Herpetological Conservation and Biology 15(2):367–371.
Submitted: 11 May 2020; Accepted: 20 July 2020; Published: 31 August 2020.

Cold Storage of Sperm From the Axolotl, *Ambystoma mexicanum*

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***Abstract*.—The development of reproductive methodologies such as techniques for storage of sperm can be valuable for conservation management of endangered amphibians. For example, storage and preservation of genetic material can assist in transferring genes from natural populations to cultured breeding colonies and can assist in increasing genetic exchange among distinct populations. For salamanders, collecting spermatophores and their subsequent cold storage is a useful method of increasing genetic variation and assisting in propagation efforts. In this study, I examined the influence of temperature on the short-term viability of sperm from spermatophores of Axolotls (*Ambystoma mexicanum*) salamanders for spermatophores stored at 0° C, 3° C, and 6° C. Sperm remained viable for 28 d when stored at 0 ° C (45.0% ± 31.2%), 3° C (61.7% + 12.6%) and 6° C (40.0 ± 45.8%). The percentage of viable sperm cells fluctuated through time (individual spermatophores assayed on days 0, 7, 14, 21, and 28), and was not significantly different. Additionally, the percentage of viable sperm cells in the three storage temperatures were not significantly different, and neither this factor nor the interaction of time × temperature resulted in a significant difference in the percent of sperm that were viable. Overall, the ability to store spermatophores for 28 d for *A. mexicanum* (critically endangered in nature) may prove useful for optimizing protocols for a range of endangered species.**

Key Words.—amphibian; reproductive methods; salamander; spermatophores

Introduction

To assure the survival of relict and fragmented species of amphibians, it has become necessary to use artificial fertilization techniques to maximize culture success. For example, storage and preservation of gametes are an effective way of addressing the differential maturation of males and females and allows the transfer of genetic resources from natural populations to breeding programs (Clulow et al. 1999; Kouba et al. 2009; Browne and Figiel 2010; Kouba et al. 2013). Collecting spermatophores of *Ambystoma* salamanders and their subsequent cold storage both refrigerated and cryopreserved (Peng et al. 2011; Figiel 2013; Guy et al. 2020), is one practical way of augmenting genetic variation and provide for ex situ propagation activities. Refrigerated sperm can remain viable for several weeks, or indefinitely when cryopreserved in liquid nitrogen and has become an essential tool for optimizing amphibian reproduction in captivity (Browne et al. 2019; Della-Togna et al. 2020 and references therein).

During *Ambystoma* mating aggregations, males deposit spermatophores (a gelatinous structure containing a sperm cap) on the pond substrate in large numbers: greater than 80 spermatophores in the Spotted Salamander (*A. maculatum*; Arnold 1976) and greater than 125 spermatophores in the Small-Mouthed Salamander (*A. texanum*; McWilliams 1992). By collecting spermatophores, culturists and researchers eliminate the need to transport live animals, potentially reduce the risk of disease transfer, and provide a non-invasive approach for harvesting viable sperm cells. Detailed knowledge of optimal handling and storage of spermatophores is lacking and there is a need to refine this process.

I collected spermatophores of the Axolotl (*Ambystoma mexicanum*) to examine the influence of temperature on the short-term viability of sperm cells. Optimizing cold storage of spermatophores could offer a reliable technique (and less expensive than cryopreservation) to assist in conservation actions for this and other endangered species of *Ambystoma*: Reticulated Flatwoods Salamander (*A. bishop*), Frosted Flatwoods Salamander (*A. cingulatum*), Santa Cruz Long-Toed Salamander (*A. macrodactylum croceum*), Blunt-Headed Salamander (*A. amblycephalum*), and Delicate-Skinned Salamander (*A. bombypellum*), as well as other salamanders with similar reproductive behavior. Although there is a small number of studies on the short-term storage of Anuran sperm at temperatures > 0° C (Rostand 1946; Hollinger and Corton 1980; Browne et al. 2001; Browne et al. 2002a), no study has examined the short-term storage of sperm or spermatophores in salamanders or caecilians.

Material and Methods

Ambystoma mexicanum is an endemic neotenic salamander (Fig. 1), from the Mexican Central Valley and has been widely used in scientific research

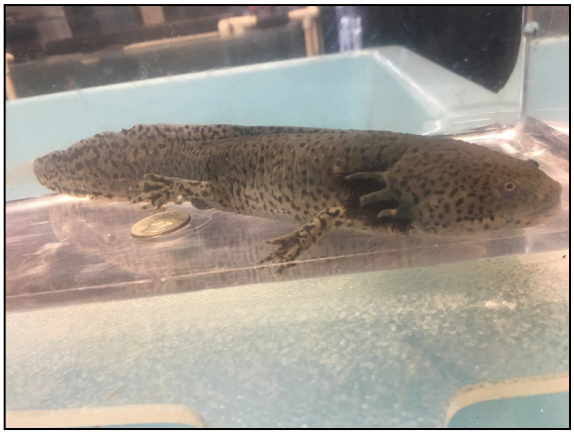

Figure 1. Male Axolotl (*Ambystoma mexicanum*) used in this study. (Photographed by Chester Figiel).

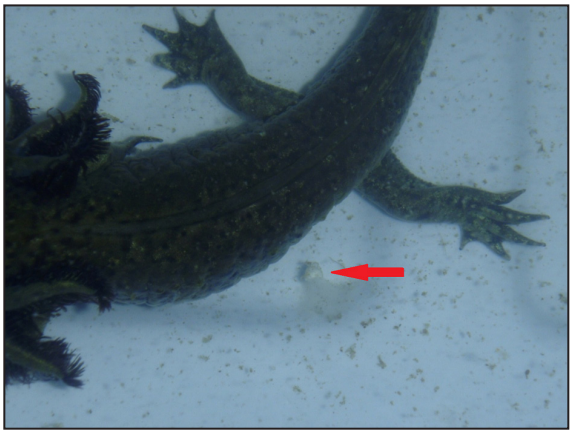

Figure 2. Female Axolotl (*Ambystoma mexicanum*) with spermatophore (red arrow) deposited by male. (Photographed by Chester Figiel).

because of their ability to regenerate body parts, ease of breeding, and large embryos (Voss et al. 2009; Reib et al. 2015). *Ambystoma mexicanum* are abundant in captivity but are imperiled and near extinction in nature because of invasive predatory fish, pollution, and urbanization (Zambrano et al. 2007; Recuero et al. 2010). This species is listed as critically endangered on the International Union for Conservation of Nature (IUCN) annual Red List (IUCN 2018).

I obtained *A. mexicanum* from the *Ambystoma* Genetic Stock Center of the University of Kentucky, Lexington, USA. Male salamanders had an average snout-vent-length (SVL) of 23.4 ± 1.7 cm (standard deviation) with a range 21.6 to 25.0 cm and an average weight of 90.4 ± 12.2 g. Females had an average SVL of 22.5 ± 0.5 cm with a range between 22.0 and 23.1 cm and an average weight 96.3 ± 12.2 g. I kept salamanders individually in aquaria (10 L and 30 L tanks) with flowing spring water (about 0.5 L/min) and fed a prepared diet at least 3 d per week. This study took place at the Warm Springs Fish Technology Center, U.S. Fish and Wildlife Service, Warm Springs, Georgia, USA.

To obtain spermatophores, I followed similar protocols to Figiel (2013). I filled six containers (46 cm length × 33 cm width × 11.5 cm height) with approximately 15 L of water and placed one male and one female together in each of these at approximately 1630. The following morning, I searched tanks and collected deposited spermatophores (Fig. 2). Unless a male had at least 13 spermatophores (to ensure one spermatophore per treatment - see below description), I did not use it in the experiment. I placed each spermatophore into a 50 ml sterile disposable centrifuge tube (Thermo Fisher Scientific, Waltham, Massachusetts, USA) filled with 45 ml of spring water. I placed these tubes in racks in three compact refrigerators (model NS-CF17BK6 Insignia, Richfield, Minnesota, USA) that were each equipped with an external thermostatic controller (Ranco, Robertshaw Co., Itasca, Illinois, USA). I installed thermostats following instructions in Tiersch and Tiersch (1993).

Right before assaying for intact sperm cells, I placed spermatophores in 2 ml collection tubes and used pipette tips and an up down motion to break the sperm cap apart. This provided access to sperm cells; however, it is unknown if using this technique affects sperm cell viability. I stained sperm on microscope slides with propidium iodide (PI) and SYBR-14 (L-7011 Molecular Probes, Inc., Eugene, Oregon, USA) to determine cell membrane integrity. When viewed with fluorescence microscopy, SYBR-14 fluoresce intact sperm a bright green, whereas, PI fluoresce damaged sperm cells red (Fig. 3; Garner et al. 1997). Use of this method indicates sperm membrane integrity, but it is unknown if sperm are viable and can fertilize eggs. I used five µL of sperm (estimated to contain between 100 to 1,000 cells; unpubl. data) and 2 µL of both dyes and I scored sperm by color. I estimated the percentage of intact sperm for

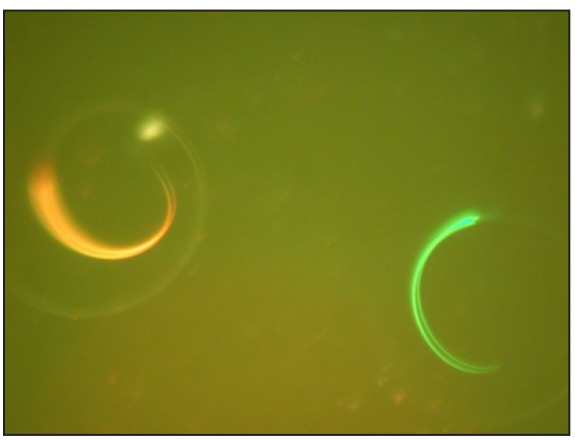

Figure 3. Sperm cells from the Axolotl (*Ambystoma mexicanum*). Viable intact sperm cells fluorescent a bright green while damaged cells fluoresce red. (Photographed by Chester Figiel).

each spermatophore until all sperm in that sample were gone. I assayed sperm by this method as sperm were not motile.

I collected 13 spermatophores from each of three males (males 1, 2, and 3) and I placed four spermatophores from male 1 in the 0° C temperature. Additionally, I place four spermatophores from male 1 in the 3° C temperature, and four spermatophores from male 1 in the 6° C temperature. I repeated this protocol for both males 2 and 3. I assayed one spermatophore from each of the three males on the day collected (day 0) and one spermatophore from each of the three males stored at each temperature on days 7, 14, 21, and 28. Each spermatophore was unique from other spermatophores. I arcsine-square root transformed the percentage of viable sperm cells (the percentage of green/green + red sperm) to minimize the heterogeneity of variances among treatments (Snedecor and Cochran 1989). I performed a Two-way ANOVA (α = 0.05) using the Excel2016.lnk software package (Microsoft, Redmond, Washington, USA) to analyze the main effects (Time, Temperature) and interactions (Time × Temperature) on the percentage of viable sperm. I report intact sperm cells as the mean ± one standard deviation.

Results

Sperm cells remained intact for 28 d when stored at 0° C (45.0% ± 31.2%), 3° C (61.7% + 12.6%) and 6° C (40.0 ± 45.8%; Table 1). The percentage of intact sperm cells varied among the time-periods (Table 1; individual spermatophores assayed on days 0, 7, 14, 21, and 28), and was not significantly different ($F_{3.24}$ = 2.739, P = 0.066; Table 1). Additionally, the percentage of intact sperm cells in the three storage temperatures also were not significantly different ($F_{2.24}$ = 2.365, P = 0.116; Tables 1), and neither this factor nor the interaction of time by temperature resulted in a significant difference in the percentage of sperm that were viable ($F_{6.24}$ = 1.084, P = 0.400; Table 1).

Table 1. Summary of the percentage of intact sperm cells (± standard deviation) of three male Axolotl (*Ambystoma mexicanum*) from spermatophores stored at three temperatures (0° C, 3° C, and 6° C), over 28 d (7, 14, 21, 28 after spermatophore deposition). One spermatophore from each male was examined at each time period that was stored at each temperature. Intact sperm cells on day of deposition = 45.0% ± 39.l% (standard deviation).

	Time Period			
	Day 7	Day 14	Day 21	Day 28
0° C	38.3 ± 22.5	31.7 ± 24.7	20.0 ± 15.0	45.0 ± 31.2
3° C	26.7 ± 2.90	45.0 ± 21.8	43.3 ± 25.2	61.7 ± 12.6
6° C	36.7 ± 20.8	65.0 ± 22.9	66.7 ± 25.2	40.0 ± 45.8

Discussion

I found that *A. mexicanum* spermatophores may be maintained unfrozen for 28 d at low temperatures, which indicates that it is feasible to use this technique for the production of salamanders in conservation breeding programs. This non-invasive method of gamete collection (i.e., the deposition of spermatophores) would prove useful when working with critically endangered species to avoid the need for the excision and maceration of testes, or hormone injections (see Xiao et al 2006; Mansour et al. 2010; Shishova et al. 2011). During propagation, asynchronicity of males and females is not uncommon; thus, storing gametes for short time periods would be beneficial for captive breeding.

Previous research has shown that storing amphibian sperm at temperatures around 0° C retained greater sperm viability compared to sperm stored at higher temperatures. For example, Armstrong and Duhon (1989) described protocols for artificial insemination of *A. mexicanum* by placing spermatophores on ice (0° C) resulting in viable sperm cells for 24 h. Browne et al. (2001) found that testis and sperm suspensions for Cane Toads (*Bufo marinus*) was better at 0° C (on ice) and survived better than at 4° C for up to 10 d. Additionally, Browne et al. (2002b) stored *B. marinus* sperm for 6 d at 0° C (on ice) before cryopreservation. Furthermore, Kouba et al. (2009) reported unpublished data that Fowler's Toad (*B. fowleri*) had a greater percentage of motile sperm at 0° C (on ice) compared to 22° C. It appears that lower temperatures may assist sperm cells or spermatophores in surviving longer intervals; however, the reason for this is not known. Potentially storing spermatophores at colder temperatures could prevent degradation of the sperm cap or the cell membrane granular and fibrous material (Zalisko et al. 1984) that aide in protecting sperm cells.

The percentage of viable sperm cells varied among males in this experiment. This conceivably resulted from variation in aspects of spermatophore characteristics (e.g., shape and size, structure of the sperm cap); however, male-male differences (e.g., age, energetic costs) and sperm cell quality cannot be ruled out. It is unknown if sperm cells and spermatophores from an individual are uniquely distinct in these facets from beginning to end of spermatophore deposition. That is, are the first few spermatophores that are deposited during courtship behavior similar to the last few spermatophores? Further studies using a greater number of males and a greater quantity of spermatophores would help address this question. Additionally, it is worth examining whether sperm from spermatophores collected from wild axolotl have similar qualities compared to these laboratory animals.

The highest priority of the Amphibian Conservation Action Plan of the International Union for the

Conservation of Nature is "to develop standardized and non-invasive methods for the collection, assessment and storage of germplasm (sperm, oocytes or embryos) from a variety of amphibian species" (Gascon et al. 2007). This is important for both conservation-breeding programs as well as for small fragmented populations in nature to prevent loss of genetic resources. The refinement and application of cold storage methods for amphibian sperm should help in that regard.

Acknowledgments.—I thank Randal Voss, Laura Muzinic, and Chris Muzinic at the *Ambystoma* Genetic Stock Center of the University of Kentucky and the National Science Foundation (NSF-DBI-0443496) grant mechanism that supported this program. I thank Natalie Calatayud and Bill Wayman for commenting on versions of this manuscript, and Katie Roberts for her help throughout this research. Any use of trade name or proprietary product does not imply endorsement by the U.S. Government. 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. Links to commercial sites are in no way an endorsement of any product or service of a vendor.

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