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Sperm cryopreservation of a live-bearing fish, *Xiphophorus couchianus*: Male-to-male variation in post-thaw motility and production of F_1 hybrid offspring ☆

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ABSTRACT

Fishes of the genus *Xiphophorus* are well-studied biomedical research models, and some species, such as *X. couchianus*, are currently listed as endangered in the wild. Sperm cryopreservation in these live-bearing fishes has begun recently. Thus far, live young have been produced with cryopreserved sperm only in one species (*Xiphophorus helleri*). In this study, the goal was to develop a practical protocol for sperm cryopreservation of *Xiphophorus couchianus*, and to produce live young with cryopreserved sperm. Sperm were collected by crushing of testis in Hanks' balanced salt solution at an osmolality of 500 mOsmol/kg (HBSS500), and were cryopreserved with 14% glycerol (v/v) as cryoprotectant at a cooling rate of 20 °C/min from 5 to −80 °C in 250-μL French straws. For artificial insemination, samples were thawed at 40 °C for 5 s in a water bath, washed once using fresh HBSS500 by centrifuging at 1000 *g* for 5 min at 4 °C, concentrated into ~5 μL, and injected into virgin females of *Xiphophorus maculatus*. The inseminated females were monitored for 90 days for subsequent discharge of live young. Results from 2006 and 2007 showed considerable male-to-male variation in post-thaw motility (from 1 to 70%). Offspring were produced by cryopreserved sperm in two tanks (of three) at 36 and 66 days after insemination in 2007. Paternity was confirmed via phenotypes (body color) and genotypes (microsatellite genetic marker) of the hybrid offspring. Overall, a practical protocol for sperm cryopreservation and artificial insemination is provided to preserve *X. couchianus*, which is an important biomedical research model, and also currently listed as an endangered species in the International Union for Conservation of Nature (IUCN) red list.

1. Introduction

Fishes of the genus *Xiphophorus* (swordtails) are valuable biomedical research models for spontaneous or induced tumor research (Kazianis and Walter, 2002; Fernandez and Browser, 2008), especially specific hybrid and back crosses between different species (Schartl, 1995; Schartl et al., 1995; Walter and Kazianis, 2001; Walter et al., 2006). Seventy years of research use has led to development of 60 pedigreed lines among 25 species, that are currently held in the *Xiphophorus* Genetic Stock Center (XGSC, www.xiphophorus.org) which was established in 1939 (Kallman, 2001). For example, *X. maculatus* line Jp 163A and Jp 163B are in their 102nd and 96th generations of inbreeding, *X. couchianus* is in its 72nd generation, and *X. helleri* is in its 57th generation (Walter et al., 2006). These valuable pedigreed research stocks and inbred lines are maintained as live animals in the XGSC, and need to be preserved in perpetuity. Gamete cryopreservation could become a useful technique for this purpose.

Sperm cryopreservation research was first reported in 2004 in live-bearing fishes (Huang et al., 2004b). Compared to most fish species with external fertilization, live-bearing (i.e., viviparous) fishes reproduce by internal fertilization, and consequently their sperm possess specialized morphology and physiology, such as a cylindrical nucleus, short thickened midpiece, an elongated axoneme (Jonas-Davies et al., 1983), formation of head-to-head agglutination bundles within the female reproductive tract (Hoar, 1969; Grier, 1975), and storage of sperm for months in the female reproductive tract (Tavolga, 1949). Additionally, *Xiphophorus* fishes are characterized by small body sizes (2–5 cm); consequently, the availability of sperm is limited to microliter volumes, and artificial insemination, difficult by itself, is further complicated by the small body size of females. Therefore, sperm cryopreservation and artificial insemination with cryopreserved sperm present significant challenges in live-bearing fishes compared to fishes with external fertilization. Thus far, sperm cryopreservation in live-bearing fishes has been reported in two *Xiphophorus* species, *X. helleri* and *X. couchianus* (Huang et al., 2004a,b,c; Yang et al., 2006,

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2007b), but currently live young have only been produced from cryopreserved sperm of *X. helleri* (Yang et al., 2007b).

For *X. couchianus*, sperm cryopreservation has been studied using post-thaw motility as the evaluation indicator (Huang et al., 2004a), but evaluation of artificial insemination with cryopreserved sperm has not been reported. For *X. helleri*, sperm suspended in hypertonic extender (500 mOsmol/kg) were osmotically immobilized (they are motile when held in isotonic buffer), and the immobilization of sperm was able to extend retention of motility during refrigerated (non-frozen) storage compared to storage of non-immobilized sperm in isotonic solutions, and did not affect the motility of sperm after thawing (Yang et al., 2006). When the immobilized sperm were used for artificial insemination after thawing, live young were produced, and occurred more frequently than for non-immobilized sperm held in isotonic solution (310 mOsmol/kg) (Yang et al., 2007b).

In the present study, the protocol developed for *X. helleri* (Yang et al., 2007b) was adopted for sperm cryopreservation and artificial insemination in *X. couchianus* including use of hypertonic (500 mOsmol/kg) extender. Herein, we report successful fertilization and offspring production by cryopreserved sperm of *X. couchianus*, and document considerable male-to-male variation on post-thaw motility. The established protocols in this study are expected to assist preservation of germplasm resources in *X. couchianus* which is an endangered species on the red list of the International Union for Conservation of Nature (http://www.iucn.org/).

2. Materials and methods

2.1. Animals

Fishes used in this experiment were all from the XGSC (Texas State University, San Marcos, TX, USA). Males used in 2006 were of the 71st generation of strain Xc (7–8 months old) which have been maintained in the XGSC since 1961; while males used in 2007 were of the 72nd generation of the same strain (Xc; 7–8 months old). Females used for artificial insemination were *Xiphophorus maculatus* of the strains JP Wild and SR. Virgin females were selected by separation from mixed-sex broods prior to maturation (at around 6 weeks of age) (Walter et al., 2006). Guidelines from the Institutional Animal Care and Use Committees (IACUC) of Louisiana State University Agricultural Center and Texas State University were followed for animal care in this study. These IACUC animal protocols and inspections are current (IACUC no. 05–05F7651F62), as is the National Institutes of Health Protection from Research Risks approval.

2.2. Sperm collection

Sperm were collected by crushing of dissected testis in this study. Before sperm collection, males were anesthetized in 0.01% (w/v) tricaine methane sulfonate (MS-222, Western Chemical Inc., Ferndale, WA, USA) until their swimming stopped, rinsed in fresh water, and blotted with a paper towel to dry the body. Before dissection of testes, the fish were measured for standard length (from snout tip to the base of the tail) and body weight, and dissected at 10-× magnification. Testes were separated from the surrounding lipid tissues, and transferred to a 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 body length (cm). Sperm were released by crushing of the testis in Hanks' balanced salt solution at an osmolality of 500 mOsmol/kg (HBSS500) with a volume of 30–40 times the testis weight. HBSS500 was prepared by adjusting the water volume from 1 L to around 600 mL for the recipe of HBSS used to produce an osmolality of 300 (0.137 M NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 1.0 mM MgSO4, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 4.2 mM NaHCO3, and 5.55 mM glucose, pH = 7.2), the osmolality was measured with a vapor pressure osmometer (Model 5520, Wescor Inc., Logan, UT, USA), and pH was measured with a pH meter (AB15, Fisher Scientific). Before use in experiments the sperm suspensions were held on ice. The fish bodies after testis dissection were preserved in 95% (v/v) ethanol for verification by genotypic analysis of parental contribution to offspring.

2.3. Motility estimation

Sperm motility was estimated by placing 1 μL of sperm suspension on a glass slide, addition of 20 μL of HBSS at an osmolality of 300 mOsmol/kg, and observation of the sperm at 200-x magnification using dark-phase microscopy (Optiphot 2, Nikon Inc., Garden City, NY, USA). The percentage of sperm which moved actively in a forward direction was recorded as sperm motility in this study. Sperm vibrating in place were not considered to be motile.

2.4. 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 in *X. helleri* (Yang et al., 2007b). Sperm cells were suspended in HBSS500 with a volume of 30–40 times the testis weight. Glycerol (final concentration of 14%, v/v) was used as cryoprotectant by mixing an equal volume of the sperm suspension with double strength glycerol-HBSS500 mixture (28%). After equilibration for 15 min, 200 μL of the sperm suspension with glycerol was loaded into 250-μL straws, frozen with a cooling rate of −20 °C/min 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 month in liquid nitrogen, the frozen straws were thawed at 40 °C for 5 s, the sperm suspensions were transferred into 1.5-mL centrifuge tubes on ice, and the motility of thawed sperm was estimated within 30 s. For concentrating and washing, the thawed sperm suspensions were centrifuged (1000 *g*) for 5 min at 4 °C; the supernatant was decanted, the sperm pellet was re-suspended by adding 100 μL of fresh HBSS500, centrifuged again (1000 *g*) for 5 min at 4 °C, and the supernatant decanted again. For artificial insemination, the sperm pellet was gently suspended into a total volume of 5–10 μL of fresh HBSS500 prior to injection.

2.5. Artificial insemination

To detect the fertility of cryopreserved sperm, artificial insemination was performed using the hybrid cross of *X. maculatus*×*X. couchianus* (female×male). 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 details in Yang et al., 2007b). The tip end of the device was filled with sperm sample, and gently pushed into the genital duct (viewed at 10-x magnification), and the sperm sample (5 μL) from each male was injected into the genital duct. After insemination, the females were returned to fresh water for recovery, and were maintained for collection of live young in aquaria systems in the XGSC. The inseminated females were maintained with the routine care (www.xiphophorus.org), and checked daily. At 90 days after insemination or when live young were collected (whichever came first), the inseminated females were dissected for examination of the reproductive tract.

In 2006 (March 16), 10 cryopreserved samples (of the 22 males) with post-thaw motility of 30–70% were used for artificial insemination, and fresh samples collected from 10 males by crushing of the dissected testis in HBSS500 with a volume (μL) of 4 times testis mass (mg) were used as control. Inseminated females were grouped as two fish per tank. In 2007 (February 23), 15 samples (of the 19 males) with post-thaw motility of 20–70% were used for artificial insemination, and fresh sperm collected from 15 males were used as the controls. Inseminated females were grouped as five fish per tank.

2.6. Phenotype and genotype confirmation of the F1 offspring

In this study, phenotypes and genotypes were both used to confirm the paternity of the live young harvested from females inseminated with cryopreserved sperm or with fresh sperm (controls). Live or ethanol-preserved (95%, v/v) adult fish and live young (9–10 months old) were used for DNA analysis. Tailfin clips from each fish were collected after the fish were briefly anesthetized in 0.01% MS-222 and photographed. All young collected in this experiment were analyzed.

Genomic DNA was extracted from the tail clips using the PureGene™ Cell and Tissue DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) 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 Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Stock DNA solutions were diluted to 5 ng/μL in 1x Tris/HCl-EDTA buffer pH 8.0 and stored at 4 °C until use.

Prior to screening of the DNA samples, several microsatellite polymerase chain reaction (PCR) primer sets (http://www.xiphophorus.org/microsats/microsat.htm) were tested using DNA from known *X. maculatus* Jp 163 B, *X. couchianus*, and F1 hybrids from *X. maculatus* Jp 163 B×*X. couchianus* to ensure discrete amplification using PCR. An optimized microsatellite primer set, Msd037, was used for analyzing the samples collected in this study. The sequence of the forward oligonucletide was 5′-TGTACAGATCATAACCTAAAACAATGTGC-3′ and the sequence of the reverse oligonucleotide was 5′-TTGCCATGGA-AACAGATGATG-3′. Genomic DNA was amplified and analyzed by agarose gel electrophoresis. Each 25-μl reaction contained 30 ng of genomic DNA, 0.2 μM of each forward and reverse oligonucleotide, 2.0 units of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), and 10 mM $MgCl_2$ in 32 mM Tris-HCl (pH 8.4), 80 mM KCl, 5% DMSO. The amplification steps consisted of initial denaturation at 94 °C for 5 min, followed by 40 cycles of: denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. 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-x Tris-acetate EDTA containing 50 ng/mL ethidium bromide. For the PCR reaction, 2 controls consisting of TE buffer, and primer but no DNA (blank in Fig. 3A) were electrophoresed alongside genomic DNA samples from two *X. maculatus* female parents, two *X. couchianus* male parents, two control offspring produced from fresh sperm, and two offspring produced from cryopreserved sperm.

2.7. Data collection

Data in this study were analyzed using SYSTAT 10 (Chicago, IL) or Excel 2003. The effects of treatments were tested by use of T-test and F-test. Percentage data were arcsine-transformed before analysis. The significance level was set at $P<0.050$.

3. Results

3.1. Basic parameters

In total, 66 males (Xc strain) were used in this study with 32 in 2006 (10 for fresh sperm and 22 for cryopreserved sperm) and 34 in 2007 (15 for fresh sperm and 19 for cryopreserved sperm). For these males, the standard body length was 2.0±0.2 cm (mean±SD), the body mass was 0.184±0.052 g, and the testis mass was 3.1±1.0 mg. By hemacytometer counting, the sperm density after crushing of testis in extender was calculated as $5.4\pm2.5\times10^6$ cells per mg testis from the 32 males used in 2006.

Correlation analysis showed that body length, body weight, and testis weight were positively correlated to each other ($P\leq0.044$), and the coefficient of determination (R^2) was 0.6852 between body weight and standard length (Fig. 1A), 0.1069 between standard length and testis weight (Fig. 1B), and 0.1275 between body weight and testis weight (Fig. 1C). Analysis of body condition factor did not show a significant correlation with testis weight ($P=1.000$) (Fig. 1D).

3.2. Post-thaw motility of cryopreserved sperm

In 2006, 22 males were used for sperm cryopreservation experiments. Sperm motility before freezing ranged from 60 to 90% (Fig. 2A).

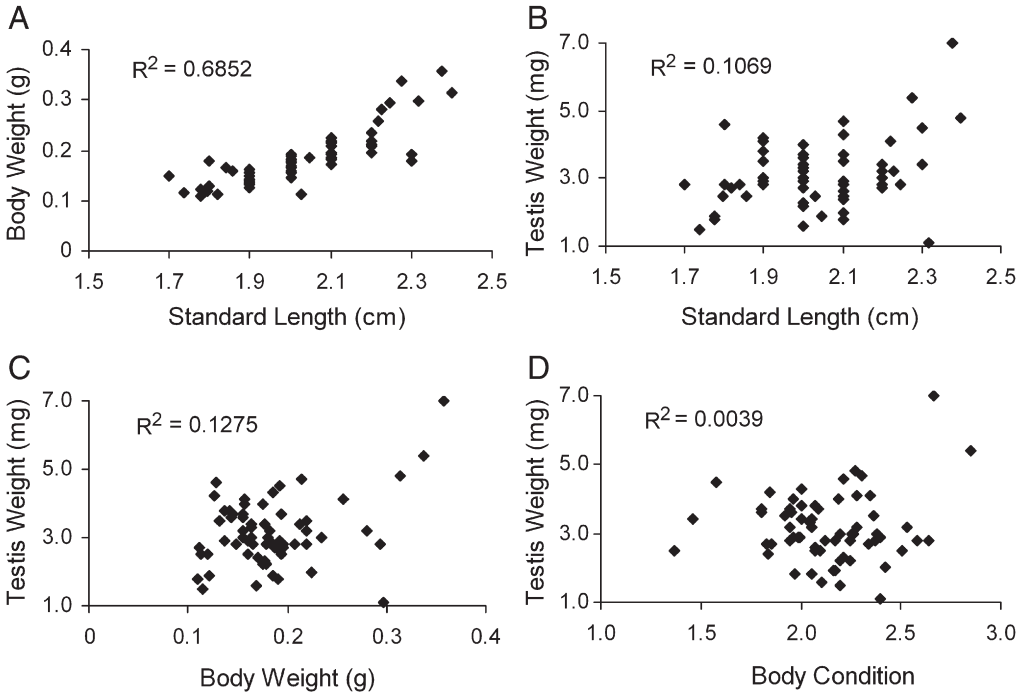

Fig. 1. The correlation of body length, body weight, testis weight, and body condition of *Xiphophorus couchianus* males used in this study (N=66). Body condition was calculated as the percentage of body weight (g) to the cube of the body length (cm).

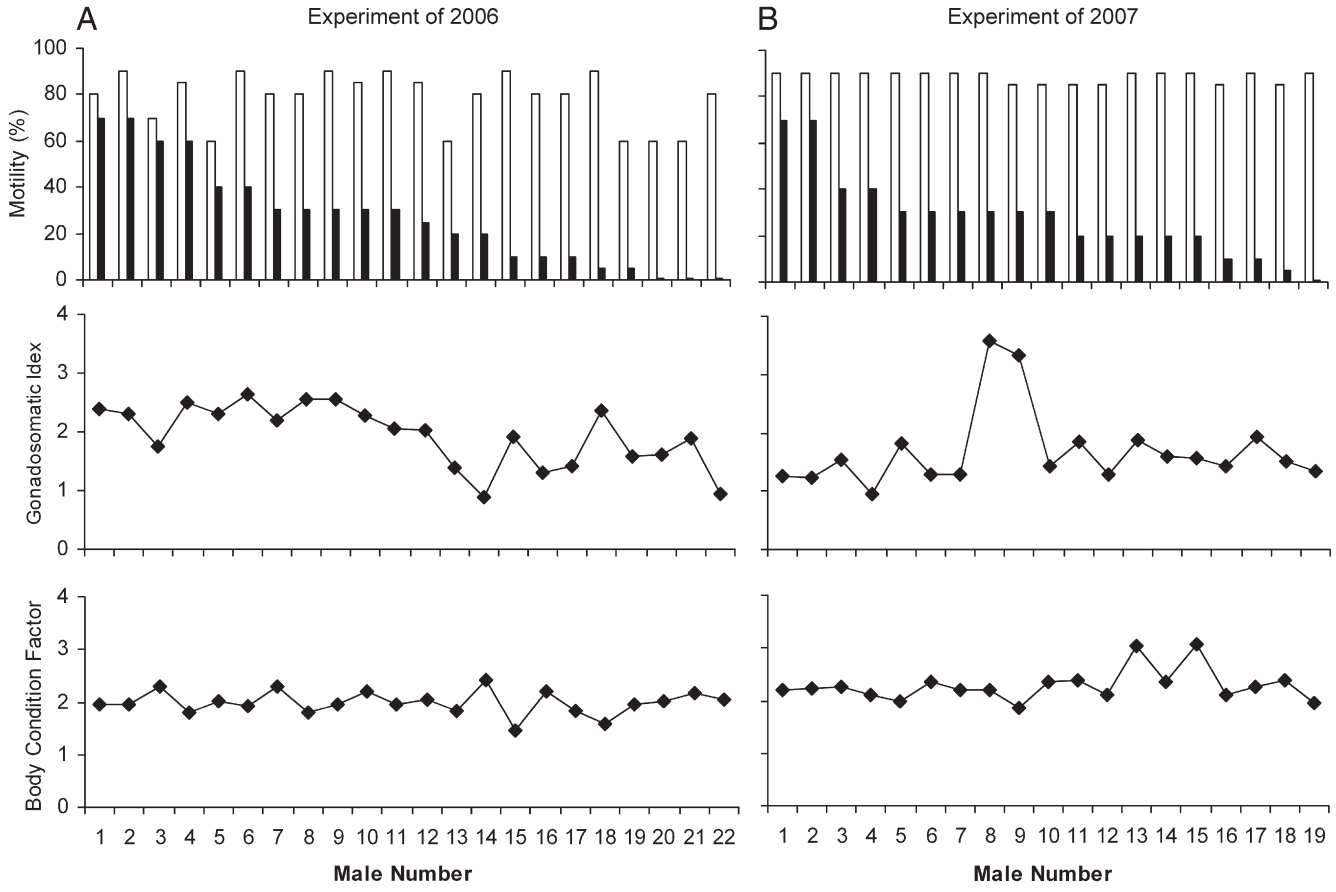

Fig. 2. Sperm motility of individual *Xiphophorus couchianus* males after thawing (black bars) and before freezing (white bars) in the experiments of 2006 (A) and 2007 (B) along with the gonadosomatic index (percentage of testis weight to the body weight) and body condition factor (percentage of body weight to the cube of the body length) of each male. Males from each year are presented in ranked order (from highest to lowest) of post-thaw motility.

After thawing, the sperm motility varied significantly from 1% to 70% among individuals (P=0.001) (Fig. 2A). Correlation analysis showed that post–thaw motility was significantly related with the gonadosomatic index (P=0.008) (Fig. 2A), but not with the body condition factor (P=0.706) (Fig. 2A).

For the 19 males studied in 2007, sperm motility before freezing ranged from 85 to 90% (Fig. 2B). After thawing, the sperm motility again varied from 1 to 70% among individuals (P=0.000) (Fig. 2B). Correlation analysis showed that post-thaw motility was not related with the gonadosomatic index (P=0.706) (Fig. 2B), nor with the body condition factor (P=0.815) (Fig. 2B). No significant difference (P=0.314) was detected between the gonadosomatic index of males used in 2006 (N=22, 1.95±0.52) and 2007 (N=19, 1.69±0.67), but a significant difference (P=0.006) was observed for the body condition factor of males in 2006 (1.99±0.23) and in 2007 (2.29±0.31).

3.3. Artificial insemination of cryopreserved sperm

In 2006, ten cryopreserved sperm samples were used for artificial insemination of 10 females of *X. maculatus*. The inseminated females were grouped into five tanks with 2 female in each. No live young were harvested from the 10 inseminated females with cryopreserved sperm, and dissection of the females at 3–4 months after artificial insemination did not show any developing embryos. Also, no live young were harvested from 10 females inseminated with fresh sperm (controls).

In 2007, fifteen cryopreserved sperm samples were used for artificial insemination. The 15 inseminated females were grouped into three tanks with 5 females in each. Live young were harvested at 36 and 66 days after insemination in two tanks (of three). Also, live young were harvested at 31 and 35 days after insemination in two control tanks inseminated with fresh sperm.

3.4. Phenotype and genotype of the live young

Phenotypic characters in adult hybrids of *X. maculatus*× *X. couchianus* include: (1) expression of the Crescent tail pattern (C) originating from *X. maculatus* (Jp wild strain) coupled with the distinct vertical barring and more elongated body shape exhibited by *X. couchianus*. These traits from both parental species were identified in the harvested offspring from cryopreserved sperm (pedigree #10916) (Fig. 3, Panel B). (2) Also observed was expression of the anal red (*Ar*) and Dot (D) pigment patterns originating from *X. maculatus* (strain SR) along with the vertical barring pattern and more elongated body shape exhibited by *X. couchianus* (pedigree #10929) (Fig. 3, Panel C). Overall, the phenotypic characteristics expected from interspecies hybrids between *X. maculatus* and *X. couchianus* were all confirmed in the live young harvested from the females inseminated with cryopreserved sperm and the fresh sperm controls.

Genotype analyses were performed by use of a polymorphic microsatellite marker to screen the DNA isolated from fins of parental fish and the interspecific hybrids (Fig. 3, Panel A). Microsatellite marker Msd037 was used to amplify the genomic DNA isolated from all putative interspecies hybrids with polymerase chain reaction and resulted in a 300-bp amplicon from *X. maculatus* DNA and a 200-bp amplicon from *X. couchianus* (Heater et al., 2004). Analysis of each F_1 live young harvested from cryopreserved sperm and controls showed the expected bands at 300 and 200 bp (Fig. 3, Panel A, lanes 7, 8, 9

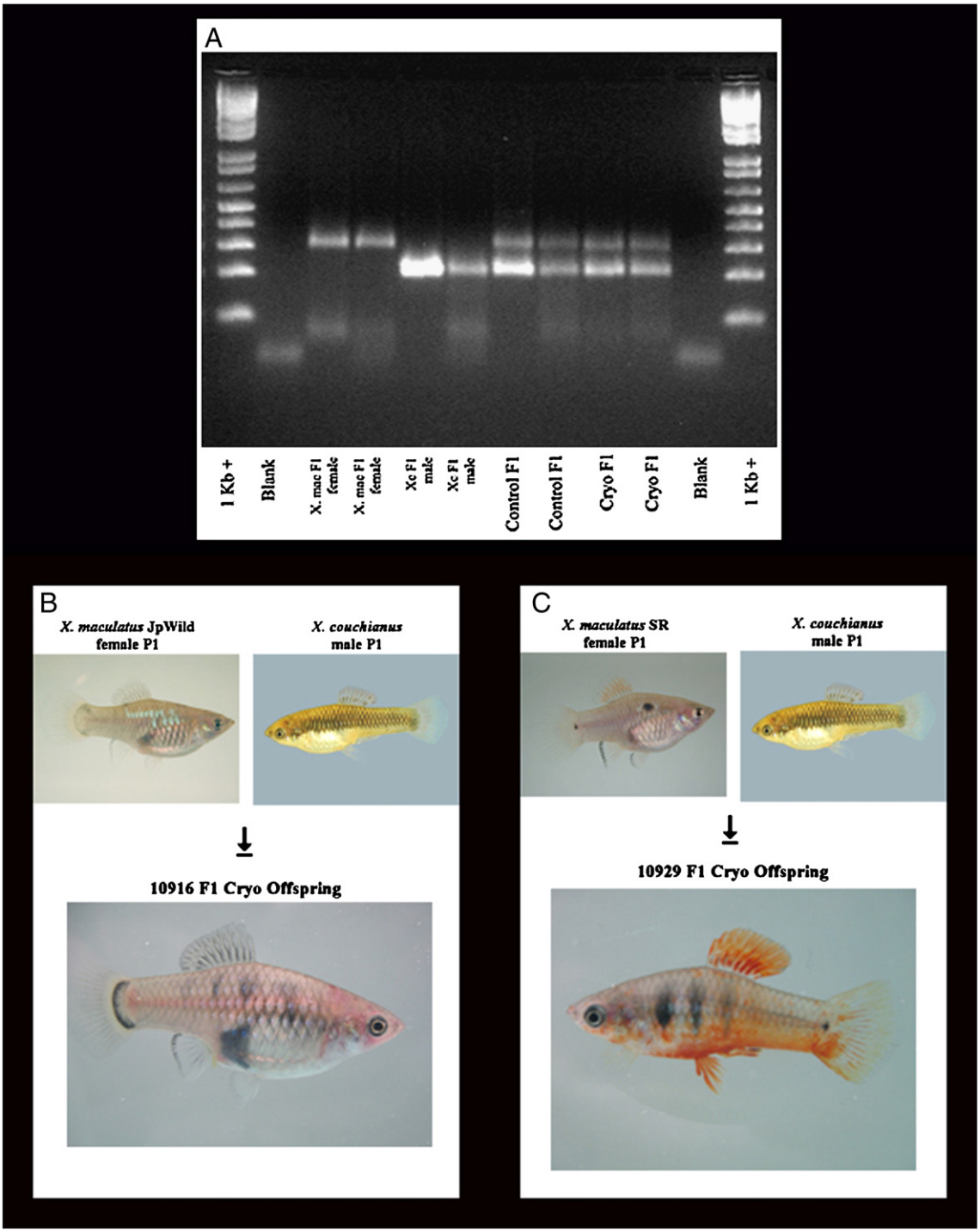

Fig. 3. Genotype (panel A) and phenotype (panels B, C) analyses of parental and interspecies hybrid offspring (8–9 months old) derived from artificial insemination of female *X. maculatus* with cryopreserved sperm from *X. couchianus* (Xc). Microsatellite marker Msd037 was used to amplify the genomic DNA isolated from all putative interspecies hybrids with polymerase chain reaction and resulted in a 300-bp amplicon from *X. maculatus* DNA and a 200-bp amplicon from *X. couchianus* DNA (Heater et al., 2004). Analysis of each F_1 offspring collected from cryopreserved sperm and controls showed the expected bands at 300 and 200 bp (lanes 7, 8, 9 and 10). The parents exhibited the single band diagnostic of each species (lanes 3, 4, 5 and 6). Blank lanes were the PCR product from a reaction with buffer and primers but without DNA template. Phenotypes of parental and interspecies hybrid identified that the hybrids (pedigree #10916, Panel B) exhibited expression of the Crescent tail pattern (C) originating from *X. maculatus* (Jp Wild strain) coupled with the distinct vertical barring and more elongated body shape originating from *X. couchianus* and that the hybrids (pedigree #10929, Panel C) also exhibited expression of the anal red (*Ar*) and Dot (D) pigment pattern originating from *X. maculatus* (strain SR) along with the vertical barring pattern and more elongated body shape exhibited by *X. couchianus*. Analysis of genotype and phenotypes indicate that all of the young collected in this experiment were interspecies hybrid offspring produced from cryopreserved sperm.

and 10). The parents exhibited the single band diagnostic of each species (Fig. 3, Panel A, lanes 3, 4, 5 and 6). This indicates that all of the young harvested in this experiment were indeed interspecfic hybrid offspring, and did not result from stored sperm originating from conspecific matings.

4. Discussion

In this study, hybrid offspring were produced with cryopreserved sperm from *X. couchianus*, the paternity was confirmed via phenotypic and genotypic detection, and considerable male-to-male variation

was observed in post-thaw motility (from 1 to 70%). A practical approach for sperm cryopreservation and artificial insemination was developed and established, and possible reasons for the male-to-male variation of cryopreserved sperm were discussed. This is the second report of the production of offspring with cryopreserved sperm from live-bearing fishes after the first in *X. helleri* (Yang et al., 2007b). The approach established in this study provides an effective avenue for genetic resource preservation in other live-bearing fishes, many of which are endangered species (www.iucnredlist.org).

4.1. Considerable male-to male variation in post-thaw motility

Sperm cryopreservation is a valuable technique used in a wide range of animals, such as humans, livestock, fish, and aquatic invertebrates. In cryopreserved sperm, viability after thawing varies widely among species and individuals (Holt, 2000b; Thurston et al., 2002; Mazur et al., 2008), such as in boar (Holt et al., 2005), rhesus monkey *Macaca mulatta* (Leibo et al., 2007), sea bream *Sparus aurata* (Cabrita et al., 2005), Pacific oyster *Crassostrea gigas* (Dong et al., 2005), and zebrafish *Danio rerio* (Yang et al., 2007a). This is not unexpected given that apparently normal samples of fresh sperm in mammals, birds, fishes, and insects display differential capability in fertilizing eggs (Holt and Van Look, 2004). In this study, significant male-to-male variation in post-thaw motility was observed, although the sperm motility of these males before freezing was not different. Variation in fertility was not quantified due to the considerable logistic difficulties of monitoring embryonic development after internal fertilization in these species.

There are several possible reasons for these variations. First, the susceptibility of spermatozoa to injury might vary based on the differences that have been identified in sperm quality such as membrane integrity and functionality, ATP content, and mitochondrial functionality (Cabrita et al., 2005). In this study, the analysis of data in 2006 showed that individual post-thaw motility was significantly correlated with gonadosomatic index, this agrees with studies about fertility of fresh sperm which concluded that large relative testis size and sperm production capacity were linked to sperm competition ability (Harcourt et al., 1981; Holt and Van Look, 2004). But this correlation did not exist in the data of 2007 although the gonadosomatic indices were not different from these of 2006. This suggests that gonadosomatic index is a measurable indicator to evaluate post-thaw motility, but is not a dominant factor. Body condition analysis in this study did not show a correlation with post-thaw motility in 2006 or 2007, but it is noticeable that body condition in 2007 was significantly higher than in 2006. Thus, it might be true that nutritional status (indicated by body condition factor) is a factor that can influence the viability of cryopreserved sperm, and may compensate for low gonadosmotic index. More investigation is necessary to evaluate specific nutrients for this purpose.

Second, a genetic basis has been suggested for variation in thawed sperm quality, and molecular technologies have been used to identify markers linked to genes influencing this variation (Watson, 1995; Holt, 2000a; Thurston et al., 2002). In this study, the fish used were the 76th (in 2006) and 77th generations (in 2007) of a highly inbred line that has been maintained in the XGSC for more than 45 years. Assuming that these fishes are nearly genetically identical (Walter et al., 2006), a strict genetic basis does not present itself as the reason for the male-to-male variation observed in post-thaw motility in this study.

Third, there are successive steps in the process of gamete cryopreservation, any one of which can result in damage or destruction of cells (Leibo, 2000). These include collection of cells, suspension in extender with addition of cryoprotectant, the cooling process, storage, the warming process, and post-thaw handling. Errors in any of the steps can lead to the loss of viable cells, For example, even with identical samples, ice nucleation can form spontaneously at different temperatures in the cooling process. Therefore, careful attention should be taken to control even small details in each step to reduce the sources of variation. Overall, the reasons for variation in cryopreserved sperm viability include different factors. Further investigation is necessary to develop more effective indicators for evaluating sperm quality, and elucidation is necessary for the genetic and environmental determinants and their interactions in relation to cryopreservation. At present, tight control should be considered in all steps for sperm handling, refrigerated storage, cryopreservation, thawing, and use. Males used for sperm collection should be provided a balanced diet to ensure good body condition and large size of testis, and future research should address enhancing male quality as an approach to minimizing variation.

4.2. Artificial insemination and fertilization efficiency of live-bearing fish

Artificial insemination is a necessary step to test the fertility of cryopreserved sperm of live-bearing fish. For fishes from the genus *Xiphophorus*, different species do not cross naturally in general, but can be induced to produce interspecies hybrid broods by forced matings or artificial insemination. The females chosen for use in this study were similar to those used for study of sperm cryopreservation in *X. helleri* (Yang et al., 2007b). Eight-month-old-female *X. maculatus* were chosen for artificial insemination, and they were segregated as presumptive virgins at about 6 weeks of age (about the earliest time that sexual phenotypes are identifiable). The interspecific cross of *X. maculatus*×*X. couchianus* is an established model for medical research (Walter and Kazianis 2001), and provides an effective screening technique for evaluation of sperm quality.

Due to the reproductive characteristics of *Xiphophorus* fishes, artificial insemination with cryopreserved sperm requires the following considerations: 1) virgin females are needed because females can store sperm sufficient for production of 4–5 broods (Tavolga, 1949); 2) the sperm volume for injection needs to be appropriate for the body size of females (2–4 cm), and thus the sperm volume for insemination is limited to 4–5 μL (Yang et al., 2007b), and 3) the technique for insemination needs a highly-trained technician, especially given that based on 10 years of experience at XGSC, artificial insemination using fresh sperm injection yields a 20–30% success rate for harvest of live young (unpublished data).

Fertilization and embryonic development in live-bearing fishes can be influenced by many factors, such as the protein level in nutrition (Chong et al., 2004), certain fatty acids (Ling et al., 2006), and water quality (Edwards et al., 2006). In live-bearing fishes, social behavior is widely recognized during sexual selection and mating (Uribe and Grier, 2005), but it is unknown to what extent social interactions can influence fertilization, development and gestation. Further investigation is needed.

In this study, artificial insemination in 2006 did not yield live young from the ten matings tested with cryopreserved sperm, nor were live young produced in the ten control matings with fresh sperm. However, in 2007 the allocation of inseminated females was changed from two females per tank to five females per tank. Live young were collected in two of the three tanks of females inseminated with cryopreserved sperm, and in the same number for the control tanks of females inseminated with fresh sperm. These trials utilized fishes of the same strains and the same protocols. This raised the possibility that there might be social interactions or hormonal influences involved among the inseminated females to synchronize or trigger pregnancy. Further study is necessary because it is difficult or impossible to identify the maternity of individual offspring in tanks with grouped inseminated females. Alternatively it could be informative to utilize non-invasive imaging technologies such as ultrasound to monitor grouped females for embryonic development prior to release of live young.

In summary, this study established protocols for sperm cryopreservation, thawing, and post-thaw sample preparation for artificial

insemination as follows: crushing of dissected testis in Hanks' balanced salt solution (HBSS) at an osmolality of 500 mOsmol/kg, mixing of sperm suspension with double-strength glycerol-HBSS to yield a final concentration of 14% and equilibration prior to freezing for 15 min, cooling of the sperm suspension at a rate of 20 °C/min from 5 to −80 °C after loading of 200 µL in 250 µL-French straws, and transferring of the frozen samples to liquid nitrogen for storage. For use in artificial insemination: thawing of samples at 42 °C for 5 s in a water bath, centrifugation at 1000 g of the thawed sperm for 5 min at 4 °C, discarding of the supernatant and re-suspension in fresh HBSS, centrifugation to pellet the sperm, discarding of the supernatant and concentration of the sperm to a volume of ~5 µL, and injection of 4–6 µL of concentrated sperm into individual virgin females for artificial insemination. This is followed by monitoring of the females (grouped five per tank) for 90 days for abdominal swelling and subsequent discharge of live young.

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References

Cabrita, E., Robles, V., Cunado, S., Wallace, J.C., Sarasquete, C., Herraez, M.P., 2005. Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5 mL macrotubes. Cryobiology 50, 273–284.

Chong, A.S.C., Ishak, S.D., Osman, Z., Hashim, R., 2004. Effect of dietary protein level on the reproductive performance of female swordtails *Xiphophorus helleri* (Poeciliidae). Aquaculture 234, 381–392.

Dong, Q., Huang, C., Eudeline, B., Tiersch, T.R., 2005. Systematic factor optimization for cryopreservation of shipped sperm samples of diploid Pacific oysters, *Crassostrea gigas*. Cryobiology 51, 176–197.

Edwards, T.M., Miller, H.D., Guillette, L.J., 2006. Water quality influences reproduction in female mosquitofish *Gambusia holbrooki* from eight Florida springs. Environ. Health Perspect. 114, 69–75.

Fernandez, A.A., Browser, P.R., 2008. Two cases of non-hybrid melanoma formation in *Xiphophorus nezahualcoyotl* Rauchenberger, Kallmann & Morizot. J. Fish Biol. 72, 292–300.

Grier, H.J., 1975. Aspects of germinal cyst and sperm development in *Poecilia latipinna* (Teleostei-Poeciliidae). J. Morphol. 146, 229–249.

Harcourt, A.H., Harvey, P.H., Larson, S.G., Short, R.V., 1981. Testis weight, body weight and breeding system in primates. Nature 293, 55–57.

Heater, S.J., Oehlers, L.P., Rains, J.D., Walter, R.B., 2004. DNA polymerase mRNA and protein expression in *Xiphophorus* fish. Comp. Biochem. Physiol. C 138, 325–334.

Hoar, W.S., 1969. In: Hoar, W.S., Randall, D.J. (Eds.), Reproduction. Fish Physiol, vol. 3, pp. 1–72.

Holt, W.V., 2000a. Basic aspects of frozen storage of semen. Anim. Reprod. Sci. 62, 3–22.

Holt, W.V., 2000b. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 53, 47–58.

Holt, W.V., Van Look, K.J.W., 2004. Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. Reproduction 127, 527–535.

Holt, W.V., Medrano, A., Thurston, L.M., Watson, P.E., 2005. The significance of cooling rates and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Theriogenology 63, 370–382.

Huang, C., Dong, Q., Tiersch, T.R., 2004a. Sperm cryopreservation of a live-bearing fish, the platyfish *Xiphophorus couchianus*. Theriogenology 62, 971–989.

Huang, C, Dong, Q., Walter, R.B., Tiersch, T.R., 2004b. Initial studies on sperm cryopreservation of a live-bearing fish, the green swordtail *Xiphophorus helleri*. Theriogenology 62, 179–194.

Huang, C., Dong, Q., Walter, R.B., Tiersch, T.R., 2004c. Sperm cryopreservation of green swordtail *Xiphophorus helleri*, a fish with internal fertilization. Cryobiology 48, 295–308.

Jonas-Davies, J.A.C., Winfrey, V., Olson, G.E., 1983. Plasma-membrane structure in spermatogenic cells of the swordtail (Teleostei, *Xiphophorus helleri*). Gamete Res. 7, 309–324.

Kallman, K.D., 2001. How the *Xiphophorus* problem arrived in San Marcos, Texas. Mar. Biotechnol. 3, S6–S16.

Kazianis, S., Walter, R.B., 2002. Use of platyfishes and swordtails in biological research. Lab Anim. 31, 46–52.

Leibo, S.P., 2000. Sources of variation in cryopreservation. In: Tiersch, T.R., Mazik, P.M. (Eds.), Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, pp. 75–83.

Leibo, S.P., Kubisch, H.M., Schramm, R.D., Harrison, R.M., VandeVoort, C.A., 2007. Male-to-male differences in post-thaw motility of rhesus spermatozoa after cryopreservation of replicate ejaculates. J. Med. Primatol. 36, 151–163.

Ling, S., Kuah, M.K., Muhammad, T.S.T., Kolkovski, S., Shu-Chien, A.C., 2006. Effect of dietary HUFA on reproductive performance, tissue fatty acid profile and desaturase and elongase mRNAs in female swordtail *Xiphophorus helleri*. Aquaculture 261, 204–214.

Mazur, P, Leibo, S.P., Seidel, G.E., 2008. Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. Biol. Reprod. 78, 2–12.

Schartl, M., 1995. Platyfish and swordtails – a genetic system for the analysis of molecular mechanisms in tumor-formation. Trends Genet. 11, 185–189.

Schartl, A., Malitschek, B., Kazianis, S., Borowsky, R., Schartl, M., 1995. Spontaneous melanoma formation in nonhybrid *Xiphophorus*. Cancer Res. 55, 159–165.

Tavolga, W.N., 1949. Embryonic development of the platyfish (*Platypoecilus*), the swordtail (*Xiphophorus*) and their hybrids. Bull. Am. Mus. Nat. Hist. 94, 161–230.

Thurston, L.M., Watson, P.F., Holt, W.V., 2002. Semen cryopreservation: a genetic explanation for species and individual variation. Cryoletters 23, 255–262.

Uribe, M.C., Grier, H.J., 2005. Viviparous Fishes. New Life Publications, Homestead, FL.

Walter, R.B., Kazianis, S., 2001. *Xiphophorus* interspecies hybrids as genetic models of induced neoplasia. J. Inst. Lab. Anim. Res. 42, 299–322.

Walter, R.B., Hazlewood, L., Kazianis, S., 2006. The *Xiphophorus* Genetic Stock Center Manual. Texas State University.

Watson, P.F., 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Dev. 7, 871–891.

Yang, H., Hazelwood, L., Walter, R.B., Tiersch, T.R., 2006. Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail *Xiphophorus helleri*. Cryobiology 52, 209–218.

Yang, H., Carmichael, C., Varga, Z.M., Tiersch, T.R., 2007a. Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in zebrafish *Danio rerio*. Theriogenology 68, 128–136.

Yang, H., Hazlewood, L., Heater, S.J., Guerrero, P.A., Walter, R.B., Tiersch, T.R., 2007b. Production of F-1 interspecies hybrid offspring with cryopreserved sperm from a live-bearing fish, the swordtail *Xiphophorus helleri*. Biol. Reprod. 76, 401–406.