of interspecies hybrids between *X. maculatus* and *X. helleri* (Fig. 2, D–F).

Genotype analyses were performed by screening DNA isolated from the fins of parental and interspecies hybrid animals by use of polymorphic microsatellite markers. PCR amplification of genomic DNA isolated from all putative interspecies hybrids by use of primers specific for the Msc053 microsatellite marker resulted in a 450-bp amplicon from *X. maculatus* Jp 163 A DNA and a 350-bp amplicon from *X. helleri* (for microsatellite markers, see http://www.xiphophorus.org/microsats/microsat.htm). Hybrids between these two parental species produced both of the expected bands at 350 and 450 bp upon PCR amplification (Fig. 3, lanes 1–3). Analysis of each F_1 progeny harvested in this study showed two bands at 350 and 450 bp (Fig. 3, lanes 4–10), whereas the parents exhibited the single-band diagnostic of each species. This indicates that all of the young harvested in this experiment were indeed interspecies hybrid individuals.

TABLE 1. The motility and density (minimum-maximum) of sperm collected from *X. helleri* and the offspring harvested from females of *X. maculatus* Jp 163 after artificial insemination with *X. helleri* sperm in the following 7 treatments.

		Sperm motility (%) (minimum-maximum)			Live young		
						Number	
Sperm group	Replicate*	Initial	After thawing	Density ($\times 10^9$ cell/ml) (minimum-maximum)†	Days after AI‡	Female	Male
Undiluted	1	90–95	—	NA	56	8	0
	2	90–95	—	NA	—	0	0
	3	90–95	—	NA	—	0	0
HBSS310	1	80–85	—	0.8–1.7	34	0	1
	2	80–85	—	1.6–7.7	—	0	0
	3	80–85	—	3.0–6.2	—	0	0
HBSS500	1	80–85	—	2.5–3.1	—	0	0
	2	80–85	—	3.4–10.2	—	0	0
	3	80–85	—	3.5–11.0	40	0	1
CryoHBSS310	1	85–90	20–60	0.5–3.6	—	0	0
	2	85–90	30–70	1.0–2.1	29	1	1
	3	80–90	30–60	1.8–5.1	—	0	0
WashCryoHBSS310	1	85–90	20–60	0.8–3.9	—	0	0
	2	85–90	30–70	1.4–4.1	—	0	0
	3§	80–90	30–60	2.5–4.8	55	7	0
					82	11	0
CryoHBSS500	1	85–95	20–40	0.5–3.6	—	0	0
	2	80–90	30–60	1.0–2.1	—	0	0
	3	80–90	20–30	1.8–5.1	27	6	0
WashCryoHBSS500	1	85–95	20–40	0.8–3.9	31	0	7
	2	80–90	30–60	1.4–4.1	45	2	0
	3	80–90	20–30	2.5–4.8	27	0	5

* Three replicates per treatment, and five males in each replicate.
† NA, Not available.
‡ AI, artificial insemination (April 26–29, 2005).
§ A single female fish produced two broods in this replicate.

DISCUSSION

In this study, hybrid offspring were obtained with cryopreserved sperm from *X. helleri,* and the paternity was confirmed via phenotypic and genotypic detection. A practical approach for sperm cryopreservation and artificial insemination was developed and established.

Choice of Female to Detect the Fertility of Cryopreserved Sperm

Fishes from different *Xiphophorus* species do not cross naturally, but they may be induced to produce interspecies hybrid broods by forced matings or through artificial insemination. The interspecies cross used in this study requires artificial insemination and is the first step in the well-studied spontaneous melanoma tumor model originally detailed by Myron Gordon and Kurt Kosswig in the late 1920s [1]. Platyfishes such as *X. maculatus* usually attain sexual maturity at 3–5 months, whereas swordtails, like *X. helleri*, may require 6 months to a year to become fully mature. However, due to development of the male gonopodium, sexual dimorphism is evident at earlier times (6 wk for platyfish to 3 mo for swordtails) [10]. After copulating with the male, female *Xiphophorus* may store sperm for months and can subsequently produce broods at approximately 30-day intervals for 4–5 mo without the presence of a male. The intervals between mating and the first few broods are irregular and vary between 26 and 90 days, but once a brood is produced by a female the intervals between successive broods can become fairly regular [10]. Because of these reproductive characteristics, presumptive virgin females of *X. maculatus* Jp 163 A and B were used in this study to detect the fertility of cryopreserved sperm from *X. helleri* by artificial insemination [17]. The inseminated females were monitored for as long as 90 days after insemination for production of live young. Based on our experience, artificial insemination using fresh sperm injection yields a 20%–30% success rate for live young harvest (unpublished XGSC data). Thus, 15 females were used for insemination with sperm from each group, and they were divided into three groups with 5 females in each as single replicates.

Number of Sperm Injected for Successful Artificial Insemination

In previous studies of sperm cryopreservation in *X. helleri*, high motility (40%–78%) of thawed sperm was obtained by use of dimethyl sulfoxide (DMSO) or glycerol as the cryoprotectant and HBSS at 310–320 mOsmol/kg as the extender [12, 14]. However, fertilization trials with thawed sperm were not successful: no embryo development was observed, and no live young were produced. In this study, injection of 1.5 μl control fresh sperm with a density of 10^9 sperm/ml resulted in fertilization and production of live young. Taking into consideration the maximum sperm volume females can receive by artificial insemination, we concentrated the cryopreserved and thawed sperm to a density of 10^9/μl and used 4–6 μl of this concentrated sperm for injection. This concentration was aimed at compensating for sperm loss during the cryopreservation process (based on the estimation of percent motility). The harvest of live young from all cryopreserved treatment groups and the controls demonstrated that use of concentrated sperm and a suitable injection volume were necessary for successful artificial insemination. Even so, further investigations are necessary to better document the relationship between injected sperm number and ultimate fertilization potential, especially for cryopreserved sperm.

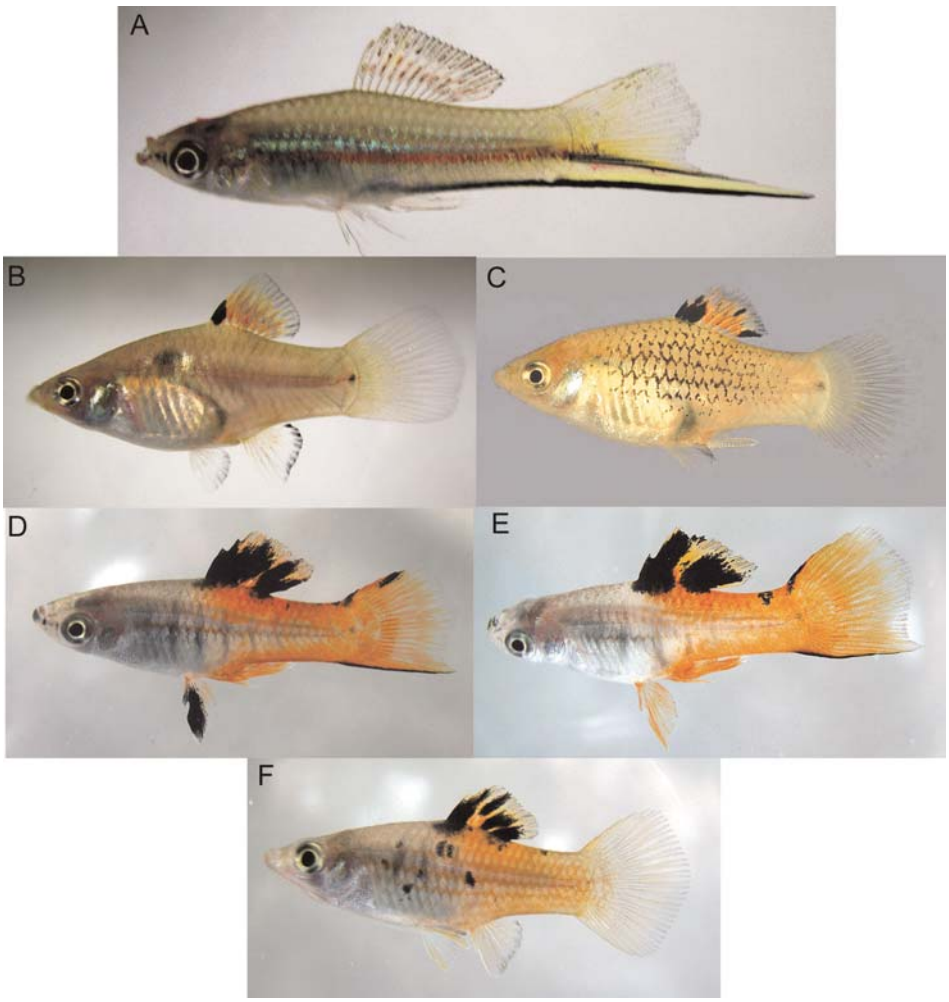

FIG. 2. Examples of parental and interspecies hybrid phenotypes. Examples of individual interspecies hybrids are from three different broods (~10 mo old; **D**, **E**, and **F**) derived from artificial insemination of female *X. maculatus* Jp 163 A (**B**) with cryopreserved sperm samples collected from *X. helleri* males (**A**). An example of a *X. maculatus* Jp 163 A male is presented in **C** (note absence of swordtail) to illustrate the differences between hybrid and parental pigment patterns. Note that hybrids (**D**, **E**, **F** from *X. helleri* strains of Jalapa, Sarabia, and BXII) exhibit enhancement of dorsal red (*Dr*) and the macromelanophore spotted dorsal (*Sd*) pigment patterns [17] compared with male or female *X. maculatus*. This F_1 phenotype has been well studied as a first step in the Gordon-Kosswig melanoma model [17]. This cross absolutely requires artificial insemination to produce interspecies hybrid progeny, and thus verifies these broods resulted from insemination with cryopreserved sperm.

Immobilized and Nonimmobilized Sperm in Cryopreservation and Artificial Insemination

Generally, sperm from oviparous fishes will remain motile only for a short time (less than a few minutes) after being activated [13, 18]. Therefore, motility inhibition is required when sperm are stored for artificial fertilization and cryopreservation. In contrast to oviparous fishes, sperm from viviparous fishes of the genus *Xiphophorus* can remain continuously motile for 3–7 days during storage at 4°C after being activated in solutions isotonic to seminal plasma (300–320 mOsmol/kg) [11, 12]. Our previous work has shown that *Xiphophorus* sperm can be immobilized in HBSS500, and that the immobilization can extend the motility of thawed sperm after cryopreservation [11]. Therefore, in this study activated motile sperm (in HBSS310) and immobilized sperm (in HBSS500) were cryopreserved and used for artificial insemination to test the hypothesis that immobilization of sperm may save energy stores during sperm sample processing and may benefit the storage of sperm in the female reproductive tract (the energy-limiting hypothesis) [11]. The harvest of live young from cryopreserved sperm in both HBSS310 and HBSS500 indicated that energy limitation is not a dominant consideration in the conditions we tested, although it could become important if sperm samples must be stored for several days prior to cryopreservation.

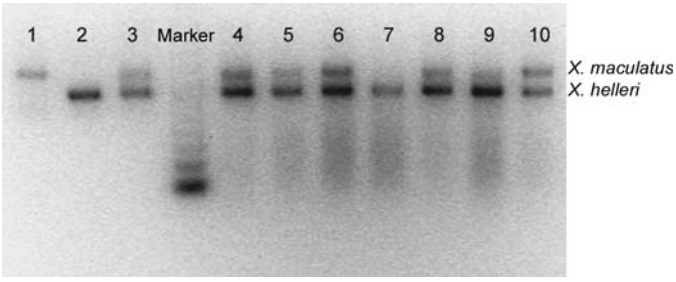

FIG. 3. *Xiphophorus maculatus* Jp 163 A females were artificially inseminated with cryopreserved sperm from *X. helleri,* and the progeny were screened with microsatellite marker Msc053. Lane 1, *X. maculatus* Jp 163 A control; lane 2, *X. helleri* control; lane 3, F1 hybrid control from *X. maculatus* Jp 163 A × *X. helleri*; lanes 4–10, F1 offspring harvested from *X. maculatus* Jp 163 A × *X. helleri*. Lane 4, undiluted sperm; lanes 5–6, WashCryoHBSS310 sperm; lane 7, CryoHBSS310 sperm; lanes 8–9, WashCryoHBSS500 sperm; lane 10, CryoHBSS500 sperm.

Sperm stored in females may derive energy from external sources, such as the metabolism of carbohydrates [19]. Due to internal fertilization of *Xiphophorus,* it is problematic to track the fate of sperm after insemination, especially physiologic changes that may occur during the storage period within the female reproductive tract prior to fertilization. One study in *X. maculatus* showed that sperm stored in inseminated females became associated with specific epithelial cells lining the oviduct in two forms: 1) sperm were stored within deep surface pits or pockets, and 2) sperm were taken up and incorporated within the cytoplasm of specific epithelial cells. These epithelial cells play an undefined role in sperm storage and could be associated with secretory or nutritive support of sperm [20]. Further investigation is necessary to evaluate such

mechanisms for storage of cryopreserved *X. helleri* sperm in *X. maculatus* females.

Washing and Nonwashing of Cryopreserved Sperm after Thawing

Most cryoprotectants, including glycerol, are generally toxic to sperm [21]. Cryoprotectant toxicity is an especially important consideration in artificial insemination in *Xiphophorus* because thawed sperm, when injected into the female reproductive tract, could be toxic to females or oocytes. Previous work has shown that washing of thawed sperm by centrifugation is an effective method to reduce cryoprotectant toxicity, and it may prolong viable sperm storage times at 4°C from 24 h to 72 h [11]. In this study, cryopreserved sperm samples from each male were split into washed or nonwashed treatments before injection into females. In each case live young were produced. This indicates that removal of glycerol from thawed sperm was not required for successful artificial insemination. However, the occurrence of live young harvested from cryopreserved sperm with washing was higher than for the nonwashed sperm cryopreserved in HBSS500 (three replicates vs. one replicate). Considering this result, washing thawed sperm should be further studied for its effect on artificial insemination with cryopreserved sperm.

Lifespan of Cryopreserved Sperm in Female Reproductive Tract

In domestic livestock it is recognized that cryopreserved sperm have a shorter lifespan within the female reproductive tract than do fresh semen [22]. In this study, a second, successive brood was harvested at 82 days from a female inseminated with cryopreserved sperm. This demonstrated that the cryopreserved sperm were still viable for fertilization after the harvest of a first brood (56 days), and showed a functional lifespan comparable to that of fresh sperm. The shortened lifespan of cryopreserved sperm in domestic livestock is suggested to be due to the unsuccessful establishment of normal sperm-epithelium interactions [22]. As stated above, sperm from *Xiphophorus* have been observed to associate with epithelial cells when stored in the female reproductive tract [20], but it is unknown to what extent this association contributes to maintaining the viability of cryopreserved sperm in *Xiphophorus*.

Summarily, in this study the protocols established for sperm cryopreservation, thawing, and postthaw sample preparation for artificial insemination were as follows: crushing of dissected testis in HBSS at an osmolality of 310 mOsmol/kg (HBSS310) or 500 mOsmol/kg (HBSS500, recommended), mixing sperm suspension with premade double-strength glycerol-HBSS to a final concentration of 14% and equilibrating for 15 min, cooling the sperm suspension at a rate of 20°C/min from 5 to −80°C after loading of 240 μl in 250-μl French straws, and transferring the frozen samples to liquid nitrogen for storage. Before artificial insemination, the steps were: thawing of samples at 42°C for 5 sec in a water bath, centrifuging ($1000 \times g$) the thawed sperm for 5 min at 4°C, discarding the supernatant and resuspending in fresh HBSS (a wash step, recommended), centrifuging to pellet the sperm again, discarding the supernatant and concentration of the sperm to a density of $\sim 10^9$ cells/ml, and injecting 4–6 μl of concentrated sperm into individual, virgin females for artificial insemination. The females were monitored for 90 days for abdominal swelling and subsequent discharge of live young.

This study reports the first successful fertilization and offspring production by cryopreserved sperm in live-bearing fishes, and it establishes a practical protocol for sperm cryopreservation in *X. helleri*. The approach established in this study provides an effective way for genetic resource preservation in *Xiphophorus* fishes, a group of species that is widely used for biomedical research and is rapidly being lost in the wild. In addition, based on the present study, *Xiphophorus* fishes could conceivably also serve as a research model for cryopreservation and reproduction in mammals.

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