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Applying cryopreservation to anuran conservation biology

Sinlan Poo | **Kristin M. Hinkson**

Department of Research and Conservation, Memphis Zoological Society, Memphis, Tennessee

Correspondence
Sinlan Poo, Department of Research and Conservation, Memphis Zoological Society, 2000 Prentiss Pl, Memphis, TN 38112.
Email: sheilapoo@gmail.com

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Abstract

Novel conservation methods have become increasingly important in protecting our remaining biodiversity in the face of unprecedented rates of species declines. One method of maintaining genetic and species diversity is through germplasm cryopreservation. However, our knowledge of cryopreservation relies heavily on studies in domestic or aquaculture species. Moreover, most studies are narrowly focused on a single species. Consequently, the broader application of these methods to wildlife conservation is often unknown or assumed. Here, we examine the response of four anuran species from two families (*Anaxyrus fowleri, Anaxyrus baxteri, Lithobates pipiens*, and *Lithobates sevosus*) to three sperm cryopreservation treatments (5, 10, and 15% N,N-dimethylformamide with 10% trehalose). Within each family, we selected a congeneric species pair with one common, nonthreatened species and one endangered species. We found that endangered species had significantly lower initial sperm quality, though recovery rate of sperm after cryopreservation was not significantly different between nonthreatened and endangered species. Overall, responses to the three treatments were consistent across species, with 5% DMFA with 10% trehalose producing the highest recovery rates in all four species. In addition, cryopreserved sperm were used to successfully produce hatchlings in both of the nonthreatened species. Our results demonstrate that standardized methods developed for anurans can be applied more widely across families and can be transferred from model species to species of conservation concern. These findings form a basis for further exploration into cryopreservation as an effective tool for wildlife conservation in amphibians.

KEYWORDS
amphibian, assisted reproductive technology, Bufonidae, cryobiology, Ranidae, sperm motility

1 | INTRODUCTION

Globally, we are currently facing unprecedented declines in biodiversity far exceeding the "normal background" rates (Johnson et al., 2017). This loss in biodiversity alters the biophysical function of ecosystems and their ability to sustain human populations (Cardinale et al., 2012). With species loss projected to increase in the near future (Johnson et al., 2017), there is a race to preserve our current biological and genetic diversity (Baillie & Butcher, 2012). One of the ways to do so is through germplasm cryopreservation. Cryopreservation has the potential to extend the genetic lifespan of endangered species through long-term storage and to preserve the genetic diversity represented within captive

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populations as a safeguard against extinction in the wild (Ballou, 1992). Perhaps not surprisingly, aside from research in humans, studies on germplasm cryopreservation have largely focused on advances in technology for livestock (e.g., Morrell & Mayer, 2017; Sieme & Oldenhof, 2015; Singh, Mal, & Singla, 2017) and aquaculture management (e.g., reviews by Asturiano, Cabrita, & Horváth, 2017; Martinez-Paramo et al., 2017; Magnotti et al., 2018). In comparison, cryopreservation technologies for wildlife and for other less commercially valuable taxa, such as birds, reptiles, and amphibians, are much less developed. As a result, use of cryopreservation as a conservation tool in these taxa is limited and is contingent upon the best available data from the closest taxonomically related species (Comizzoli, Songsasen, Hagedorn, & Wildt, 2012). However, whether the same methods can be applied across different levels of taxonomic relatedness is often unknown (Comizzoli et al., 2012). Consequently, there is a critical gap between the development of cryopreservation tools and its application in biodiversity conservation.

In amphibians, investigations into the freezability and cryo-tolerance of gametes began with collections from whole or macerated testes (Beesley, Costanzo, & Lee, 1998; Browne, Clulow, & Mahony, 2002; Browne, Clulow, Mahony, & Clark, 1998; Mugnano, Costanzo, Beesley, & Lee, 1998), which allowed researchers to circumvent the issue of sperm activation which occurs during natural spermiation. However, cryopreservation of testes macerates has limited applicability to wildlife conservation beyond terminal collections due to its invasive nature. In the recent decade, increasing attention has been given to developing less invasive cryopreservation techniques by collecting sperm using hormonal induction (Shishova, Uteshev, Kaurova, Browne, & Gakhova, 2011; Uteshev, Shishova, Kaurova, Manokhin, & Gakhova, 2013). In doing so, animals are relatively unharmed during the process and multiple collections can be done on a single individual. Together, these few studies form a valuable basis for our current knowledge of sperm cryopreservation in amphibians. Thus far, studies using hormonally induced sperm have generally focused on a single species. The lack of cross-species comparisons impedes the development of cryotechniques in amphibians because sperm cell differences (i.e., size and structural variations) can influence freezability. For example, the sperm of several species of toad and caecilian possess a unique structure termed the mitochondrial vesicle, which is lacking in ranid frogs (George et al., 2005; Kouba, Vance, Frommeyer, & Roth, 2003). This fragile structure facilitates sperm movement, but is often harmed in the cryopreservation process (Kouba & Vance, 2009). Given these differences, the main question of whether protocols for cryopreservation can be generalized across amphibian species remains unanswered. More importantly, can cryopreservation techniques developed using nonthreatened, model species be successfully applied to threatened or endangered target species that are in need of conservation efforts?

One way to fill this gap is to systematically assess different methods of amphibian sperm cryopreservation across taxonomical levels. In particular, priority should be given to comparisons of sperm cryopreservation between common, model species and targeted, endangered species to validate its use as a conservation tool. To do so, we chose to study two pairs of congeneric anuran species (*Anaxyrus fowleri, Anaxyrus baxteri, Lithobates pipiens,* and *Lithobates sevosus*) from two of the most diverse anuran families in North America (IUCN, 2018, Bufonidae and Ranidae, respectively). Collectively, bufonids and ranids represent over 50% of all North American anurans (IUCN, 2018). Within each congeneric pair, we selected a common, nonthreatened species that is often used as a model system, *A. fowleri* (Fowler's toad) and *L. pipiens* (northern leopard frog), and an endangered species that is the focus of substantial conservation efforts involving multiple governmental and private organizations, *A. baxteri* (Wyoming toad) and *L. sevosus* (dusky gopher frog). In the bufonid pair, the Fowler's toad is a widespread species commonly found in the Eastern U.S. (Powell, Conant, & Collins, 2016; IUCN, 2018: Least Concern). In comparison, the Wyoming toad is a federally listed, endangered species with only a few reintroduced populations in the Laramie Basin of Wyoming (IUCN, 2018: Extinct in the Wild). Though substantial efforts have been made to establish wild populations, the species is still dependent on releases from the captive breeding programs (U.S. Fish and Wildlife Service, 2016). Similarly, in the ranid pair, the northern leopard frog is commonly found in the Eastern U.S. (Powell et al., 2016; IUCN, 2018: Least Concern). Whereas the dusky gopher frog is a federally listed, endangered species with only around 200 individuals remaining in the wild in coastal Mississippi (IUCN, 2018: Critically Endangered). Like the Wyoming toad, the dusky gopher frog is still reliant on ongoing conservation management efforts for natural recruitment and captive breeding colonies for genetic maintenance. Together, these species provide the perfect opportunity to explore the effectiveness of sperm cryopreservation methods using less invasive sperm collection methods. Moreover, comparisons between these species allow us to gain insight into the applicability of cryopreservation methods across species with varying conservation priorities within the same genus and across different families.

Herein, we examine how four anuran species, namely, Fowler's toads, Wyoming toads, northern leopard frogs, and dusky gopher frogs, respond to three different concentrations of cryoprotectants. We hypothesize that the most effective treatment for each species will differ due to species-specific responses, with more similar responses found between congeneric species compared to species from other anuran

families. In addition, we assess the fertilization rate of cryopreserved sperm from the two nonthreatened anuran species, Fowler's toad and northern leopard frog. Finally, we highlight potential areas in amphibian cryopreservation research that merit more attention in the future.

2 | METHODS

2.1 | Animals

We conducted experiments from June 2017 to August 2018. Captive-bred Wyoming toads were housed indoors at the Leadville National Fish Hatchery (Leadville, Colorado) as part of the Wyoming toad captive breeding program. Wild-caught Fowler's toads were collected during the breeding season (April – May) in Shelby County, Tennessee. Wild-caught northern leopard frogs were obtained commercially through Carolina Biological Supply (Burlington, North Carolina). Captive-bred and wild-caught dusky gopher frogs were housed indoors at the Memphis Zoo (Memphis, Tennessee) as part of the dusky gopher frog captive breeding program. We maintained all individuals in groups of one to five in either acrylic enclosures (98 cm L × 46 cm W × 34 cm H) or 10 gal glass aquaria (51 cm L × 25 cm W × 31 cm H). We fitted enclosures with organic substrates (coconut shaving or sphagnum moss), cover, and water, and cleaned enclosures daily. We fed study animals a variety of insects (ex. crickets, cockroaches, mealworms, and superworms) *ad libitum*. All animal procedures were approved by the Memphis Zoo Animal Care and Use Committee and all necessary state or federal agencies (Fowler's toad: Approval 16–101, TWRA Permit 1,315; Wyoming toad: Approval 17–102, USFWS Permit TE704930-1; northern leopard frog: Approval 18–102; dusky gopher frog: Approval 16–102, USFWS Permit TE171493-1). Sample size was determined by the number of individuals available for research use according the permitting agencies and the institutes in charge of captive colonies.

2.2 | Sperm collection

We administered exogenous hormones to induce the release of spermic urine in all study animals. Because response to hormone treatments varies across taxa (Della Togna et al., 2017; Kouba & Vance, 2009), we used different hormones and hormone concentrations for the males of each study species following either established protocols within breeding programs or previous experience with the species (Poo, Hinkson, & Stege, 2018). Specifically, we administered 0.2 μg/g body mass of GnRH (des-Gly^{10}, D-Ala^{6}; Sigma-Aldrich, St. Louis, Missouri) to Wyoming toads, 7.5 IU/g body mass hCG (human chorionic gonadotropin; Sigma-Aldrich) to Fowler's toads, and 10 IU/g hCG + 0.4 μg/g body mass of GnRH to northern leopard frogs and dusky gopher frogs. All injections were given using a 0.3 mL syringe and 29 gauge ½” needle.

Immediately following injections, we placed each animal individually in 2.4 L plastic boxes filled with 1 cm of aged water to promote urine production. We collected urine at 4 hr post-injection for Wyoming toads and Fowler's toads, and 1 hr post-injection for northern leopard frogs and dusky gopher frogs. Collection time was determined based on previous findings (Browne, Seratt, Vance, & Kouba, 2006; Kouba, Vance, & Willis, 2009; Obringer et al., 2000) combined with our experiences with these species. If urination did not naturally occur within 5 min, we facilitated the collection by inserting medical-grade, plastic catheter tubing (0.86 mm inner diameter × 1.32 mm outer diameter, Scientific Commodities, Inc., Lake Havasu City, Arizona) into the cloaca.

2.3 | Cryopreservation and thawing

We divided sperm samples from each individual equally into three cryoprotectant treatments with varying concentrations of DMFA (N,N-dimethylformamide; Sigma-Aldrich) and trehalose (Sigma-Aldrich). We chose these reagents because, in amphibians, solutions consisting of both a permeating and nonpermeating cryoprotectant have proven to be most successful (Mansour, Lahnsteiner, & Patzner, 2009; Shishova et al., 2011; Uteshev et al., 2013). DMFA and trehalose have preliminarily shown promising results (Langhorne et al., 2013), but due to the potential toxicity of DMFA at high concentrations, variations of the protocol developed by Langhorne et al. (2013) were tested. Specifically, we prepared three cryoprotectant solutions with distilled water and gradually diluted 1:1 with fresh spermic urine to create sperm cryopsuspensions with final concentrations of 5% DMFA +10% trehalose, 10% DMFA +10% trehalose, and 15% DMFA +10% trehalose (hereafter, treatments DT 5-10, DT 10-10, and DT 15-10, respectively). We then loaded samples into 0.25 cc cryostraws (Reproduction Resources, Walworth, Wisconsin) and equilibrated samples at 4°C for 10 min. Following equilibration, we cooled samples to −90°C in nitrogen vapor for 10 min in an insulated polystyrene box. We then immersed samples into liquid nitrogen (−196°C) to complete the cryopreservation process. To assess the sperm quality after cryopreservation, we thawed samples for 10 s in room temperature (23°C) followed by 10 s in a 40°C water bath. Finally, we diluted thawed samples 1:10 with distilled water.

2.4 | Sperm assessments

Upon collection, we immediately evaluated each fresh spermic urine sample for percent total motility, percent forward

progressive motility, and concentration at 400 × using an Olympus CX41 phase-contrast microscope. Similarly, we evaluated thawed sperm samples for percent total motility and percent forward progressive motility immediately upon dilution. For all samples, we determined percent total motility by counting all cells with flagellar movement within 100 cells. We determined percent forward progressive motility by counting all cells exhibiting forward movement within 100 cells. Visual counts are a common method used in amphibian sperm analysis (Della Togna et al., 2017; Poo et al., 2018; Shishova et al., 2011) and were conducted with the same observer to reduce potential observer bias. We calculated recovery rate (RR) for motility and forward progressive motility as RR = (FV/IV) × 100, where IV = initial value of fresh samples and FV = final value after samples were cryopreserved and thawed. Finally, we determined sperm concentration using a Neubauer-ruled chamber hemocytometer.

2.5 | Fertilization ability

To assess the fertilization ability of cryopreserved sperm, we conducted *in vitro* fertilization trials (IVF) in Fowler's toads and northern leopard frogs. Sperm samples with a concentration of more than 5,000,000 sperm cells/mL were preserved using DT 5-10 cryoprotectant and thawed for IVF. To induce oviposition, we administered two priming doses 72 hr apart, followed by an ovulation dose 24 hr after the second priming dose. Priming and ovulation doses were 2.5 IU hCG/g body mass and 12.5 IU hCG + 0.5 μg GnRH/g body mass, respectively, for Fowler's toads and 3.7 IU/g body mass and 13.5 IU hCG + 0.4 μg GnRH/g body mass, respectively, for northern leopard frogs. Females were held over a cell culture dish (150 × 25 mm), so that eggs could be deposited directly on to the dish. Immediately after eggs were deposited, thawed sperm samples were placed on eggs for fertilization. After 5 min, the petri dish was filled with aged tap water to submerge the eggs. Eggs were monitored daily and hatching success was recorded. Fertilization ability was calculated as the number of hatchlings produced per 1,000,000 sperm cells.

2.6 | Statistical analyses

Assumptions of data normality were tested using the Shapiro–Wilk test. For fresh sperm samples, we tested the effects of conservation status (nonthreatened species or endangered species) and taxonomical group (Bufonidae or Ranidae) on sperm motility, forward progressive motility, and concentration using generalized linear models (GLMs). For sperm motility and forward progressive motility, we used GLMs with underlying quasibinomial distribution and a logit link function (for over-dispersed proportional data), while for sperm concentration we used a GLM with underlying quasi-Poisson distribution and log link function (for over-dispersed count data). For thawed sperm samples, we tested the explanatory variables: conservation status, taxonomical group, and cryoprotectant treatment on the recovery rate of motility and forward progressive motility using GLMs with underlying quasibinomial distribution and a logit link function. For all GLMs, we used F tests to test the significance of explanatory variables. In addition, we used *post hoc* Tukey's Honest Significant Difference tests to determine differences between cryoprotectant treatments. We conducted all statistical analyses in the R programming environment (v. 3.5.1) using a significance level of $\alpha = 0.05$.

3 | RESULTS

We collected samples from 14 Wyoming toads, 20 Fowler's toads, 17 northern leopard frogs, and 13 dusky gopher frogs. Average mass for the four species were 20.61 (SE 0.75), 22.68 (SE 0.73), 38.53 (SE 1.77), 43.85 (SE 2.98) g, respectively. For fresh sperm samples, motility, forward progressive motility, and concentration were significantly different between species with different conservation statuses (Table 1), with nonthreatened species exhibiting higher sperm quality in all three sperm metrics compared to their congeneric endangered species (Figure 1, Table 2). Taxonomical group had an effect on two of the three sperm quality metrics (Table 1), with bufonids exhibiting higher forward progressive motility but lower concentration compared to ranids. No significant differences were found for motility between taxonomic groups (Table 1, Table 2).

Comparing sperm quality before and after cryopreservation, we found that recovery rates in motility and forward progressive motility for all four species followed the same trends in response to the three cryoprotectant treatments (Figure 2). In particular, significantly higher sperm quality was retained in the DT 5-10 treatment compared to DT 10-10 and DT 15-10 (Table 2, Table 3). Recovery rates in motility and forward progressive motility were not significantly different between endangered and nonthreatened species (Table 1), though ranids showed higher recovery rates in both sperm metrics compared to bufonids (Figure 2, Table 2). The overall average motility and forward progressive motility recovered across all four species in the cryoprotectant with the highest recovery rate (DT 5-10) was 37.10% (SE 3.49) and 32.35% (SE 4.06), respectively.

Fertilization ability of cryopreserved sperm cells was tested using five pairs of Fowler's toads and two pairs of northern leopard frogs. Overall, sperm cryopreserved in DT 5-10 was deposited on 10,920 Fowler's toad and

TABLE 1 Generalized linear models examining explanatory factors associated with sperm quality in fresh sperm samples and recovery rate after cryopreservation

	Explanatory factors		
	Conservation status	Taxonomical group	Cryoprotectant treatment
Fresh sperm			
Motility	<.001	.078	—
Forward progressive motility	<.001	.002	—
Concentration	.002	<.001	—
Recovery rate			
Motility	.279	.025	<.001
Forward progressive motility	.320	.015	<.001

Note: Numbers represent *p* values.

328 northern leopard frog oocytes, resulting in 1,704 and 43 hatchlings, respectively. On average, cryopreserved Fowler's toad sperm cells were able to produce 27.41 (SE 31.01) fertilized eggs per 1,000,000 sperm cells applied. In comparison, cryopreserved northern leopard frog sperm cells were able to produce 71.46 (SE 20.07) fertilized eggs per 1,000,000 sperm cells applied.

4 | DISCUSSION

In this study, we investigated the response of sperm to cryopreservation in two congeneric species pairs from different anuran families, with one nonthreatened and one endangered species in each pair. Contrary to our hypotheses, response to different cryoprotectant treatments in all four species followed the same trend, regardless of their taxonomy or conservation status. Specifically, the treatment that produced the highest recovery rate, DT 5-10, was the same across species, though ranids showed a higher recovery rate in general. Moreover, conservation status did not have a significant effect on the recovery rate of sperm after cryopreservation, despite the fact that initial quality of fresh sperm was lower in endangered species compared to their nonthreatened counterparts. The key finding in this study, namely the consistency of responses across species to a standardized protocol, is a substantial breakthrough in amphibian sperm cryopreservation research, with implications that transcend our particular study species. The significance of these findings is two tiered; first, in terms of transferring methods between nonthreatened and endangered species, and second, in terms of the wider applicability of methods across anuran families. Despite the fact that both of these findings appear to be self-evident in retrospect, empirical studies that provide cross-taxa comparisons, especially with species of different levels of conservation priority, are still lacking in many regards.

First, similar responses between common, ecologically resilient species and endangered species provide empirical evidence showing that model species are indeed effective and reliable tools for developing and refining amphibian sperm cryopreservation techniques. While gamete cryopreservation studies regularly cite their potential use for species conservation, few have shown the successful transfer of techniques to at-risks species. Consequently, there has been a historical gap between the study of cryopreservation technologies and its applications for wildlife conservation (reviewed in Holt, 2008; Jewgenow, Braun, Dehnhard, Zahmel, & Goeritz, 2017). For domestic species, the goal is often to develop tailored protocols to maximize sperm recovery rate in a small number of species with high economic value (e.g., Iaffaldano, Di Iorio, Cerolini, & Manchisi, 2016; Martinez-Paramo et al., 2017; Purdy, 2006). In comparison, however, the focus of developing sperm cryopreservation as a tool for wildlife conservation is understandably different. Because of the large number of threatened and endangered species within each taxon, the primary goal in wildlife conservation is to develop methods in model species that can be transferred to species in need of intervention. For example, cryopreservation methods for domestic cats have been successfully applied to a number of endangered species within the felid family (reviewed in Amstislavsky, Kozhevnikova, Muzika, & Kizilova, 2017). Similarly, a comparative study showed that a single, simple method can be successfully applied across a number of endangered pheasant species (Saint Jalme, Lecoq, Seigneurin, Blesbois, & Plouzeau, 2003). With our findings, we provide an example of how methods of sperm cryopreservation established in nonthreatened species can be applied to preserve the diversity of threatened or endangered amphibian species. Moreover, results from IVF indicate that sperm cryopreservation can be used to successfully produce offspring in both nonthreatened

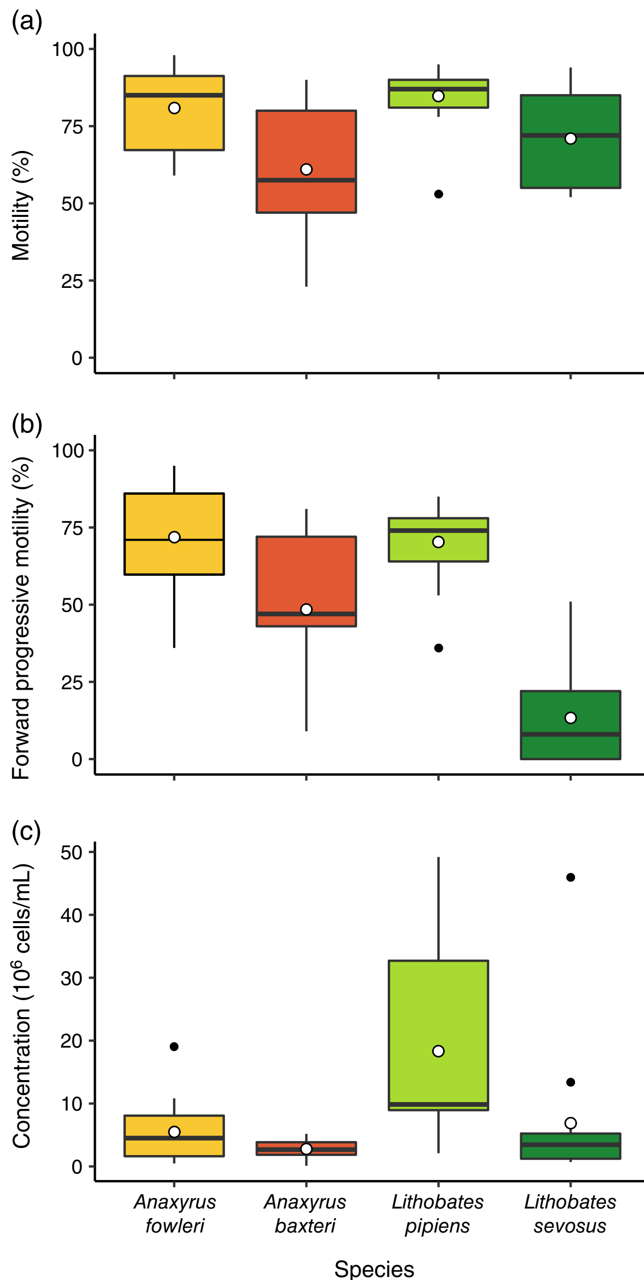

FIGURE 1 Boxplot of (a) fresh sperm motility, (b) forward progressive motility, and (c) concentration in two common, nonthreatened (*Anaxyrus fowleri* and *Lithobates pipiens*) and two endangered species (*Anaxyrus baxteri* and *Lithobates sevosus*) of amphibians. Lower and upper hinges of box plot correspond to the first and third quartiles, horizontal line denotes median, white dot denotes mean, and black dots denote outlying points

species, further highlighting the potential for these methods to be utilized in conservation efforts.

Second, consistent responses to cryoprotectant treatments across families indicate that methods of cryopreservation can be more widely applied within anurans. In the past, one of the things that has hindered the progress of developing standard methods is the fact that most studies focus on a single species and are often restricted by small sample sizes ($N \leq 5$) (e.g., Mansour, Lahnsteiner, & Patzner, 2010; Mugnano et al., 1998; Shishova et al., 2011). Consequently, their applicability beyond the focal species is often unknown or assumed. As our findings suggest, methods for sperm cryopreservation within amphibians appear to be transferable at least between bufonids and ranids, if not beyond. Interestingly, recovery rates in ranids were higher than that of bufonids, which may be due to the presence of the mitochondrial vesicle in the latter. However, despite these differences, both bufonids and ranids clearly responded better to the DT 5-10 treatment compared to the two other cryoprotectants. Beyond the two families examined, it is possible that other species within the same suborder, Neobatrachia, which is the lowest taxonomical level that links the bufonids and ranids (Cannatella, Ford, & Bockstanz, 2008), would respond similarly to these cryopreservation methods. This suborder of frogs includes roughly 95% of all extant frog species (IUCN, 2018), and contains over 1,400 endangered or critically endangered species (IUCN, 2018). Though our study focuses on two specific genera, these results demonstrate that it is possible for standardized methods developed using a small number of species to be applied more broadly across different anuran families.

Given the high diversity within anurans alone, an order that consists of over 7,000 species (Frost, 2019), it would be unrealistic and inefficient to develop sperm cryopreservation protocols on a species level. This is particularly apparent in light of the fact that 44% of anurans are currently listed as vulnerable, endangered, or critically endangered (IUCN, 2018), and could potentially benefit from assisted reproductive technologies in the near future. Therefore, it is imperative to develop and assess methods, such as those presented in our study, that can be applied more widely within anurans (Comizzoli et al., 2012). Yet, until now, it was unclear whether methods for cryopreservation of hormonally induced sperm could be transferred across species within the same genus, family, or order or if methods from model species can be successfully applied to threatened or endangered species. Our data help to elucidate these questions and grow our collective understanding of the applicability of generalized anuran sperm cryopreservation methods on a broader scale.

An unexpected finding from our study was that the endangered species had a significantly lower initial (fresh) sperm quality compared to the congeneric, nonthreatened species. One possibility is that the lower sperm quality observed was a result of sampling from captive-bred individuals (e.g., Locatello et al., 2018; Morato et al., 2001; Zupa et al., 2017). However, a previous study showed that sperm quality is not significantly different between captive and wild Wyoming toads (Poo et al., 2018). Therefore, although

TABLE 2 Quality of fresh sperm samples and recovery rate after cryopreservation under three cryoprotectant treatments

	Nonthreatened	Endangered	Bufonid	Ranid
Fresh sperm				
Motility (%)	82.649 (SE 1.885)	65.815 (SE 3.737)	72.676 (SE 3.356)	78.800 (SE 2.565)
Forward progressive motility (%)	71.162 (SE 2.428)	31.556 (SE 5.307)	62.265 (SE 4.020)	45.600 (SE 5.828)
Concentration (10^6 cells/mL)	11.384 (SE 2.010)	4.751 (SE 1.663)	4.353 (SE 0.673)	13.383 (SE 2.670)
Recovery rate				
Motility (%)				
DT 5-10	42.628 (SE 3.864)	28.631 (SE 6.285)	26.581 (SE 4.042)	51.230 (SE 4.862)
DT 10-10	3.866 (SE 0.771)	6.942 (SE 1.498)	6.725 (SE 1.063)	2.849 (SE 0.939)
DT 15-10	0.365 (SE 0.144)	3.438 (SE 1.735)	2.326 (SE 1.198)	0.519 (SE 0.198)
Forward progressive motility (%)				
DT 5-10	31.103 (SE 3.951)	34.368 (SE 8.604)	18.651 (SE 4.231)	50.275 (SE 6.023)
DT 10-10	0.950 (SE 0.403)	5.308 (SE 1.898)	2.758 (SE 1.074)	2.441 (SE 1.247)
DT 15-10	0.000 (SE 0.000)	4.458 (SE 4.344)	3.016 (SE 2.940)	0.000 (SE 0.000)

Note: Numbers represent mean and standard error.

the effects of captivity cannot be discarded without nutritional and genetic analyses, it is unlikely to be the main driver of reduced sperm quality. Instead, the most likely reason for our findings is a higher degree of inbreeding in the endangered species. Inbreeding has been linked with a reduction in sperm quality in a number of endangered species. For instance, a review of 20 mammal species indicated that inbreeding is associated with lower ejaculate quality in endangered mammal species (Fitzpatrick & Evans, 2009). Similarly, inbreeding results in lower sperm quality in the endangered Iberian lynx (*Lynx pardinus* Ruiz-Lopez et al., 2012) and three species of gazelles (Roldan et al., 2006). Coincidentally, the latter study also showed lower sperm quality affects semen cryopreservation success in the gazelle species (Roldan et al., 2006). As with many at-risk species, both the Wyoming toad and the dusky gopher frog have extremely reduced population sizes with evidence of population bottlenecks (Lewis, Baxter, Johnson, & Stone, 1985; Richter, Crother, & Broughton, 2009). A number of studies have documented a decrease in genetic variability and an increase in inbreeding in both species (Hinkson & Richter, 2016; Martin, 2010; Richter et al., 2009). Regardless of the exact cause of lower sperm quality in these two endangered species, the fact that both species do exhibit lowered reproductive health is cause for concern, as initial sperm quality can influence the subsequent ability to endure stresses associated with freezing and thawing. Therefore, the species in the most need of cryobanking and assisted reproductive technologies may also be the ones that face the most challenges when these tools are applied.

In many ways, conservation cryobiology is still a young and rapidly developing field. As such, research efforts are urgently needed in a number of different areas, from molecular biology and physiology, to organismal and conservation biology. One particular area worth highlighting for future efforts is research examining the fitness of individuals developed from cryopreserved gametes compared to their naturally bred counterparts. Since the goal of developing cryopreservation as a conservation tool is to produce individuals using cryopreserved gametes, it is imperative to assess variables that influence the overall fitness of these individuals, including their survival, development, behavior, and reproductive output (Mendelson III. & Altig, 2016). These studies are few and far between, with only a few examples found in aquaculture. For instance, studies have found little difference in the development and survivorship between fry produced from cryopreserved sperm and from fresh sperm (duration of observations = 35–112 days, *Brycon insignis* Viveiros, Isau, Caneppele, & Leal, 2012; *Puntius sarana* Akter, Hassan, Nahiduzzaman, & Hossain, 2016; *Mastacembelus armatus* Rahman, Ali, Sarder, Mollah, & Khan, 2016). Unfortunately, there is a paucity of such studies in other taxa and in long-term monitoring of individuals past early stages of development. Given the relative ease of maintaining and monitoring large numbers of amphibians, research that builds on the findings of the current study would be particularly valuable in adding to our knowledge of the influence of gamete cryopreservation methods in wildlife conservation.

As species continue to exhibit unprecedented rates of global decline, novel conservation methods will become increasingly important in protecting the remaining genetic and species diversity. Within the field of *ex situ* conservation, germplasm cryopreservation has been repeatedly

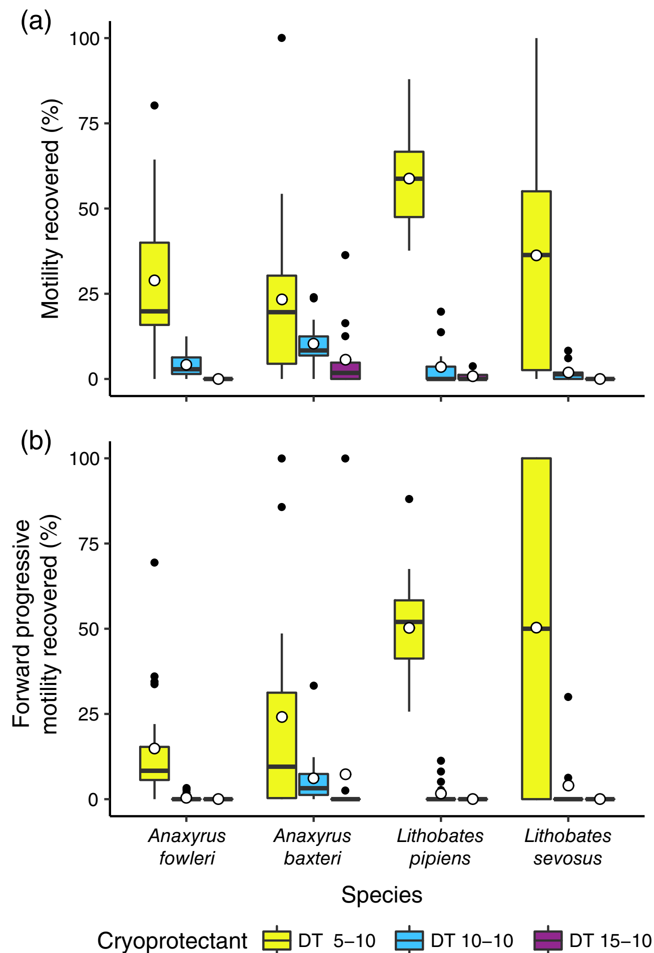

FIGURE 2 Boxplot of recovery rate after cryopreservation for (a) sperm motility and (b) forward progressive motility in two common, nonthreatened (*Anaxyrus fowleri* and *Lithobates pipiens*) and two endangered species (*Anaxyrus baxteri* and *Lithobates sevosus*) of amphibians. Lower and upper hinges of box plot correspond to the first and third quartiles, horizontal line denotes median, white dot denotes mean, and black dots denote outlying points

suggested as a new and important tool to for species conservation (Ballou, 1992; Jewgenow et al., 2017; Martinez-Paramo et al., 2017). However, the application of cryopreservation methods on a larger scale has been historically limited by a lack of comparative studies and reproducibility between studies (Martinez-Paramo et al., 2017; Torres & Tiersch, 2018). Our study provides empirical evidence from a direct comparison showing that, within anurans, standard methods can in fact be applied across families and can be transferred from nonthreatened to endangered species. Additionally, sperm preserved through these methods are capable of producing fertilized embryos and hatchlings. These findings form a basis for further explorations in a number of areas, such as optimizing sperm cryopreservation methods and assessing the fitness of offspring produced using cryopreserved gametes. Collectively, it is our hope that these multidisciplinary studies will contribute to making sperm cryopreservation an accessible tool that can be applied more widely and effectively in wildlife conservation.

TABLE 3 Tukey's honest significant difference tests comparing the effects of cryopreservation treatments on the recovery rate of sperm quality after cryopreservation

	Cryoprotectant treatment comparisons		
	DT 5-10: DT 10-10	DT 5-10: DT 15-10	DT 10-10: DT 15-10
Recovery rate			
Motility	<.001	<.001	.075
Forward progressive motility	<.001	<.001	.901

Note: Numbers represent *p* values.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

S.P. and K.M.H. conceived and collected data for the study. S.P. carried out data analyses and wrote the manuscript, with significant contributions from K.M.H.

DATA ACCESSIBILITY STATEMENT

All data from this research is openly available from Harvard Dataverse at https://dataverse.harvard.edu/dataverse/harvard.

ETHICS STATEMENT

All animal procedures in this study were approved by the Memphis Zoo Animal Care and Use Committee and all necessary state or federal agencies.

ARTICLE IMPACT STATEMENT

Sperm cryopreservation methods can be applied across amphibian families and transferred to at-risk species for wildlife conservation.

ORCID

Sinlan Poo https://orcid.org/0000-0002-7767-8173

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