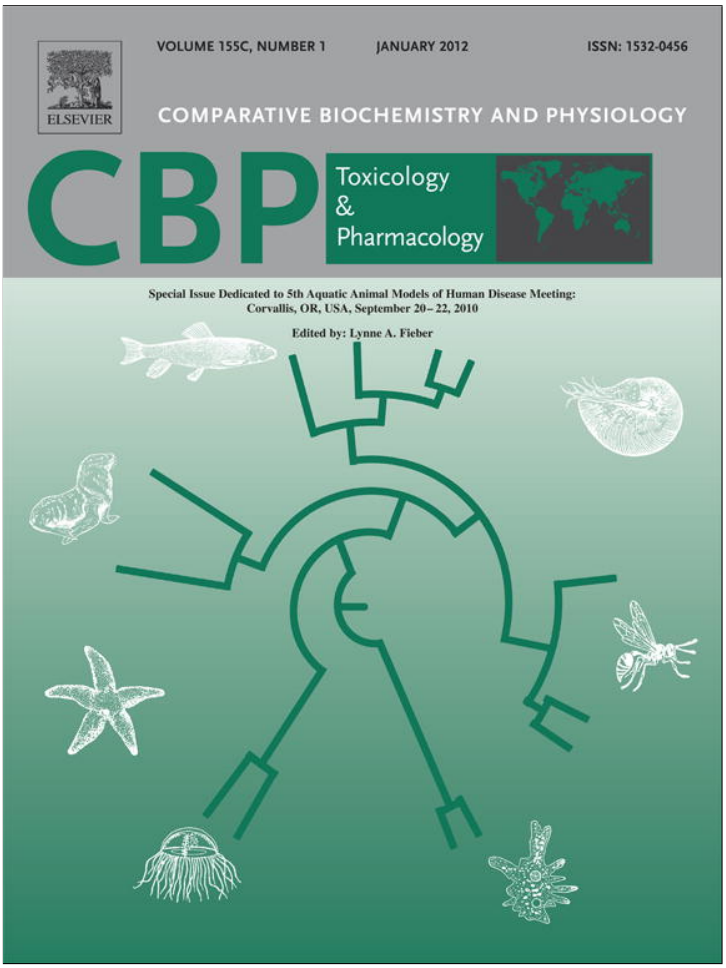

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Offspring production with cryopreserved sperm from a live-bearing fish *Xiphophorus maculatus* and implications for female fecundity ☆

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ABSTRACT

Xiphophorus fishes are well-established models for biomedical research of spontaneous or induced tumors, and their use in research dates back to the 1930s. Currently, 58 well-pedigreed lines exist among 24 *Xiphophorus* species housed as live animals at the *Xiphophorus* Genetic Stock Center. The technique of sperm cryopreservation has been applied to preserve these valuable genetic resources, and production of offspring has been reported with cryopreserved sperm in two species (*X. helleri* and *X. couchianus*). The goal of this research was to establish protocols for sperm cryopreservation and artificial insemination that yield live young in *X. maculatus*, a widely used research species. The objectives were to: 1) collect basic biological characteristics of males, and quantify the sperm production yield after crushing of dissected testis; 2) cryopreserve sperm from *X. maculatus* by adapting as necessary the protocols for sperm cryopreservation of *X. helleri* and *X. couchianus*; 3) use cryopreserved sperm to inseminate virgin females of *X maculatus* and other species (*X. helleri* and *X. couchianus*), and 4) compare experimental trials over a 3-year period to identify opportunities for improving female fecundity. In total, 117 males were used in this study with a standard length of 2.5 ± 0.3 cm (mean ± SD), body weight of 0.474 ± 0.149 g, and dissected testis weight of 7.1 ± 3.7 mg. Calculation of sperm availability showed $5.9 \pm 2.8 \times 10^6$ sperm cells per mg of testis weight. Offspring were produced from cryopreserved sperm. Male-to-male variation (1–70%) was observed in post-thaw motility despite little variation in motility before freezing (60–90%) or genetic variation (~100 generations of sib-mating). Comparisons of biological factors of males did not have significant correlations with the production of live young, and the influence of females on production of young was identified from the comparison of artificial insemination over 3 years. Overall, this study describes offspring production from cryopreserved sperm in a third species of *Xiphophorus* fishes, and identifies the opportunities for improving female fecundity which is essential for establishment of germplasm repositories for *Xiphophorus* fishes.

1. Introduction

The use of *Xiphophorus* fishes as a research model dates back to the 1930s, and led to establishment of the *Xiphophorus* Genetic Stock Center (XGSC, www.xiphophorus.org) (Kallman, 2001). Currently there are 58 pedigreed lines among 24 *Xiphophorus* species held in the XGSC. Live-bearing fishes from the genus *Xiphophorus* are valuable because of their use in the following three areas. 1) They are well-established in biomedical research of spontaneous or induced tumors (Schartl et al., 1995; Kazianis and Walter, 2002), especially specific hybrid backcrosses between different species (Walter and Kazianis, 2001; Walter et al., 2006a). In addition, *Xiphophorus* fishes are used as research model for a broad range of studies including evolution (Basolo, 1998), behavioral ecology (Basolo, 1990), and sex determination (Kallman, 1983). 2) They are major species for the ornamental fish trade because of their unique colorations, behavior and morphology (Monks, 2007). As such, swordtails, platyfish, and the closely related guppies and mollies make up the "big four" of the live-bearing tropical fish groups in ornamental aquarium fish culture. 3) *Xiphophorus* unfortunately includes threatened and endangered species. These fishes inhabit freshwater drainages in Mexico, Guatemala, Belize, and Honduras. Due to human impact on their natural habitats, many wild populations of these fishes have become imperiled. Four species of this genus (*X. clemenciae, X. couchianus, X. gordoni, and X. meyeri*) are listed as rare, endangered, or critically endangered by the Red List of the International Union for Conservation of Nature (www.iucn.org/). Gamete cryopreservation is a technique that can be used to preserve unique genetic resources and is especially relevant for application in *Xiphophorus* fishes to address the three areas listed above. This technique can be employed to preserve valuable pedigreed lines for biomedical research that are currently held as live animals in XGSC and elsewhere, to preserve individuals with commercial value due to unique

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phenotypic traits, and to assist conservative programs for use in preservation and management of endangered wild populations of fishes.

Individuals of the Southern platyfish, *Xiphophorus maculatus*, display a wide array of color variations and pigment pattern markings. Currently the XGSC houses 17 strains of *X. maculatus* which have differences in collection site, history, and in alleles that regulate pigment patterns and sexual differentiation. Eleven of these strains are maintained for chromosomes that can be crossed with stocks of *X. maculatus* Jp163A or Jp163B (Gordon, 1952; Walter et al., 2006a). The mechanism of sex determination in *Xiphophorus* fishes has been characterized as involving at least three sex chromosomes (X, Y, and W) that originated and interact over broad geographic range (Gordon, 1952; Kallman, 1965; Gutbrod and Schartl, 1999). Interspecies *Xiphophorus* crosses involving *X. maculatus* as the non-recurrent parent in backcrosses with *X. helleri*, *X. couchianus*, or others, have become well-established as valuable research models for spontaneous and induced tumor development (Gordon and Gordon, 1952; Kazianis and Walter, 2002). Due to their utility in tumor biology, many individual *Xiphophorus* strains have been maintained in closed colonies or by sibling matings continuously for many decades. For example the *X. maculatus* Jp 163 strain was originally collected from the Rio Jamapa, in Veracruz, Mexico in 1939, and later split into Jp163 A and Jp163 B lines after 9 generations in the laboratory. These highly inbred lines have been maintained by sib-mating since 1968. Currently, the Jp 163 A line is in its 109th inbred generation, and Jp 163 B line is in its 102nd inbred generation (Walter et al., 2006a). As such, these fishes are among the most valuable of inbred vertebrate models.

Xiphophorus fishes are characterized by internal fertilization and the production of live young. Artificial insemination using fresh sperm is difficult and the use of cryopreserved sperm is even more problematic. However, to date, sperm cryopreservation in *Xiphophorus* fishes has been reported to produce live young in two species: *X. helleri* (Yang et al., 2007b) and *X. couchianus* (Yang et al., 2009). In this study, we report successful fertilization and offspring production by cryopreserved sperm from a third species, *X. maculatus.* The protocol developed for *X. helleri* (Yang et al., 2007b) was adopted for sperm cryopreservation and artificial insemination in *X. maculatus* including the use of hypertonic (500 mOsmol/kg) extender. Females of *X. maculatus* and other species (*X. couchianus* and *X. helleri*) were used for artificial insemination to test the fertility of cryopreserved sperm during a 3-year period of research. The goal of this research was to establish protocols for sperm cryopreservation and artificial insemination that yield live young in *X. maculatus*. The objectives were to: 1) collect basic biological characteristics of males, and quantify the sperm production yield after crushing of dissected testis; 2) cryopreserve sperm from *X. maculatus* by adapting as necessary the protocols for sperm cryopreservation of *X. helleri* and *X. couchianus*; 3) use cryopreserved sperm to inseminate virgin females of *X. maculatus* and other species (*X. helleri* and *X. couchianus*), and 4) compare experimental trials over a 3-year period to identify opportunities for improving female fecundity which is essential for establishment of germplasm repositories for *Xiphophorus* fishes.

2. Materials and methods

2.1. Animals

The male fishes used in this study were of *Xiphophorus maculatus* strain Jp 163 A (103rd, 104th and 105th inbred generations), Jp 163 B (94th, 95th and 96th inbred generations) or Jp Wild at 6–7 months of age. All of these strains originated as described above from Rio Jamapa drainage in Veracruz, Mexico. They were cultured in 20-L tanks with static water at a photoperiod of 14 h light: 10 h dark, fed twice daily with live *Artemia* larvae and beef liver paste prepared at the XGSC (Walter et al., 2006a, 2006b). The fish were shipped from the XGSC to the Aquaculture Research Station of the Louisiana State University Agricultural Center (Baton Rouge, LA) by overnight shipping. 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, USA), and were fed twice daily with commercial flakes (Aquatic Eco-system) and live *Artemia salina* 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 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 approval for the National Institutes of Health Protection from Research Risks.

2.2. Sperm collection

Sperm were collected by crushing of dissected testis. Before sperm collection, males were anesthetized in 0.01% (w/v) tricaine methanesulfonate (MS-222, Western Chemical Inc., Ferndale, WA, USA) until swimming motions ceased, and were blotted with a paper towel to remove excess water from the body. Before dissection, the fish were measured for standard length (from the tip of the snout 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 tared 1.5-mL centrifuge tubes for weighing. Gonadosomatic index (GSI) was calculated as the percentage of testis weight in relation 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 released by crushing of the testis in Hanks' balanced salt solution prepared at an osmolality of 500 mOsmol/kg (HBSS500) with a volume of 30–40 times the testis weight, and sperm concentration was obtained by counting the sperm number with a hemocytometer (Bright-line, Hausser Scientific, Horsham, PA, USA). HBSS500 was prepared by adjusting the water volume from 1 L to around 600 mL for the standard recipe of HBSS (used to produce a final osmolality of 300 mOsmol/kg) (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 = 7.8). The osmolality was measured with a vapor pressure osmometer (Model 5520, Wescor Inc., Logan, UT, USA), and pH was measured with a meter (AB15, Fisher Scientific, Pittsburgh, PA, USA). The sperm suspensions were held on ice before and during use in experiments.

2.3. Motility estimation

Xiphophorus sperm are distinct from those of other fishes in that motility is activated by isotonic solutions (Huang et al., 2004; Yang et al., 2006). Accordingly, sperm motility was estimated by placing 1 μL of sperm suspension on a glass slide, with activation by addition of 20 μL of HBSS300, and observation of the sperm at 200-× magnification using dark-phase microscopy (Optiphot 2, Nikon Inc., Garden City, NY, USA). The percentage of sperm that actively moved 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) and *X. couchianus* (Yang et al., 2009). Sperm cells were suspended in HBSS500 with a volume of 30 to 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 French straws (IMV Technologies USA, Maple Grove, MN, USA), frozen at a cooling rate of 20 °C/min from 5 °C to −80 °C (based on probe temperature within a straw) in a computer-controlled programmable freezer (Kryo 10 series II; Planer Products, Sunbury-on-Thames, UK), and transferred into liquid nitrogen for storage.

After storage for 1 month in liquid nitrogen, the frozen samples 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 sperm was estimated within 30 s of thawing. Based on the study in *X. helleri* (Yang et al., 2007b), the thawed samples were washed and concentrated before insemination. The thawed sperm suspensions were centrifuged (1000×*g*) for 5 min at 4 °C; the supernatant was removed by pipette, the sperm pellet was re-suspended by adding 100 μL of fresh HBSS500, was centrifuged again (1000×*g*) for 5 min at 4 °C, and the supernatant again removed by pipette. For artificial insemination, the sperm pellet was gently suspended into a total volume of 5–10 μL of fresh HBSS500 prior to injection into females.

2.5. Artificial insemination

Artificial insemination was performed at the XGSC. Virgin females were used in this study for insemination because *Xiphophorus* females can store sperm sufficient for production of 3 to 5 broods without further mating (Tavolga, 1949). Virgin females were selected by separation from mixed-sex broods at around 6 wks of age which is 2 months prior to maturation (Walter et al., 2006a). Before insemination, the females were anesthetized in 0.01% MS-222, 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-× magnification), and the sperm sample from each male was injected into the genital duct. After insemination, the females were returned to fresh water for recovery, and monitored for 3 months for collection of live young in aquaria systems at the XGSC. The inseminated females were maintained with routine care (Walter et al., 2006a), and were checked daily. At 90 d after insemination, or when live young were collected (whichever came first), the inseminated females were dissected for examination of the reproductive tract.

2.6. Experimental design

In 2006 (March 16), cryopreserved sperm from 10 individuals (of a total of 22 males) with post-thaw motility of ≥30% were used for artificial insemination. Females of *X. couchianus* were used for these trials because this cross produces distinctive hybrid offspring verifying paternity (Walter et al., 2006a), and a 3-μL sample volume was used for injection. Fresh samples collected from 10 males by crushing of dissected testis (suspended in HBSS500 at a volume (μL) of four times the testis weight (mg)) were used as a control treatment for insemination of females from the same population. Inseminated females were grouped as two fish per tank for monitoring of live young production.

In 2007 (February 23), cryopreserved sperm from 15 individuals (of a total of 19 males) with post-thaw motility of ≥20% were used for artificial insemination. Again, the females used were *X. couchianus*, and a 3-μL sample volume was used for injection. Fresh sperm collected from 15 males were used as controls. Inseminated females were grouped as five fish per tank for monitoring of live young production.

In 2008 (July 29), cryopreserved sperm from 20 individuals (of a total of 26 males) with post-thaw motility of ≥30% were used for artificial insemination. In this trial, the procedure was different from that used in 2006 and 2007, as each sperm sample was divided into halves and used to inseminate females of *X. maculatus* and *X. helleri* (Doce strain) with a 5-μL sample volume. Fresh sperm collected from 15 males were used as the controls, and each control sample was also separated into two for insemination of *X. maculatus* and *X. helleri*. Inseminated females were grouped as five fish per tank for monitoring of live young production.

2.7. Phenotype confirmation of the F_1 offspring

In this study, phenotypes were used to confirm the paternity of the live young harvested from females inseminated with cryopreserved sperm or with fresh sperm (controls). All live young collected in this experiment were photographed and analyzed. For *Xiphophorus* fishes, most natural interspecies matings can produce hybrids except for crosses between *X. helleri* and *X. maculatus* which require artificial insemination. For the hybrid offspring of *X. helleri* (female) and *X. maculatus* (male), the diagnostic phenotypes are: Jp Wild exhibit tail patterns of twin spot with or without crescent, and a blue-green iridescence in the shoulder region. Females of *X. helleri* Doce were scored for orange or green tail color and rubra litoralis (Walter et al., 2006a). The hybrid offspring exhibit phenotypes from both parents (Fig. 1).

2.8. Data collection

Data in this study were analyzed using SYSTAT 13 (Systat Software, Inc., Chicago, IL). 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 tested by use of T-test, ANOVA or non-parametric Kruskal–Wallis test when the normality and homogeneity of variance requirements of the data were not satisfied. Percentage data were arcsine-square-root transformed before analysis, and body length data were logarithm transformed. The significance level was set at $P<0.050$.

3. Results

3.1. Basic male characteristics of X. maculatus

In total, there were 117 males used in experiments over 3 yrs. The standard length of the males was 2.5 ± 0.3 cm (mean ± SD), body mass was 0.474 ± 0.149 g, and dissected testis weight was 7.1 ± 3.7 mg. The calculated GSI of the males was 1.51 ± 0.64, and body condition factor was 2.92 ± 0.32. There were significant correlations among body weight, standard length and testis weight ($P\leq0.001$) (Fig. 2. A, B and C), but there were no significant correlations between body length and GSI or condition factor ($P\geq0.127$), nor between body weight and GSI or condition factor ($P\geq0.304$). For body condition factor, no significant correlations were found with testis weight ($P=0.197$), or GSI ($P=1.000$) (Fig. 2D).

For sperm production, the total number of sperm obtained from each male was calculated from sperm concentration and sample volume from 30 individual males in 2006 and 27 males in 2008, and these values were divided by the testis weight from each male, yielding on average $5.88\pm2.81\times10^6$ sperm cells per mg of testis weight (N = 57).

3.2. Post-thaw motility of cryopreserved sperm and correlation analysis with biological parameters

In 2006, post-thaw motility of sperm from 20 individuals varied from 1 to 60% (Table 1). No correlations were found between the post-thaw motility and body weight, body length, testis weight, GSI, body condition factor ($P\geq0.366$), or fresh sperm motility ($P=0.074$). The

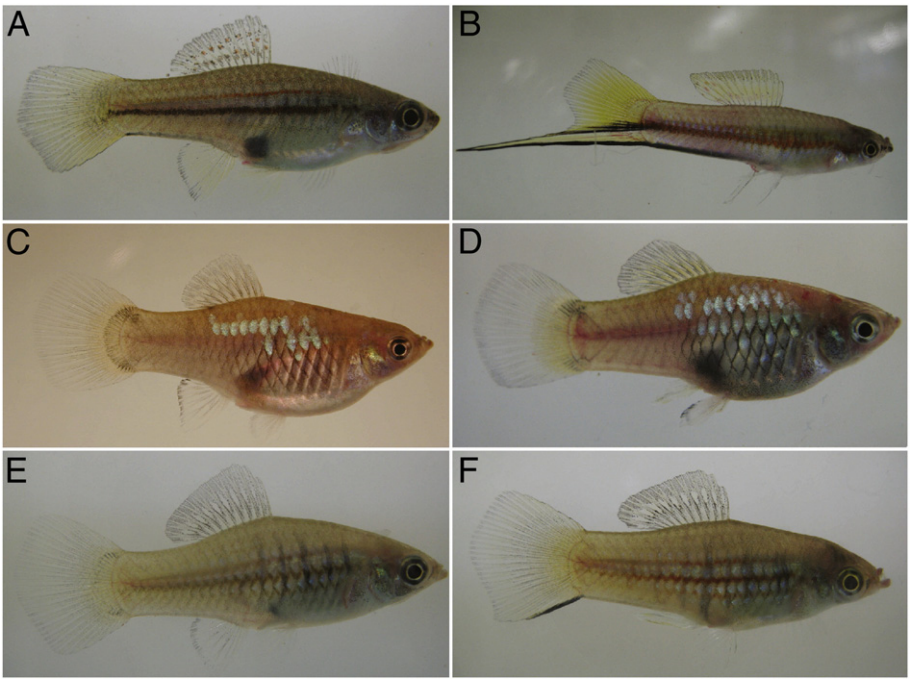

Fig. 1. Phenotypes of *X. helleri* Doce female (A) and male (B) and *X. maculatus* Jp wild female (C) and male (D) and their hybrid offspring female (E) and male (F). The phenotypes of female hybrid offspring (E) included crescent tail pattern, blue-green iridescence, and a more elongated body type with the vertical barring seen in *X. helleri* Doce; male hybrid offspring (F) exhibited blue-green iridescence as well as the more elongated body type and vertical barring. These phenotypes verified that the offspring collected in this experiment were interspecies hybrids produced from cryopreserved sperm, and not storage of conspecific sperm.

concentrations of sperm samples for injection were 0.5–1.5×10^9 cells/mL (sample volume for injection was 3 μL) (Table 1). In 2007, post-thaw motility of sperm from 30 individuals ranged from 1 to 70% (Table 2). No correlations were found between the post-thaw motility and body weight, body length, testis weight, GSI, body condition factor ($P\geq0.071$), or fresh sperm motility ($P=0.247$). In 2008, post-thaw motility of sperm from 26 individuals ranged from 5 to 80% (Table 3), but was significantly correlated with the motility of fresh sperm ($P=0.000$) and body condition factor ($P=0.008$), but not with body weight, body length, testis weight, or GSI ($P\geq0.158$).

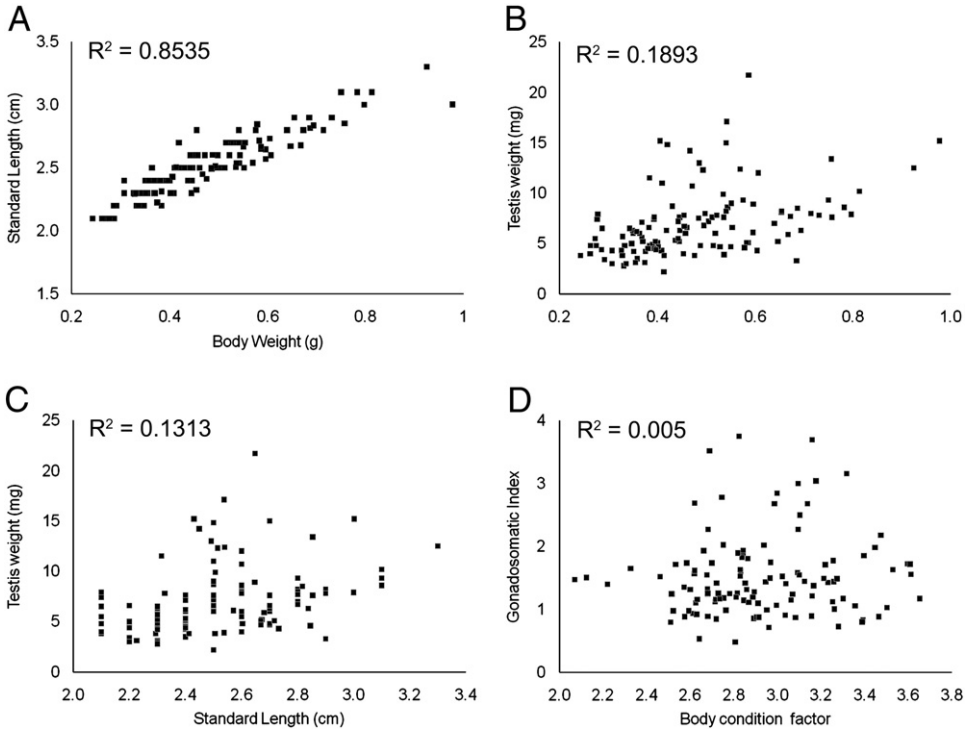

Fig. 2. Correlation analysis of body length, body weight, testis weight, and the gonadosomatic index (percentage of testis weight to the body weight) of the *Xiphophorus maculatus* males used in this study (N = 117).

Table 1
Cryopreservation and artificial insemination of *X. maculatus* in 2006. Sperm from individual fish (numbers 1–20) were cryopreserved, and sperm from fish numbers 1–10 with post-thaw motility equal or greater than 30% were used for artificial insemination with virgin females of *X. couchianus*. The volume for injection was 3 μL at the listed concentrations. Inseminated females were grouped as 2 fish per tank for monitoring of offspring production. No offspring were harvested in females inseminated with cryopreserved sperm, but offspring were harvested in females in one tank (of five) at day 34 after insemination with fresh sperm.

Fish	Length (cm)	Weight (g)	Testis (mg)	Motility (%)		Concentration ($\times 10^9$ cells/mL)	Group	Offspring production
				Fresh	Thawed			
Cryopreserved sperm (Fish # 1–10 were used for artificial insemination)								
1	2.2	0.3828	6.6	80	30	0.5	1	No
2	2.3	0.3678	5.6	70	50	1.5		
3	2.6	0.5434	8.6	85	20	1.2	2	No
4	2.5	0.5049	7.2	80	30	0.3		
5	2.5	0.4946	6.8	80	30	0.4	3	No
6	2.4	0.3844	4.8	90	30	0.9		
7	2.5	0.4457	6.3	90	50	1.5	4	No
8	2.3	0.3698	5.2	85	60	0.9		
9	2.4	0.3894	4.9	85	30	0.7	5	No
10	2.1	0.2426	3.8	90	35	1.1		
11	2.4	0.4451	7.6	80	1	–	–	–
12	2.4	0.3928	7.4	80	15	–	–	–
13	2.4	0.3931	7.6	80	10	–	–	–
14	2.3	0.4083	4.3	70	2	–	–	–
15	2.2	0.3326	4.8	80	15	–	–	–
16	2.4	0.4001	5.1	80	1	–	–	–
17	2.2	0.347	5.0	80	20	–	–	–
18	2.3	0.4017	4.7	80	1	–	–	–
19	2.3	0.4443	5.2	80	20	–	–	–
20	2.4	0.3953	5.2	80	15	–	–	–
Fresh sperm (used as control for insemination)								
21	3.0	0.7969	7.9	80	–	0.7	1	No
22	3.1	0.7825	8.6	85	–	0.5		
23	2.7	0.5277	4.7	80	–	0.8	2	Yes
24	2.9	0.6845	3.3	85	–	1.2		
25	2.9	0.6537	8.1	75	–	1.4	3	No
26	2.7	0.5182	6.0	80	–	0.5		
27	2.6	0.4977	8.0	85	–	0.9	4	No
28	2.6	0.6059	12.0	90	–	1.2		
29	2.7	0.5141	4.8	85	–	1.0	5	No
30	2.8	0.639	7.0	80	–	0.8		

Overall, data analysis of post-thaw motility showed a significant increase in variance compared to that in fresh sperm in 2006 ($P = 0.000$), 2007 ($P = 0.000$), and 2008 ($P = 0.001$).

3.3. Artificial insemination of cryopreserved sperm and harvest of live young

In 2006, after insemination, the females of *X. couchianus* were grouped as 2 fish per 20-L tank for monitoring of live young production. No young were collected from the 10 females inseminated with the 10 cryopreserved samples with post-thaw motility ≥30%. But live young were harvested from females in one tank (of five) at day 34 after insemination with fresh sperm (controls).

In 2007, after insemination, the females of *X. couchianus* were grouped as 5 fish per 20-L tank for monitoring of live young production. No young were harvested from the 15 females inseminated with 15 cryopreserved samples (of 30) with post-thaw motility ≥20%. Also, no live young were harvested from the 15 females inseminated with fresh sperm (controls).

In 2008, after insemination, the females were grouped as 5 fish per 20-L tanks. Live young were harvested from females of *X. helleri* inseminated with cryopreserved sperm in all of the four tanks, and phenotype analysis showed that the live young were hybrids derived from cross of *X. helleri* and cryopreserved sperm of *X. maculatus* (Fig. 1). In addition, in three tanks 2 to 3 broods were harvested at intervals of 2 to 4 d, indicating more than one female produced babies in each tank (Table 3). However, the 20 *X. maculatus* females (in four tanks) inseminated with the same cryopreserved sperm used for insemination with *X. helleri* did not produce any live young. For fresh sperm (controls), females of *X. helleri* produced young in two of three tanks (Table 3), and a second brood was harvested from one tank. In addition, females of *X. maculatus* in one tank (of three) produced live young.

Table 2
Cryopreservation and artificial insemination of *X. maculatus* in 2007. Sperm from 30 individual fish were cryopreserved, and samples from fish numbers 1–15 were selected for artificial insemination with virgin females of *X. couchianus* with a volume of 3 μL for injection. Inseminated females were grouped into 5 fish per tank for monitoring of offspring production. No offspring were harvested in females inseminated with cryopreserved sperm or fresh sperm.

Fish	Length (cm)	Weight (g)	Testis (mg)	Motility (%)		Group	Offspring production
				Fresh	Thawed		
Cryopreserved sperm (sperm from fish numbers 1–15 were used for insemination)							
1	2.3	0.349	6.3	80	20	Cryo 1	No
2	2.3	0.329	5.7	80	30		
3	2.1	0.272	5.5	85	30		
4	2.3	0.343	6.5	85	50		
5	2.7	0.536	7.6	80	70		
6	2.1	0.262	4.0	85	50	Cryo 2	No
7	2.1	0.277	7.4	85	30		
8	2.5	0.460	6.6	80	70		
9	2.7	0.540	25.0	60	30		
10	2.5	0.430	8.7	50	50		
11	2.8	0.575	9.3	90	50	Cryo 3	No
12	2.7	0.418	6.3	90	40		
13	2.6	0.471	10.7	90	30		
14	2.9	0.654	8.2	90	30		
15	2.8	0.455	6.7	90	20		
16	2.5	0.364	6.0	85	5	–	–
17	2.5	0.420	14.8	80	5	–	–
18	2.4	0.375	4.2	80	10	–	–
19	2.5	0.410	11.0	85	5	–	–
20	2.5	0.413	2.2	80	10	–	–
21	2.4	0.359	3.5	80	15	–	–
22	2.1	0.278	7.9	85	10	–	–
23	2.6	0.453	4.0	85	10	–	–
24	2.1	0.263	4.8	80	5	–	–
25	2.1	0.275	4.8	85	10	–	–
26	2.8	0.540	8.2	85	10	–	–
27	2.1	0.287	6.5	90	10	–	–
28	2.4	0.350	6.0	90	15	–	–
29	2.3	0.326	4.3	90	10	–	–
30	2.4	0.307	4.3	90	5	–	–
Fresh sperm (used as control for insemination)							
31	2.8	0.686	8.5	85		Control 1	No
32	2.6	0.596	8.9	80			
33	2.6	0.587	21.7	90			
34	2.9	0.757	7.6	80			
35	2.5	0.485	13.0	85			
36	2.9	0.756	13.4	85		Control 2	No
37	2.3	0.383	11.5	85			
38	2.5	0.493	12.3	85			
39	2.5	0.569	12.4	90			
40	3.0	0.977	15.2	85			
41	2.4	0.467	14.2	85		Control 3	No
42	2.4	0.405	15.2	85			
43	2.3	0.454	7.8	80			
44	2.5	0.542	17.1	85			
45	2.5	0.535	9.9	85			

In addition, dissection of the females in this study at 90 d after insemination did not show the presence of embryos (i.e., fertilized eggs), only immature oocytes.

3.4. Comparison of the results over three years and the need for improving the female fecundity to increase the efficiency of fertilization and live young production

Comparison of sperm cryopreservation and artificial insemination over the 3 year (Table 4) showed that the males used in 2007 and

Table 3
Cryopreservation and artificial insemination of *X. maculatus* in 2008. Sperm from individual fish 1–26 were cryopreserved, and samples from fish 1–20 were split and used for artificial insemination with virgin females of *X. maculatus* and *X. helleri* with a volume of 5 μL. Inseminated females were grouped into 5 fish per tank for monitoring of offspring production. Offspring were harvested in females inseminated with cryopreserved and fresh sperm.

Fish	Length (cm)	Weight (g)	Testis (mg)	Motility (%)		Concentration $\times 10^9$ cells/mL	Groups for AI	Offspring production	
				Fresh	Thawed			*X. helleri*	*X. maculatus*
Cryopreserved sperm (samples from fish numbers 1–20 were used for AI with two female species									
1	3.1	0.813	10.2	80	30	1.5	Cryo 1	2 at day 37 3 at day 40	No
2	3.1	0.750	9.3	70	30	0.6			
3	2.4	0.368	7.1	80	70	0.7			
4	2.4	0.393	4.5	80	50	1.0			
5	2.8	0.714	8.0	70	40	1.1			
6	2.5	0.485	7.5	65	30	1.2	Cryo 2	1 at day 34 2 at day 35 3 at day 37	No
7	2.5	0.442	7.2	75	45	1.2			
8	2.4	0.437	5.3	70	30	0.9			
9	2.4	0.357	6.2	70	55	0.6			
10	2.4	0.381	4.5	80	70	0.9			
11	2.2	0.291	3.4	70	50	0.6	Cryo 3	1 at day 50	No
12	2.3	0.355	3.1	70	40	0.6			
13	2.2	0.285	4.4	80	35	0.5			
14	2.5	0.511	7.6	85	70	1.2			
15	2.7	0.553	6.6	80	70	0.9			
16	2.6	0.522	7.8	85	70	1.3	Cryo 4	4 at day 55 1 at day 71	No
17	2.5	0.552	9.0	90	80	1.7			
18	2.9	0.731	7.8	90	70	1.5			
19	2.8	0.672	7.7	90	80	1.4			
20	2.4	0.397	4.4	85	70	0.6			
21	2.6	0.457	6.0	60	25	–	–	–	–
22	2.3	0.307	3.0	50	5	–	–	–	–
23	3.3	0.925	12.5	80	10	–	–	–	–
24	2.3	0.331	2.8	60	20	–	–	–	–
25	2.6	0.442	5.5	50	15	–	–	–	–
26	2.3	0.351	4.2	80	20	–	–	–	–
Fresh sperm (used as control for insemination with the sample female populations as the cryopreserved sperm)									
27	2.2	0.374	3.1	80		–	Control 1	4 at day 41	No
28	2.6	0.595	6.1	75		–			
29	2.7	0.646	5.2	80		–			
30	2.4	0.475	3.8	70		–			
31	2.7	0.667	5.9	75		–			
32	2.7	0.586	5.1	80		–	Control 2	1 at day 36 9 at day 52	No
33	2.7	0.550	4.7	75		–			
34	2.6	0.488	4.8	75		–			
35	2.7	0.583	5.1	80		–			
37	2.8	0.693	6.3	80		–			
38	2.5	0.413	3.8	80		–	Control 3	No	2 at day 48
39	2.3	0.328	3.8	70		–			
40	2.7	0.604	4.3	70		–			
41	2.5	0.536	3.9	75		–			
42	2.8	0.578	4.6	75		–			

2008 had similar body length, body weight, and testis weight which were significantly larger than those for the males in 2006. For GSI, the males used in 2007 had significantly higher values than did the males in 2006 and 2008, but no significant differences were detected for body condition factors. Overall, from those comparisons none of these biological factors was linked with the production of live young in 2008 (and not in 2006 and 2007).

However, the influence of females on offspring production was identified from the comparison of artificial insemination over 3 years, especially that in 2008, where the same cryopreserved samples were

Table 4
Comparison of experiments on sperm cryopreservation and artificial insemination of *Xiphophorus maculatus* in 3 years by use of ANOVA or Non-parametric Krukal Wallis Test.

Year	*Xiphophorus maculatus* Males						Females used for insemination		
	Number of male	Length (cm)	Weight (g)	Testis weight (mg)	GSI	Body Condition Factor	Species	Number per tank	Live young
2006	20	2.4 ± 0.1	0.404 ± 0.065	5.8 ± 1.3	1.45 ± 0.23	3.08 ± 0.27	*X. couchianus*	2	No
2007	30	2.4 ± 0.2	0.393 ± 0.104	7.2 ± 4.2	1.84 ± 0.81	2.68 ± 0.25	*X. couchianus*	5	No
2008	27	2.6 ± 0.3	0.493 ± 0.174	6.4 ± 2.4	1.29 ± 0.26	2.85 ± 0.26	*X. maculatus*	5	No
							X. helleri	5	Yes
P values for comparison of males									
2006 vs 2007		0.000	0.000	0.000	0.000	0.790			
2006 vs 2008		0.000	0.000	0.000	0.564	0.916			
2007 vs 2008		0.794	0.908	0.059	0.000	0.999			

*GSI, Gonadosomatic Index, the percentage of testis weight out of total body weight.

used for insemination of two different species of females, and live young produced in females of one species (*X. helleri*) but not in another (*X. maculatus*) (Table 4).

4. Discussion

4.1. Male-to-male variation of post-thaw motility and fertility

Male-to-male variation in post-thaw sperm motility and fertility has been observed in most species studied (Holt, 2000; Mazur et al., 2008) including mammals such as boar *Sus scrofa* (Holt, 2000) and rhesus monkey *Macaca mulatta* (Leibo et al., 2007), and in aquatic species such as sea bream *Sparus aurata* (Cabrita et al., 2005), channel catfish *Ictalurus punctatus* (Christensen and Tiersch, 2005), zebrafish *Danio rerio* (Yang et al., 2007a), *X. couchianus* (Yang et al., 2009), and eastern oyster *Crassostrea virginica* (Paniagua-Chavez and Tiersch, 2001). In this study, male-to-male variation in post-thaw motility was observed in each experiment over a 3-year period. The quantitative relationship and variability between post-thaw motility and fertility are unknown because percent fertilization was not quantifiable in these species due to the logistic difficulties of monitoring embryonic development after internal fertilization. With respect to the sources of male-to-male variation in post-thaw motility, three possibilities have been previously discussed (Yang et al., 2009): 1) the susceptibility of spermatozoa to injury during cryopreservation might vary due to physiological (non-genetic) reasons, 2) the series of steps in the cryopreservation process produces interactions and can accumulate defects, and as such sub-optical conditions at any step would lead to poor viability in post-thaw sperm, and 3) there may be slight differences in genetic composition across males. Each hypothesis has its supporting proofs but cannot serve as the sole explanation. In this study, the males used were from highly inbred lines, and theoretically the genetic backgrounds would be essentially identical among individuals. Therefore, as concluded previously the study of *X. couchianus* (Yang et al., 2009), the present study points to non-genetic mechanisms for the variability. Therefore, the variations in thawed sperm among individuals were likely derived from the interaction of multiple factors such as fish health, age, nutrition, and sample handling during cryopreservation.

4.2. Approaches to minimize male-to-male variation in cryopreserved sperm

To produce uniform and reliable cryopreserved samples, it is necessary to minimize the variation in sperm viability that could involve different factors such as those discussed above. Further investigation is necessary with the following aspects: 1) Development of effective indicators for evaluating sperm quality and prediction of the output of cryopreserved sperm. Practical standards for quality control of fresh sperm can potentially be used to predict the outcome of cryopreservation, and enable screening of the samples prior to freezing. This could provide reliable quality control in frozen samples, which is critical for a sperm repository for offspring recovery. Computer-assisted sperm analysis (CASA) is an instrument that can be used to simultaneously quantify multiple sperm motility characteristics, such as velocity and fragellar beat frequency, and has been use to evaluate fish sperm quality (Kime et al., 2001). A recent research report utilizing *X. helleri* showed that sperm swimming velocity is related to the fertilization success (Gasparini et al., 2010). Flow cytometry is another technique for analysis of sperm cell characteristics such as membrane integrity and mitochondrial potential by use of fluorescent dyes (Martinez-Pastor et al., 2010). In addition, other techniques that have been used for sperm analysis include identification of bio-markers that are related to sperm quality such as two-dimensional protein analysis (Jobim et al., 2004) and amplified fragment length polymorphism (Thurston et al., 2002). 2) Standardization of cryo-preservation procedure to ensure uniform post-thaw sperm quality. As indicated above, cryopreservation comprises a series of interacting steps (Tiersch and Mazik, 2000), and accumulated errors at any steps will affect post-thaw sperm quality (Leibo et al., 2000; Tiersch, 2011). Standardization of procedures for sperm cryopreservation can minimize the possibilities of errors (Tiersch et al., 2007). This has been demonstrated for sperm cryopreservation of blue catfish *Ictalurus furcatus* with standardized procedures (Hu et al., 2011). 3) Enhancement of male quality by attention to age and reproductive condition, and providing a balanced diet and optimal culture conditions. This can produce enhancement of the gonadosomatic index (James and Sampath, 2004; Ling et al., 2006), and sperm from fishes with higher gonadosomatic indices and body condition factors could produce sperm with better motility after cryopreservation (Yang et al., 2007a).

4.3. The effect of females on offspring production of cryopreserved sperm

To ensure the production of offspring from cryopreserved sperm, the availability of high-quality eggs is essential and equal in importance to the quality of thawed sperm. To assess quality, direct observation of egg color, size, and shape after collection is commonly used for fishes with external fertilization. In zebrafish, for example, yellowish, granular eggs are considered to be of good quality (Westerfield, 2005), and fertilization of an aliquot of eggs with known-quality sperm can be used as an egg quality control treatment (Yang et al., 2007a; Yang et al., 2010). For fishes with large body sizes, ovarian maturation can be monitored by measuring hormone levels in blood samples or by techniques such as catheterization (Babin et al., 2007) or ultrasound (Novelo et al., 2011). However, for *Xiphophorus* fishes it is difficult to use these approaches because the eggs are confined within the female for internal fertilization and small body size constrains blood sampling and other analyses. Thus, the quality and developmental stage of oocytes in the females used for artificial insemination in these experiments represent un-controlled variables. In addition, virgin females were needed to test the fertility of cryopreserved sperm because *Xiphophorus* females can store sperm and produce several broods from a single mating (Tavolga, 1949). The fecundity of virgin females is poorly understood but could be low compared to larger females that have already produced broods. These factors make study of fertilization and offspring production with cryopreserved sperm in *Xiphophorus* fishes difficult compared to study of externally fertilizing fishes.

In this study, cryopreserved sperm did not produce offspring in the experiments of 2006 and 2007 when females of *X. couchianus* were used. In the experiments of 2008, females of *X. maculatus* and *X. helleri* were inseminated with paired cryopreserved samples coming from the same males with the same procedures. Offspring were produced from females of *X. helleri*, but not from *X. maculatus*. These results suggest that largely unexplained female factors can play an important role in offspring production following artificial insemination. For *Xiphophorus* fishes, sexual maturity and fecundity are genetically controlled (Kallman and Borkoski, 1978; Schreibman and Kallman, 1978), but are also influenced by size (Sohn and Crews, 1977), age (Mckenzie et al., 1983), and social interactions (Sohn, 1977). In this study, the females used were all more than 6 months old, and had reached the minimum age (6 to 8 months) and size (body length of >2.1 cm for *X couchianus* and *X. maculatus* and 3.0 cm for *X. helleri*) associated with sexual maturity (Walter et al., 2006a).

4.4. Approaches for improving the efficiency of offspring production with cryopreserved sperm

To date in all studies of *Xiphophorus*, only 10–20% of females inseminated with cryopreserved sperm have produced offspring (Yang et al., 2007b; Yang et al., 2009), but offspring production was 40% in this study. Even for females inseminated with fresh sperm, the occurrence of pregnancy is typically only 20% based on more than

30 years of data from XGSC encompassing several thousand artificial inseminations (unpublished observation). From this it appears that to increase the occurrence of fertilization and production of offspring, the fecundity of female *Xiphophorus* needs to be improved for artificial insemination. This is especially necessary for establishment of sperm repositories because each sample should guarantee offspring production in single-species crosses. Possible approaches include:

1) An expanded understanding of the relationship of species and strain fecundity. Ovarian regression and infertility have been observed in highly inbred lines of *X. maculatus* Coatzacoalcos (Cp) in approximately 25% of females, and physiological lesions were suggested as being responsible for this regression which involved the processes that control determination and differentiation of germ cells (Burns and Kallman, 1985). Therefore, it is essential to know the reproductive physiology of species and strains before attempting artificial insemination.
2) Choice of females that have already produced broods. These females would be of known fertility, and may be more suitable for artificial fertilization with cryopreserved sperm than virgin females. *Xiphophorus* females can store sperm for months and produce several broods, but the interval between broods from the stored sperm is usually 28–30 d (Tavolga, 1949). Thus, females which do not produce live young for an extended period (e.g. >60 d) could be considered as having depleted sperm stores. In addition, the development of precise molecular markers in *Xiphophorus* fishes (Walter et al., 2004; Walter et al., 2006b) provides tools for paternity confirmation of offspring. Therefore, it could be possible to use proven breeders to increase fecundity for cryopreserved sperm.
3) Hormone treatment of females to enhance oogenesis. Reproductive physiology in fish has been studied since the early 1960s and the pathways for controlling gonadal development and ovulation are fairly clear (Okuzawa, 2002; Rhen and Crews, 2002). Based on these principles, hormone treatment is a commonly used practice in aquaculture fishes for sex reversal, improvement of egg quality and quantity, and control of artificial egg collection (Foran and Bass, 1999). As in most fishes, reproduction of *Xiphophorus* fishes is under control of the brain–pituitary–gonadal axis (Schreibman and Charipper, 1962; Schreibman and Kallman, 1977). The release of pituitary secretions has been studied in *Xiphophorus* fishes (Weiss, 1965), and estrogen-target cells were identified in the pituitary (Kim et al., 1979a; Kim et al., 1979b). The application of male sex hormones (testosterone and 11-ketotestosterone) was shown to stimulate sperm production and advance sexual maturation (Schreibman et al., 1986). These studies indicated that *Xiphophorus* fishes can respond to sex hormones, and thus it could be possible to apply gonodotropic hormones to enhance the oogenesis and ovulation in *Xiphophorus* females to trigger an increase of embryonic development after insemination with cryopreserved sperm.
4) Holding of males with inseminated females. Courtship behavior exists in *Xiphophorus* fishes and reports indicate mating preferences such as for males with long swordtails (Basolo, 1990), for females with a large gravid spot (a darkened region on the abdomen near the urogenital opening) (Benson, 2007), and these male and female mating preferences have the potential to influence one another. It was observed that females display behavioral signs (e.g. headstands and pecks) that provide information to males about their willingness to mate, and these signs were only performed in the presence of males, especially in the presence of large males (Fernandez et al., 2008). This indicates that the presence of males can be used to stimulate the willingness of females to mate which is related to fertilization and pregnancy. More investigation is needed on this topic to improve the efficiency of offspring recovery for cryopreserved sperm. Artificial insemination is time consuming and sperm samples can be rare or valuable. Repository development will place greater demands for artificial insemination and, at present cryopreservation protocols offer fertility compensable to fresh sperm. Future, advancement will reply on improvement of female fecundity.
5) Holding of females in groups. In the experiment of 2006 two females were grouped per tank for monitoring of offspring production, but five were grouped in 2007 and 2008. We hypothesized that larger groups would encourage synchronization of fertilization and release of live young perhaps as evolutionary mechanism to minimize individual risk of predation. Fertilization occurred in groups of five females, although this made it more difficult to identify the individual brooders.

5. Summary

This study reported offspring production from cryopreserved sperm in a third species of *Xiphophorus* fishes (the other two were *X. helleri* (Yang et al., 2007b) and *X. couchianus* (Yang et al., 2009). Male-to-male variation (1–70%) was observed in post-thaw motility despite little variation in motility (60–90%) or genetic variation (over 100 generations of sib-mating) before freezing. Briefly, the protocols for sperm cryopreservation, thawing, and post-thaw sample preparation for artificial insemination were: 1) crushing of dissected testis in Hanks' balanced salt solution at an osmolality of 500 mOsmol/kg; 2) mixing of sperm suspensions with double-strength glycerol-HBSS to yield a final concentration of 14% and equilibration for 15 min prior to freezing; 3) cooling of the sperm suspension at a rate of 20 °C/min (programmed sample cooling rate, monitored by probe in straw) from 5 to −80 °C after loading of 200 μL in 250 μL-French straws; 4) transfer of the frozen samples to liquid nitrogen for storage; 5) thawing of the samples at 42 °C for 5 s in a water bath; 6) centrifuging of the thawed sperm for 5 min at 4 °C (×1000 g); 7) discarding of the supernatant and re-suspension in fresh HBSS to a volume of ~5 μL; 8) injection of 5 μL of concentrated sperm into individual virgin females for artificial insemination (3 μL when females of the small species *X. couchianus* was used), and 9) monitoring of the inseminated females (grouped five per tank) for 90 d for abdominal swelling and subsequent discharge of live young.

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References

Babin, P.J., Cerdà, J., Lubzens, E., 2007. The Fish Oocyte: from Basic Studies to Biotechnological Applications. Springer, Dordrecht, The Netherlands.
Basolo, A., 1990. Female preference for male sword length in the green swordtail, *Xiphophorus helleri* (Pisces, Poeciliidae). Anim. Behav. 40, 339–349.
Basolo, A.L., 1998. Evolutionary change in a receiver bias: a comparison of female preference functions. Proc Royal Soc London Ser B-Biol Sci 265, 2223–2228.
Benson, K.E., 2007. Enhanced female brood patch size stimulates male courtship in *Xiphophorus helleri*. Copeia 212–217.
Burns, J.R., Kallman, K.D., 1985. An ovarian regression syndrome in the platyfish, *Xiphophorus maculatus*. J. Exp. Zool. 233, 301–316.
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 5ml macrotubes. Cryobiology 50, 273–284.
Christensen, J.M., Tiersch, T.R., 2005. Cryopreservation of channel catfish sperm: effects of cryoprotectant exposure time, cooling rate, thawing conditions, and male-to-male variation. Theriogenology 63, 2103–2112.
Fernandez, A.A., Fernandez, L.R., Toth, L., 2008. Head over heels: an examination of a possible mating signal in female swordtails, *Xiphophorus cortezi*. Anim. Behav. 76, 1073–1081.

Foran, C.M., Bass, A.H., 1999. Preoptic GnRH and AVT: axes for sexual plasticity in teleist fish. Gen. Comp. Endocrinol. 116, 141–152.
Gasparini, C., Simmons, L.W., Beveridge, M., Evans, J.P., 2010. Sperm swimming velocity predicts competitive fertilization success in the green swordtail *Xiphophorus helleri*. PLoS One 5, e12146.
Gordon, M., 1952. Genetic evidence for 2 opposite mechanisms for sex-determination, Xx-Xy and Wz-Zz (or Wy-Yy), in the same species of platyfish, *Xiphophorus* (Platypoecilus) *maculatus*, from different geographical populations. Anat. Rec. 113, 537–538.
Gordon, H., Gordon, M., 1952. The frequencies of 5 genes for macromelanophores (cells capable of producing tumors) in 7 natural populations of the platyfish, *Xiphophorus (Platypoecilus) maculatus*. Genetics 37, 586-586.
Gutbrod, H., Schartl, M., 1999. Intragenic sex-chromosomal crossovers of Xmrk oncogene alleles affect pigment pattern formation and the severity of melanoma in *Xiphophorus*. Genetics 151, 773–783.
Holt, W.V., 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 53, 47–58.
Hu, E., Yang, H., Tiersch, T.R., 2011. High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): establishment of an approach for commercial-scale processing. Cryobiology 64, 74–82.
Huang, C., Dong, Q., Walter, R.B., Tiersch, T.R., 2004. Initial studies on sperm cryopreservation of a live-bearing fish, the green swordtail *Xiphophorus helleri*. Theriogenology 62, 179–194.
James, R., Sampath, K., 2004. Effect of feed type on growth and fertility in ornamental fish, *Xiphophorus helleri*. Isr J Aquacult-Bamidgeh 56, 264–273.
Jobim, M.I.M., Oberst, E.R., Salbego, C.G., Souza, D.O., Wald, V.B., Tramontina, F., Mattos, R.C., 2004. Two-dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. Theriogenology 61, 255–266.
Kallman, K.D., 1965. Sex determination in teleost *Xiphophorus maculatus*. Genetics 52, 450 &.
Kallman, K.D., 1983. The sex determining mechanism of the Poeciliid fish, *Xiphophorus montezumae*, and the genetic control of the sexual-maturation process and adult size. Copeia 755–769.
Kallman, K.D., 2001. How the *Xiphophorus* problem arrived in San Marcos, Texas. Mar. Biotechnol. 3, S6–S16.
Kallman, K.D., Borkoski, V., 1978. Sex-linked gene controlling onset of sexual maturity in female and male platyfish (*Xiphophorus maculatus*), fecundity in females and adult size in males. Genetics 89, 79–119.
Kazianis, S., Walter, R.B., 2002. Use of platyfishes and swordtails in biological research. Lab Anim. 31, 46–52.
Kim, Y.S., Sar, M., Stumpf, W.E., 1979a. Estrogen target-cells in the pituitary of platyfish, *Xiphophorus maculatus*. Cell Tissue Res. 198, 435–440.
Kim, Y.S., Stumpf, W.E., Sar, M., 1979b. Topographical distribution of estrogen target-cells in the forebrain of platyfish, *Xiphophorus maculatus*, studied by auto-radiography. Brain Res. 170, 43–59.
Kime, D.E., Van Look, K.J.W., McAllister, B.G., Huyskens, G., Rurangwa, E., Ollevier, F., 2001. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp. Biochem. Physiol. C 130, 425–433.
Leibo, S.P., Tiersch, R.T., Mazik, P.M., 2000. Sources of variation is cryopreservation, Cryopreservation in aquatic species. World Aquaculture Society, Baton Rouge, Louisiana. 75–83.
Leibo, S.P., Kubisch, H.M., Schramm, R.D., Harrison, R.M., VandeVoort, C.A., 2007. Problems of freezing spermatozoa of different species. 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.
Martinez-Pastor, F., Mata-Campuzano, M., Alvarez-Rodriguez, M., Alvarez, M., Anel, L., de Paz, P., 2010. Probes and techniques for sperm evaluation by flow cytometry. Reprod. Domest. Anim. 45, 67–78.
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.
Mckenzie, W.D., Crews, D., Kallman, K.D., Policansky, D., Sohn, J.J., 1983. Age, weight and the genetics of sexual-maturation in the platyfish, *Xiphophorus maculatus*. Copeia 770–774.
Monks, N., 2007. Poeciliid fishes: Mollies, platies, guppies, swordtails. http://www.wetwebmedia.com/FWSubWebindex/poeciliids.htm.
Novelo, N., Kuenz, D., Green, C., Tiersch, T.R., 2011. Ultrasonographic mornitoring of channel catfish ovarian development, In: Tiersch, T.R., Green, C. (Eds.), Cryopreservation in Aquatic Species, 2 ed. World Aquaculture Society, Baton Rouge, LA, pp. 134–144.
Okuzawa, K., 2002. Puberty in teleosts. Fish Physiol. Biochem. 26, 31–41.
Paniagua-Chavez, C.G., Tiersch, T.R., 2001. Laboratory studies of cryopreservation of sperm and trochophore larvae of the Eastern oyster. Cryobiology 43, 211–223.
Rhen, T., Crews, D., 2002. Variation in reproductive behaviour within a sex: neural systems and endocrine activation. J. Neuroendocrinol. 14, 517–531.
Schartl, A., Malitschek, B., Kazianis, S., Borowsky, R., Schartl, M., 1995. Spontaneous melanoma formation in nonhybrid *Xiphophorus*. Cancer Res. 55, 159–165.
Schreibman, M.P., Charipper, H.A., 1962. Studies on pituitary gland of platyfish (Xiphophorus maculatus. Anat. Rec. 142, 326 &.
Schreibman, M.P., Kallman, K.D., 1977. Genetic-control of pituitary-gonadal axis in platyfish, *Xiphophorus maculatus*. J. Exp. Zool. 200, 277–293.
Schreibman, M.P., Kallman, K.D., 1978. Genetic-control of sexual maturation in teleost, *Xiphophorus maculatus* (Poeciliidae): Review. Annal Biol Anim Biochim Biophys 18, 957–962.
Schreibman, M.P., Margolisnunno, H., Halpernsebold, L.R., Goos, H.J.T., Perlman, P.W., 1986. The influence of androgen administration on the structure and function of the brain–pituitary–gonad axis of sexually immature platyfish, *Xiphophorus maculatus*. Cell Tissue Res. 245, 519–524.
Sohn, J.J., 1977. Socially induced inhibition of genetically determined maturation in platyfish, *Xiphophorus maculatus*. Science 195, 199–201.
Sohn, J.J., Crews, D., 1977. Size-mediated onset of genetically determined maturation in platyfish, *Xiphophorus maculatus*. Proc. Natl. Acad. Sci. U S A 74, 4547–4548.
Tavolga, W.N., 1949. Embyonic development of the platyfish (*Platypoecilus*), the swordtail (*Xiphophorus*) and their hybrids. Bull. Am. Mus. Nat. Hist. 94, 161–230.
Thurston, L.M., Siggins, K., Mileham, A.J., Watson, P.F., Holt, W.V., 2002. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol. Reprod. 66, 545–554.
Tiersch, T.R., 2011. Process pathways for cryopreservation research application and commercialization, In: Tiersch, T.R., Green, C. (Eds.), Cryopreservation in Aquatic Species, 2 ed. World Aquaculture Society, Baton Rouge, LA, pp. 646–671.
Tiersch, T.R., Mazik, P.M., 2000. Introduction, Cryopreservation in Aquatic Species. World Aquacultrue Society, Baton Rouge, LA. xix-xxvi.
Tiersch, T.R., Yang, H., Jenkins, J.A., Dong, Q., 2007. Sperm cryopreservation in fish and shellfish. In: Roldan, E.R.S., Gomendio, M. (Eds.), Spermatology (Society of Reproduction and Fertility supplement 65). Nottingham University Press, Nottingham, U.K, pp. 493–508.
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., Rains, J.D., Russell, J.E., Guerra, T.M., Daniels, C., Johnston, D.A., Kumar, J., Wheeler, A., Kelnar, K., Khanolkar, V.A., Williams, E.L., Hornecker, J.L., Hollek, L., Mamerow, M.M., Pedroza, A., Kazianis, S., 2004. A microsatellite genetic linkage map for *Xiphophorus*. Genetics 168, 363–372.
Walter, R.B., Hazlewood, L., Kazianis, S., 2006a. The *Xiphophorus* Genetics Stock Center Manual. Texas State University, San Marcos, TX.
Walter, R.B., Ju, Z., Martinez, A., Amemiya, C., Samollow, P.B., 2006b. Genomic resources for *Xiphophorus* research. Zebrafish 3, 11–22.
Weiss, M., 1965. Release of pituitary secretion in platyfish *Xiphophorus maculatus* (Guenther). Z. Zellforsch. Mikrosk. Anat. 68, 783 &.
Westerfield, M., 2005. The Zebrafish Book, A Guide for the Laboratory Use of Zebrafish *Danio rerio*, 5 ed. University of Oregon Press, Eugene, OR.
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.
Yang, H., Hazlewood, L., Walter, R.B., Tiersch, T.R., 2009. Sperm cryopreservation of a live-bearing fish *Xiphophorus couchianus*: male-to-male variation in post-thaw motility and production of F_1 hybrid offspring. Comp. Biochem. Physiol. C 149, 233–239.
Yang, H., Norris, M., Winn, R., Tiersch, T.R., 2010. Evaluation of cryoprotectant and cooling rate for sperm cryopreservation in the euryhaline fish medaka *Oryzias latipes*. Cryobiology 61, 211–219.