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## CRYOPRESERVATION OF SPERM FROM THE AXOLOTL *AMBYSTOMA MEXICANUM*: IMPLICATIONS FOR CONSERVATION

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**Abstract.**—Amphibians are declining globally at an alarming rate. For the sustainable management of amphibians, the development of reproductive technologies and in particular field-friendly methods for cryopreservation of sperm are required. Successful cryopreservation preserves genetic material, provides for the transfer of genes from wild populations to captured stock, and assists in dispersing genetic material among populations in nature. Collecting spermatophores of salamanders and their subsequent cryopreservation could be an effective method of perpetuating genetic variation and provide for *ex situ* propagation and reintroduction efforts. This study investigates the practicality of using spermatophores from the Axolotl, *Ambystoma mexicanum*, to assess the effects of cooling rates, thawing rates, and cryodiluents on the post-thaw viability of sperm within salamander spermatophores. In a first experiment, overall post-thaw sperm viability was  $64.7\% \pm 29.5\%$  (mean  $\pm 1$  SD; as assessed by a live/dead stain) while in the second experiment, overall post-thaw sperm viability was  $86.7\% \pm 8.0\%$ . There were no significant effects of cryodiluents, cooling rates, thawing rates or the interaction effects on sperm viability ( $P > 0.05$ ). The unique aspects of this study – cryopreserving sperm within spermatophores – potentially influenced the cooling and thawing process and likely affected the ability of water and cryodiluents to enter sperm cells. A model species such as the Axolotl (critically endangered in nature) that represents a number of *Ambystoma* species with similar breeding habitats, may prove useful for developing field cryopreservation protocols for these and other endangered species.

**Key Words.**—Axolotl; *Ambystoma*; amphibian; cryopreservation; spermatophore; salamander; conservation

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### INTRODUCTION

Amphibians are facing worldwide population declines, range contractions, and species extinction. Within the last 30 years, over 200 species have become extinct and close to one-third of the world's amphibians are imperiled (IUCN, International Union for Conservation of Nature. 2010. IUCN red list of threatened species. Available from <http://iucnredlist.org> [Accessed 29 March 2011]; Stuart et al. 2004; Pounds et al. 2006; see Semlitsch 2003 for a review). For many of these amphibian species, it has become necessary to establish conservation-breeding programs that assure the survival of relict and often fragmented populations. These programs must ensure genetic variation is not lost, and that augmentation programs maintain genetic variation of evolutionarily significant populations. Establishing and maintaining a comprehensive gene bank that represents the genetic variation of species contributes to these goals and are among the objectives of conservation plans to conserve threatened amphibians (Gascon et al. 2007). To assist with this effort, it is essential to develop reproductive technologies for amphibians and in particular, field-friendly methods for cryopreservation of

sperm.

Cryopreservation of sperm preserves genetic material, enables the transfer of genes from natural populations to those in conservation breeding programs, and can maintain population genetic variation during augmentation programs. Cryopreservation of amphibian sperm also may provide a practical method for holding genetic material until researchers develop techniques to control emerging infectious diseases and parasites.

Collection of spermatophores is a non-invasive method for recovering viable sperm from many salamander species. For example during *Ambystoma* mating aggregations, males deposit spermatophores throughout the pond substrate with up to 81 spermatophores per courtship in *A. maculatum* (Arnold 1976) and as many as 128 spermatophores per courtship event in *A. texanum* (McWilliams 1992). Thus, collecting spermatophores and their subsequent cryopreservation could be an effective method for the management of threatened salamanders in conservation breeding programs, through perpetuating genetic variation to provide for *ex situ* propagation and reintroduction efforts. In the present study, I investigate the applicability of using spermatophores from the Axolotl, *A. mexicanum*, to develop a practical and reliable

method for cryopreservation of sperm from the field. I examined the effects of cooling rates, thawing rates, and cryodiluents on Axolotl sperm post-thaw viability.

### MATERIALS AND METHODS

**Model animal.**—The Axolotl is a neotenic mole salamander belonging to the Tiger Salamander complex (*A. tigrinum*) that originated from Lake Texcoco, Mexico City, Mexico. Wild Axolotls are imperiled and near extinction due to pollution, introduction of non-native predatory fish, and urbanization (Recuero et al. 2010), and are on the International Union for Conservation of Nature’s annual Red List of threatened species (IUCN. 2010. *op. cit.*). However, researchers use Axolotls extensively in scientific laboratories because of their ability to regenerate body parts, ease of breeding, and their large embryos. I also chose the Axolotl to represent other endangered *Ambystoma* spp. found in North America (e.g., *A. bombyppella*, *A. ambycephalum*, *A. bishopi*, *A. cingulatum*, and *A. macrodactylum croceum*) as well as a range of species that have similar breeding behavior.

I obtained mature Axolotls (males SVL 24.5 ± 1.7 cm [mean ± 1 SD], mass 95.8 ± 14.7 g;

females SVL 22.1 ± 0.9 cm, mass 96.4 ± 11.8 g) from the *Ambystoma* Genetic Stock Center at the University of Kentucky and kept these animals individually in holding containers and aquaria (between 10 L to 30 L) at the Warm Springs Regional Fisheries Center, U. S. Fish and Wildlife Service, in Warm Springs, Georgia, USA. Spring water flowed through the tanks at approximately 0.5 L/min and water temperature varied from 15° C to 22° C depending on ambient seasonal temperatures. I fed Axolotls at least three days per week with prepared diet obtained from the Center.

**Collection of spermatophores.**—Salamander spermatophores are generally conical-shaped and consist of a cap containing sperm and a gelatinous structure that anchors the spermatophore to a substrate. Doyle et al. (2011) estimated the number of sperm per spermatophore in *A. maculatum* varies widely between  $3.4 \times 10^4$  and  $8.0 \times 10^7$ . To obtain spermatophores, I placed one male and one female in a container (46 cm length × 33 cm width × 11.5 cm height) with approximately 15 L of water between 1600–1700. I revisited animals the next morning (0630), and searched the bottom of the container for deposited

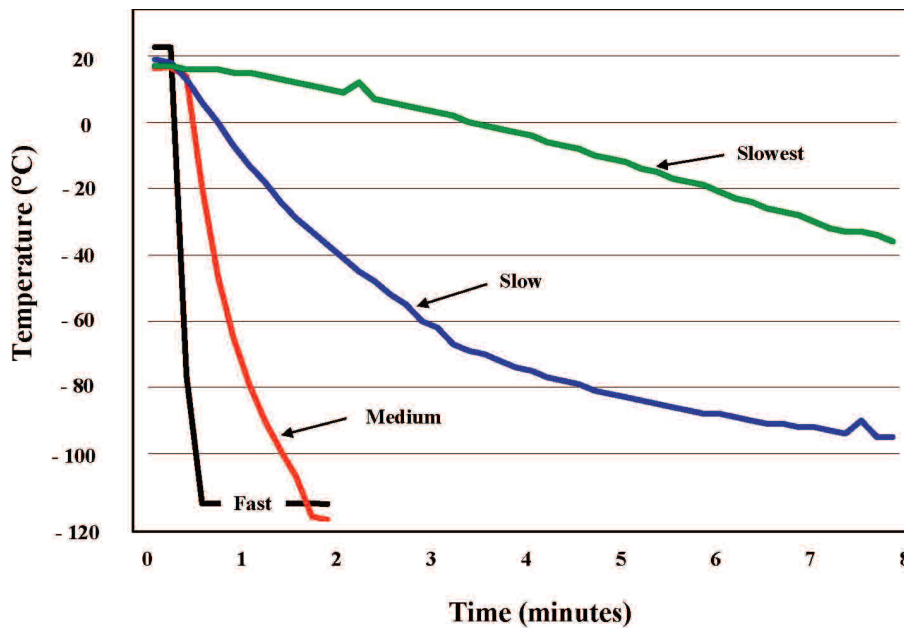


FIGURE 1. The four cooling rates used to freeze spermatophores of the Axolotl, *Ambystoma mexicanum*. Cooling rates are: Fast (black line) = ~300° C/min; medium (red line) ~108° C/min; slow (blue line) = ~24° C/min.

spermatophores. At least six spermatophores from each male were required for each experiment; if there were fewer than six spermatophores, I observed salamanders over the next 4 h to see if salamanders deposited additional spermatophores. This ensured that a male had one spermatophore per treatment effect ( $3 \times 2$  experimental designs). I placed all spermatophores into a cryule vial (1 ml CryoELITE™ Cryogenic Vials, Wheaton Science Products, Millville, New Jersey, USA) and added one of two cryodiluents: 10% sucrose solution or Simplified Amphibian Ringers (SAR). I added these to the 1 ml line before cooling in experiment 1, while I added water as a medium in experiment 2. I did not assess the percentage of viable sperm before cryopreservation to ensure the structure of the spermatophores remained intact during the cryopreservation process.

**Cooling rates.**—I froze and stored spermatophores below  $-100^{\circ}\text{C}$  by using a nitrogen-vapor shipping dewar (Taylor-Wharton model, Theodore, Alabama, USA). These units have an absorbent material that prevents liquid nitrogen spills and can keep the temperature inside the shipping cavity below  $-100^{\circ}\text{C}$ . Researchers designed cryogenic dewars for transporting cryopreserved material; however, fishery biologists have used these specifically for the cryopreservation of fish sperm in the field (Wayman et al. 1996; Tiersch et al. 1998; see Wayman and Tiersch (2000) for cooling procedures). In the present study, I froze spermatophores at four cooling rates by using a five-channel data logger (OM-550, Omega Engineering, Inc. Stamford, Connecticut, USA) that had inputs from type-T thermocouples (TMISS-040G-12, Omega). Specifically, I determined cooling rates by using the five-channel data logger, thermocouples, and cryule vials to record cooling rates as temperature change in a nitrogen-vapor shipping dewar through time. I used four methods to obtain these cooling rates: (1) Cryule vials were dropped directly to the bottom of the dewar for fast cooling of  $\sim 300^{\circ}\text{C}/\text{min}$ ; (2) cryule vials in goblets were placed into the dewar for a medium cooling rate of  $\sim 108^{\circ}\text{C}/\text{min}$ ; (3) visotubes, goblets, and canisters together were used to obtain a slow rate of  $\sim 24^{\circ}\text{C}/\text{min}$ ; and (4) foam insulation, visotubes, goblets, and canisters were used for the slowest cooling rate of  $\sim 10^{\circ}\text{C}/\text{min}$

(Fig. 1).

**Sperm viability.**—I assessed the viability of thawed sperm by using a Live/Dead Sperm Viability Kit (L-7011, Molecular Probes, Inc. Grand Island, New York, USA). This kit uses the fluorescent stains SYBR-14 and propidium iodide (PI) to assess membrane integrity and sperm cell viability. The membrane-permeant stain (SYBR-14) binds DNA of intact sperm and fluoresce a bright green, while the PI is a membrane-impermeant stain that binds DNA in sperm with leaky membranes, and fluoresce red (Garner et al. 1997; Fig. 2).

Cryopreserved spermatophores were thawed and broken up in the cryule vials using pipette tips. I placed five  $\mu\text{L}$  of the sperm on a microscope slide, added 2  $\mu\text{L}$  of both dyes, and viewed this under a microscope to assay the percentage of live intact sperm. I repeated this process several times per spermatophore to obtain a reliable percentage of live intact sperm (with a minimum of 100 sperm counted).

**Experiment one.**—I examined the effects of cooling rate and cryodiluents on post-thaw sperm viability in a  $3 \times 2$  factorial design. I used either a fast or slow cooling rate (see above, Figure 1) and I added either water or one of two diluents: a 10% sucrose solution at 341

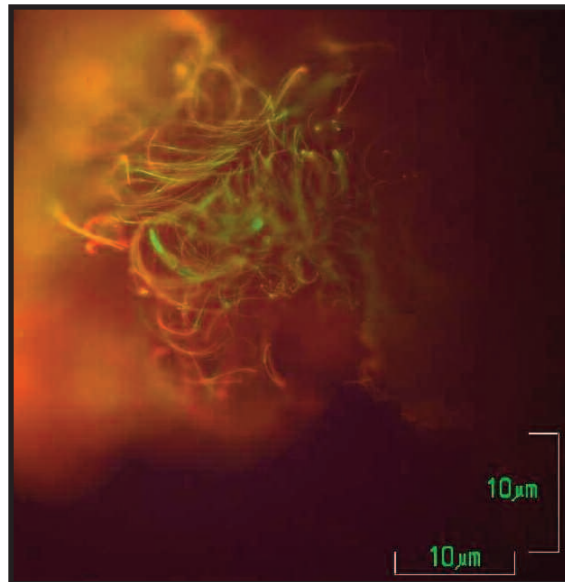


FIGURE 2. Photograph of sperm cells from the Axolotl, *Ambystoma mexicanum* after cryopreservation in spermatophores. Intact sperm cells fluoresce a bright green while damaged fluoresce red. (Photographed by Chester R. Figiel, Jr.).

mOsmol/kg; and SAR (consisting of 113.0 mM NaCl, 1 mM CaCl<sub>2</sub>, 2.0 mM KCl, 3.6 mM NaHCO<sub>3</sub>), at 287 mOsmol/kg). Researchers have utilized both diluents for sperm storage and cryopreservation in other studies (Browne et al 1998, 2001; Peng et al. 2011). The experiment began with six pair of Axolotls (male/female); however, only four males produced enough spermatophores to complete the study design. I assigned six spermatophores from each male (of four males) without bias to each of the six treatments: Sucrose-Fast, Sucrose-Slow, SAR-Fast, SAR-Slow, Control-Fast, Control-Slow. I froze spermatophores following cooling protocols and kept in the dewar below -100° C. After 48 hours, I thawed frozen spermatophores one at a time by placing cryule vials in a 25° C water bath for 5 minutes. I obtained the percent of viable sperm as described above.

**Experiment two.**—I examined the effects of cooling rate (medium, slow and slowest; Fig. 1) and thawing method (High thawing rate, Low thawing rate) on post-thaw sperm viability in a 3 × 2 factorial design. To obtain thawing rates, I placed vials containing spermatophores in a water bath at 25° C for 5 min (High thawing rate) or in a water bath at 40° C for 3.3 min (Low thawing rate). Four pair of Axolotls were placed together (male/female, different individuals from experiment 1); however, only three males produced the needed spermatophores to complete the study design. I assigned six spermatophores from a male (three males) to one of the six treatments: Medium cooling rate + High thawing rate, Medium cooling rate + Low thawing rate, Slow cooling rate + High thawing rate, Slow cooling rate + Low thawing rate, Slowest cooling rate + High thawing rate, and Slowest cooling rate + Low thawing rate. I obtained the percent of viable sperm (as

described above) after cooling spermatophores below -100° C for at least three days.

**Statistical analysis.**—The percent of viable sperm (the percent of green/green + red sperm) were arcsine square root transformed to minimize the heterogeneity of variances among treatments (Snedecor and Cochran 1989) and data were analyzed by a two-way ANOVA for the main effects and interactions in both experiments ( $P = 0.05$  level of significance). No differences were found among the males in sperm viability in experiment one ( $df = 3, F = 1.076685, P > 0.05$ ); or experiment two ( $df = 2, F = 2.846558, P > 0.05$ ); thus, males were used as replicates ( $n = 4$  males in experiment one;  $n = 3$  males in experiment two). In experiment one, male 2 in the control-slow treatment and male 5 in the SAR-fast treatment had zero sperm in the cryule vial, and thus were not included in the analysis. I used the S-Plus 7.0.6 software package (Insightful Corporation, Seattle, Washington) for all analyses.

**RESULTS**

**Experiment one.**—Post-thaw sperm viability overall was 64.7% ± 29.5% (mean ± 1 SD). There were no significant effects of cryodiluents, cooling rate, or the interaction of cryodiluent and cooling rates on sperm viability (Table 1). There were no significant statistical differences between sperm viability in the slow cooling rate (74.8% ± 25.0%) compared to the fast cooling rate (55% ± 31.3%). Further, spermatophores frozen with spring water had similar percent viability (75.0% ± 27.1%) compared to those frozen with SAR (68.3% ± 30.6%) or those frozen with 10% sucrose (52.5% ± 29.9%).

**Experiment two.**—Neither cooling rate or

**TABLE 1.** Summary of ANOVA on the post-thaw percent of the viable sperm of *Ambystoma mexicanum* after cryopreservation using three cryodiluents (10% sucrose, simplified amphibian ringers solution, and spring water) and cooling at two rates (~300° C/min, ~24° C/min).

Source of Variation	df	MS	F	P
Cooling rate	2	0.0066	0.466	0.6386
Thawing rate	1	0.0049	0.349	0.5654
Cooling × thawing rate	2	0.0340	2.403	0.1325
Error	12	0.0141		

**TABLE 2.** Summary of ANOVA on the post-thaw percent of the viable sperm of *Ambystoma mexicanum* after cryopreservation using three cryodiluents (10% sucrose, simplified amphibian ringers solution, and spring water) and cooling at two rates (~300° C/min, 24° C/min).

Source of Variation	df	MS	F	P
Cryodiluent	2	0.1039	1.472	0.2589
Cooling rate	1	0.2788	3.982	0.0633
Cryodiluent × cooling rate	2	0.1511	2.1580	0.1480
Error	16	0.0700		

thawing rate or the interaction effects of cooling and thawing rates significantly influenced the percentage of post-thaw viable sperm (Table 2). Sperm post-thaw viability over all treatments was  $86.7\% \pm 8.0\%$ . Sperm viability did not differ significantly when cooled at the slowest rate ( $89.2\% \pm 8.0\%$ ) compared to the slow ( $85.8\% \pm 7.4\%$ ) and medium rates ( $85.0\% \pm 9.5\%$ ). Further, thawing rate did not influence the percent of viable sperm (25° C for 5 minutes =  $85.6\% \pm 8.5\%$ ; 40° C for 3.3 min =  $87.8\% \pm 7.9\%$ ).

## DISCUSSION

This is the first study to report methods for the cryopreservation of sperm in salamander spermatophores. To date, cryopreservation of amphibian sperm is limited to less than 50 anuran species, and few (if any) caecilians or caudata species (Browne and Figiel 2011; Browne et al. 2011). Sperm cryopreservation of salamander species that fertilize externally has been reported only for the Chinese Giant Salamander, *Andrias davidianus* (Peng et al. 2011), and for North American Hellbenders, *Cryptobranchus alleganiensis alleganiensis* and *C. a. bishopi* (Unger et al. 2013). Sperm cryopreservation protocols on species that do not produce spermatophores (e.g., Cryptobranchidae) are similar to techniques used for anuran species (see review Browne and Figiel for review 2011) and differ from cryopreservation techniques with urodeles that have internal fertilization (e.g., *Ambystoma* spp.) mainly because of differences in sperm physiology and spermatophore morphology. Often sperm can only be obtained from salamanders or other amphibians through the excision and maceration of testes; however, inducing individuals to produce sperm through

hormone injection has been successful (Xiao et al 2006; Mansour et al. 2010; Shishova et al. 2011) and may prove necessary especially when working with critically endangered species. The techniques of this study avoided the need for testes sampling or hormonal induction with the Axolotl and may be extended to other internally fertilizing salamanders.

In the present study, spermatophores frozen at slower rates of ~24° C/min and ~10° C/min resulted in elevated post-thaw sperm viability although not significantly so. Other studies found that a continuous linear cooling rate of ~10° C/min was the best cooling rate for the anurans *Xenopus laevis* and *X. tropicalis* (Sargent and Mohun 2005), whereas a stepped cooling rate of less than ~5° C/min lead to moderate to high sperm motility and fertility in *Bufo marinus* (Browne et al. 1998). Additionally, Mansour et al. (2009, 2010) found that a cooling rate between ~5 and ~7° C/min produced greater success for sperm of *X. laevis* and *Rana temporaria*. In contrast, with *Cryptobranchus* sperm, rapid cooling results in the recovery of high rates of motility (Robert Browne, pers. com.).

The rapid thawing of cells after cryopreservation can minimize the damage associated with a longer period of recrystallization. I found no difference in the effects of thawing rates on Axolotl post-thaw sperm viability. Few amphibian cryopreservation studies have examined the effect of thawing rates (but see Browne et al. 1998, Hopkins and Herr 2004, Sargent and Mohun 2005) with none finding a significant influence on sperm quality.

The unique aspects of this study, cryopreserving sperm within spermatophores, created a unique cooling and thawing process and likely affected the ability of water and

cryodiluents to enter sperm cells (as it does in the Cryptobranchids; Unger et al. 2013). Post-thaw sperm viability was not influenced by cryodiluents potentially because the internal environment of the spermatophore provides protection and prevents the degradation of sperm. The sperm cap of spermatophores is composed of a mass of nebulous material in which the sperm are imbedded (Organ and Lowenthal 1963) and are surrounded by a membrane-like structure containing fibrous material and cytoplasmic droplets (Zalisko et al. 1984). These components may bind or retain diluents. Presently, it is not known if permeating (e.g., glycerol, dimethyl sulfoxide [Browne et al. 1998]; dimethylformamide [Shishova et al. 2011]) or non-permeating cryoprotectants (e.g., sugars; Browne et al. 2002) can penetrate the sperm cap or whether compounds found in the sperm cap improve sperm cryopreservation in Axolotl spermatophore, those of other *Ambystoma* species, or in other internally fertilizing salamanders. Understanding the effects of cooling and thawing (e.g., the formation of ice crystals, cell dehydration, damage to cell integrity), on spermatophores and cells within, would prove useful for the development of protocols for field use.

A priority in amphibian management and recovery efforts is the development of non-invasive methods for the collection and storage of gametes (Gascon et al. 2007; Browne et al. 2011). A multidisciplinary approach that includes sperm cryopreservation should assist in the production of salamanders with suitable genetic variation for augmentation programs and in conservation breeding populations. A model species such as the Axolotl that can represent a range of amphibians may prove useful for developing field cryopreservation protocols for critically imperiled species. Further work on the capability of cryopreserved spermatophores to produce healthy offspring is needed in that regard.

*Acknowledgements.*—Special thanks to Randal Voss, Laura Muzinic, and Chris Muzinic at the *Ambystoma* Genetic Stock Center, University of Kentucky. The National Science Foundation (NSF–DBI–0443496) grant mechanism supports the Center. I thank Jaci Zelko, Gregory Scull, Christie Jackson, Cody Meshes, and Nicole Rankin for assistance in care of animals. I

especially thank Robert Browne, Gregory Scull, and William Wayman for reviewing earlier versions of this manuscript. References to trade name or proprietary product does not imply endorsement by the U.S. Government. Links to external servers do not imply any official U.S. Fish and Wildlife Service endorsement of the opinions or ideas expressed therein, or guarantee the validity of the information provided. The findings and conclusions in this article are those of the author and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

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Figiel.—Axolotl sperm cryopreservation.



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