



Short communication

Effects of aeration and antibiotics on short-term storage of Fowler's toad (*Bufo fowleri*) spermJennifer M. Germano ^{a,*}, Lucía Arregui ^{a,b}, Andy J. Kouba ^a^a Conservation and Research Department, Memphis Zoo, 2000 Prentiss Place, Memphis, TN 38112, USA^b Department of Biology, Universidad Autónoma de Madrid, 28049 Madrid, Spain

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ABSTRACT

The importance of developing assisted reproductive technologies (ART) for captive assurance colonies of threatened amphibians is increasing as *ex situ* management of amphibians has been challenged with low reproductive outputs for some species. One hurdle to developing ART in amphibians is the short-term storage of gametes for *in vitro* fertilization. This study tested the application of two common aquaculture techniques, aeration or the addition of antibacterial solutions, to spermic urine to determine if this would improve the longevity and quality of non-invasively collected sperm samples using hormone therapy. Spermic urine samples were collected non-invasively from Fowler's toads (*Bufo fowleri*) and either left alone, aerated for 30 min a day, or treated with penicillin–streptomycin. All samples were kept refrigerated at 4 °C. Sperm motility declined within each treatment group over time ($P < 0.01$); however, aerated samples retained significantly greater motility (70%) during the first 24 h after collection than samples in both the control (57%) and antibacterial group (51%). The addition of penicillin–streptomycin solution to spermic urine had a negative effect on viability; with significantly fewer sperm still alive 2–4 days post collection ($P \leq 0.032$). Sperm viability was highly correlated with motility on all days and with forward progression 1–4 days post collection ($P \leq 0.002$). Our results show that aerating amphibian spermic urine samples may provide a simple and low-cost method to improve sperm storage that could be used for *in vitro* fertilization and *ex situ* management of threatened amphibians. Antibiotics appeared to have a negative effect on sperm viability, suggesting that further research on bacterial contamination and antibiotic doses is necessary.

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1. Introduction

The worldwide amphibian crisis has led to the establishment of a large number of *ex situ* conservation programs for threatened species as a hedge against extinction in the wild. However, with limited knowledge of these animals' physiology and reproductive ecology, successful breeding of animals in captive assurance colonies has been a challenge. With low reproductive output and short generation times it has become imperative to intervene and develop new technologies for maintaining original founders and breeding stock for future reintroductions. Assisted reproductive techniques (ART) are valuable tools for assisting captive management of threatened species, maintaining genetic diversity and providing a means to link *ex situ* and *in situ* conservation programs (Howard et al., 2003; Kouba et al., 2009; Pukazhenth and Wildt, 2004; Wildt et al., 1997). Yet, compared to mammals and fish, the development of ART for amphibians is still in its infancy with only a limited amount of knowledge in a

few common anuran (frog and toad) species or laboratory models, with nothing known about urodeles or caecilians. As in many fish species, amphibian ART has several advantages over mammalian systems due to high fecundity, external fertilization and large easily manipulated gametes.

Although relatively new, research into amphibian ART has increased over the past decade with the vast majority of studies focusing on developing hormone therapy protocols for inducing ovulation, spermiation, and natural mating (Kouba et al., 2009). There have also been several studies aimed at storing genetic material in cryobanks for long-term management and conservation of threatened species, primarily for toads and frogs (Browne et al., 1998, 2002a; Kouba and Vance, 2009; Mansour et al., 2009; Shishova et al., 2011). As the ability to induce spermiation in a variety of anuran species is now possible using live animals (Browne et al., 2006a; Obringer et al., 2000; Rowson et al., 2001; Silla, 2010), scientific breakthroughs are needed to improve the storage of these gametes both in the short-term through refrigeration and for the long-term through cryopreservation. The storage of spermatozoa will allow transfer of genetic material between locations instead of the current necessity of moving animals between facilities to maintain genetic diversity, which can lead to stress, illness, or even death of an animal.

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Moreover, sperm storage will also help support the development of *in vitro* fertilization (IVF) systems, since asynchrony in gamete release is often a problem.

Currently, there are only a handful of reports documenting fertilization using cryopreserved sperm collected non-invasively from live animals (Kouba et al., 2009; Shishova et al., 2011). Unfortunately, cryopreservation of amphibian sperm is not yet at the stage where it can be implemented into captive management programs due to low viability following freezing. On the other hand, refrigeration has been shown to be effective for maintaining motility over several days in cane toad (*Bufo marinus*) sperm collected from testes macerates (Browne et al., 2001) and from hormonally-induced spermatozoa of the dusky gopher frog (*Rana sevosia*), Fowler's toad (*Bufo fowleri*), Wyoming toad (*Bufo baxteri*), boreal toad (*Bufo boreas boreas*), and American toad (*Bufo americanus*) (Kouba et al., 2009). In *B. fowleri*, the greatest decline in sperm motility (30% loss) for samples stored at 4 °C occurs within the first 24 h then increases to 50% over the next five days (Germano et al. unpublished data). Thus, it may be possible to store *Bufonid* spermatozoa for extended periods of time at 4 °C and use such samples for IVF, depending on when eggs are finally ovulated from the female. In order to optimize IVF for threatened amphibians there is a need to maintain the quality of the freshly chilled sperm for as long as possible; the key to extend sperm viability may lie in the technologies that have been developed for commercial aquaculture species, in particular fish.

The majority of anurans are aquatic breeders and display external fertilization; hence, ART developed for fish have been explored for their application to amphibians (Browne et al., 2006b; Kouba et al., 2009; Trudeau et al., 2010). In general, amphibian sperm motility is short lived when deposited into a hypoosmotic environment, very similar to fish sperm, which lasts seconds to minutes (Costanzo et al., 1998; Hollinger and Corton, 1980; Raisman et al., 1980; Wolf and Hendrick, 1971); thus, similar mechanisms that may extend the viability, motility, and metabolism of fish sperm stored *in vitro* may also work for amphibians. Two such promising techniques include modifications on the gaseous environment and the addition of antibiotics (Bencic et al., 2000b; Billard et al., 2004; Jenkins-Keeran et al., 2001; Stoss and Holtz, 1983).

Daily supplements of an atmosphere of pure oxygen produced longer storage times for motile sperm of both channel catfish (*Ictalurus punctatus*) and rainbow trout (*Salmo gairdneri*) (Büyükhapoglu and Holtz, 1978; Christensen and Tiersch, 1996). Additionally, semen from striped bass (*Morone saxatilis*) also responded positively to oxygen, with sperm stored under oxygen having greater motility than those stored under ambient air (Jenkins-Keeran et al., 2001). However, Bencic et al. (2000b) found that ambient air was as effective as 100% oxygen in maintaining viability and motility of salmonid sperm, suggesting that this simpler and safer method may have a similar desired effect. In addition, amphibian spermic urine often contains bacteria due to its passage through the cloaca where it coincides with fecal material. When storing spermic urine, bacteria can multiply to produce high contamination levels over time. Loss of motility in fish spermatozoa has been associated with bacterial growth, possibly due to a negative effect of waste product or enzymes produced by bacteria (Christensen and Tiersch, 1996). Although antibacterial solutions are used routinely for the short-term storage of fish sperm (Bencic et al., 1999, 2000a; Stoss and Holtz, 1983) the addition of antibacterial solutions to amphibian sperm has never been tested.

In this study, it was hypothesized that *B. fowleri* sperm exposed to atmospheric air or treated with antibiotics would retain motility and viability longer than control samples when stored for four days at 4 °C. Not only will this investigation provide some insight into techniques that might extend the lifespan of stored toad sperm for IVF but also compare physiological mechanisms that might be similar between fish and amphibian sperm metabolism.

2. Materials and methods

2.1. Animals

B. fowleri were caught from the wild in Shelby Co., TN (35.1494° N, 90.0489° W), during 2008 and 2010 (Tennessee Wildlife Resources Agency Permit #3550). All animals were adult males (snout to vent length = 62.1 ± 0.7 mm; weight = 22.5 ± 0.9 g) determined to be in breeding condition by the presence of a dark skin patch covering the throat and indicative of the male vocal sac. Toads were housed at the Memphis Zoo in large plastic tubs (178×81×36 cm [L×W×H]; Waterland tubs, Orange, CA, USA) with 10–15 animals per tub. Retreat sites and water were provided at all times and animals were kept under ambient lighting through skylights and additional UV lamps on timers to simulate natural light cycles. Toads were maintained at a constant environmental temperature between 21 and 26 °C. The toads were fed mealworms and adult crickets, dusted with Reptocal (Tetrafauna, Melle, Germany) and gut loaded with Hi-Cal Cricket Monster Diet (Zeigler Bros, Inc., Gardners, PA, USA) 1–2 times a week. At the end of the study, toads were retained as part of the Memphis Zoo amphibian research colony.

2.2. Spermic urine collection

Male toads were placed in plastic boxes (34.3×20.3×12.7 cm [L×W×H]), with 1 cm of tap water. Each toad was given an intraperitoneal injection of 300 IU of human chorionic gonadotropin (Chorulon, Intervet Inc., Millsboro, DE, USA) diluted in 200 µL of phosphate-buffered saline solution. Spermic urine samples were then collected for analysis 4–6 h later according to methodology previously described (Browne et al., 2006b). Toads were briefly dried and then held over a 150 mm Petri dish until the animal urinated, a natural defensive mechanism for *Bufonids* upon handling. The spermic urine volume was measured to the nearest 10 µL using a pipette and then 300 µL of spermic urine was transferred into a 1.5 mL Eppendorf tube, sperm quality assessed, and samples stored at 4 °C for 4 days. Sperm concentration was measured on the first day using a Neubauer hemocytometer.

2.3. Experimental treatments

Toads were divided into three treatment groups with 15 animals per treatment. Hormone administration and spermic urine collection protocols were consistent across groups. No extender solutions were added to any of the three treatments and all samples were stored at 4 °C throughout the entire experiment. Concentration was not controlled for as dilution of samples with water or urine affected motility; however, the initial volume of 300 µL was kept consistent across all groups.

The treatments tested were as follows: controls, aeration of spermic urine, and addition of antibiotics to samples. The control group was stored with no other handling other than the removal of a small sample for daily sperm quality assessments. For the aeration group, spermic urine samples were gently aerated for 30 min per day using a battery-powered aquarium pump (Azoo Battery Air Pump, Seven Ports Inc., Whittier, CA, USA) that was operated within a refrigerator. Atmospheric air flowed from the pump through 4 mm diameter airline tubing and a sterile plastic pipette tip, which extended to just above the bottom of a 1.5 mL Eppendorf tube and was immersed into the spermic urine (Fig. 1). Immediately following 30 min of aeration, the pipette tips were removed and the Eppendorf tubes were capped. For the antibacterial group, the samples were supplemented with 600 mU of penicillin–streptomycin solution (P0781, Sigma Aldrich Inc., St Louis, MO, USA) within 4 h of collection. This dose was determined from the literature on short-term storage of fish semen (Babiak et al., 2006; Christensen and Tiersch, 1996; Riley et al., 2008).

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