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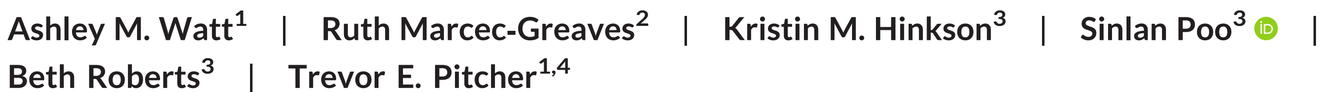
Effects of age on sperm quality metrics in endangered Mississippi gopher frogs (*Lithobates sevosus*) from captive populations used for controlled propagation and reintroduction efforts



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Abstract

A decline in sperm quality with age is a common prediction of senescence-based hypotheses and empirical studies. While widely studied across taxa, there is little known on the effect of ageing on sperm quality in amphibians, especially in captive populations used for controlled propagation and reintroduction efforts. Here, we investigated variation in sperm quality metrics (i.e., motility, concentration, and morphology) in the endangered Mississippi gopher frog (*Lithobates sevosus*) among males of three age categories using individuals from captive breeding populations housed at three different zoological institutions. Different aged males across the species expectant lifespan (1–9, 1–2, 3–4, and 8–9-year-old subcategories) were chosen in an attempt to identify an optimal breeding age relevant for captive breeding programs. Moreover, we explored and statistically controlled for potential differences in sperm quality which may be attributed to the type of induction hormones and source populations that differed among institutions. Results indicated that males of different ages did not differ in sperm motility or concentration. However, we did find that older males (8–9 years old) had significantly longer sperm than other age categories and younger males (1–2 years old) had significantly more atypical sperm than other age categories. Furthermore, we found no significant differences in any sperm quality metrics between the different induction hormones or source populations used at the different institutions. Within a captive breeding program, this information is especially valuable as our results indicate that males that have only recently sexually matured may not be ready to breed, while older males maintain sperm quality metrics presumably related to fertilization success.

KEYWORDS

anuran, captive breeding, fertilization success, induction, motility

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1 | INTRODUCTION

In response to the amphibian extinction crisis, institutions globally have established captive breeding programs to serve and assist conservation efforts for species at risk (Carrillo et al., 2015; Mendelson et al., 2006). Despite considerable investment, reproductive management remains a main concern for these programs, as low fertilization rates often occur (Kouba et al., 2009; Silla & Byrne, 2019). One of the limiting factors that leads to low fertilization rates is the quality and quantity of sperm that is produced by males in captivity. Sperm quality metrics include those involved in the proper functioning of cellular systems to achieve fertilization success and are typically measured via sperm motility, forward progressive motility, concentration, and morphology (Della Togna et al., 2020). In captivity, however, the quality of sperm is highly variable and can be influenced by a suite of factors including source population origin and reproductive technologies (Browne et al., 2015; Poole & Grow, 2012). For this reason, while a better understanding of sperm quality has gained attention, the production of high-quality sperm remains an issue for captive breeding programs despite the significant efforts made (Ananjeva et al., 2017; Silla & Byrne, 2019).

One explanation for poor sperm quality may be attributed to an animal's biological age. Ageing, or senescence, is defined as the decline in performance and function with age (Saino et al., 2002), which can be expressed as a decline in sperm quality in later years (e.g., Gasparini et al., 2010). Across taxa, data on the effect of age on sperm quality are inconsistent, with some studies reporting a decline in sperm quality with age (e.g., Striped Bass [*Morone saxatillis*], Vuthiphandchai & Zohar, 1999; Brown Norway rat [*Rattus norvegicus*], Syntin & Robaire, 2001; Asian elephant [*Elephas maximus*], Thongtip et al., 2008), while others report no decline or show an increase in sperm quality with age (e.g., White shrimp [*Litopenaeus vannamei*], Ceballos-Vázquez et al., 2003; Bluegill [*Lepomis macrochirus*], Casselman & Montgomerie, 2004; Domestic dog [*Canis lupus familiaris*], Rijsselaere et al., 2007; Barn swallows [*Hirundo rustica*], Møller et al., 2009; Guppy [*Poecillia reticulata*], Gasparini et al., 2010; Zebrafish [*Danio rerio*], Kanuga et al., 2011). For example, Syntin and Robaire (2001) found that male age has a significant effect on sperm motility and velocity in the Brown Norway rat (*Rattus norvegicus*), with both metrics steadily declining with age. In contrast, a study in the domestic dog (*Canis lupus familiaris*) found no difference in sperm quality between males of different ages (Rijsselaere et al., 2007). To date, only a handful of studies have examined ageing with respect to sperm quality in amphibians (see Easley et al., 1979; Hettyey et al., 2012; Roth et al., 2010), in which, findings are variable. In captive breeding programs, age-associated challenges might arise as older amphibians may be more prevalent due to the complexity of acquiring new animals from declining wild source populations (Snyder et al., 1996). As such, these programs may be limited by the biological age of the animals available to breed, which may pose a cause for concern for the viability of captive breeding programs.

Apart from age, sperm quality may also be influenced by a variety of other factors including the source of the population used (e.g., wild-caught or captive-bred), which can affect their reproductive fitness, and the method of sperm induction (e.g., induction hormones), which often differ amongst captive breeding facilities. Acquiring individuals from reliable source populations with consideration of genetic diversity is an important first step in reproductive management of captive populations as it is often the case that inbreeding can reduce the reproductive capacity of individuals due to the expression of inbreeding depression (Poole & Grow, 2012; Schulte-Hostedde & Mastromonaco, 2015). Due to small population sizes and isolation, many imperilled species with captive breeding programs often exhibit reduced genetic variation as a consequence of inbreeding between close relatives (reviewed in Allentoft & O'Brien, 2010; Fitzpatrick & Evans, 2009). Consequently, inbreeding can lead to inbreeding depression and result in a reduction in reproductive capacity; a negative relationship between sperm quality metrics (including sperm motility and morphology) and inbreeding has often been observed across and within taxa in wild and in captive populations (Asa et al., 2007; Fitzpatrick & Evans, 2009; Gage et al., 2006; Hinkson & Poo, 2020; Opatová et al., 2016; Zajitschek & Brooks, 2010). Furthermore, natural breeding events in captivity may be hindered due to reproductive dysfunction—an impairment of an organism's reproductive system (Synder et al., 1996), which is thought to arise in captivity from a mismatch of environmental cues, behavioral incompatibility, or inbreeding (reviewed in Schulte-Hostedde & Mastromonaco, 2015). To mitigate these challenges, the use of reproductive technologies has been implemented in captive breeding protocols to stimulate gametogenesis. However, difficulties often arise as the efficacy of induction hormones tend to be species-specific and sperm quality has been shown to vary across different types of hormones protocols (Della Togna et al., 2017; Hinkson et al., 2019; Kouba & Vance, 2009; Kouba et al., 2009, 2012b; Watt et al., 2019).

In this study, we examined sperm quality as it relates to age in Mississippi gopher frogs (*Lithobates sevosus*), a species that often experiences low reproductive success in captivity (Poole & Grow, 2012). As a consequence of habitat destruction, the Mississippi gopher frog is endangered in the United States and exists as one population (with an estimated population size ranging from 100 to 200 individuals) in the DeSoto National Forest, Mississippi (Hammerson et al., 2004; Lannoo, 2005; USFWS, 2012). This remnant wild population exhibits inbreeding levels that would arise via full sibling mating's (Hinkson & Richter, 2016). Due to their small population size, captive populations were established at zoological institutions which focus on captive breeding and reintroduction of this species. Breeding individuals in these captive breeding programs are either sourced directly as juveniles from the wild or they are individuals born in captivity. In captivity, the Mississippi gopher frog has rarely been observed to breed naturally and requires assisted reproductive technologies (i.e., exogenous hormones and in vitro fertilization) for breeding success (Kouba et al., 2012a; Watt et al., 2019).

Given the Mississippi gopher frog's low reproductive success in captivity as well as in the wild (Richter et al., 2003), it would benefit captive breeding programs to investigate potential causes of poor sperm quality. The effects of age, induction hormones and source population of breeding individuals may act independently or together and thus have the potential to influence sperm quality. In light of this critical knowledge gap, targeted research efforts are needed to better understand underlying factors which influence the quality of sperm zoos can collect for captive breeding. In this study, our primary focus was to examine the effects of age on sperm quality and quantity metrics (i.e., motility, concentration, and morphology). The results of this study could have a direct application on improving sperm quality in captive breeding programs and may ultimately improve fertilization success for an endangered species to produce more offspring for reintroduction efforts.

2 | METHODS

2.1 | Age and captive breeding source

A total of 30 male Mississippi gopher frogs ranging in age from 1 to 9 years from three different institutions (Dallas Zoo, Detroit Zoo, and Memphis Zoo) with captive breeding programs were used in this study (see Table 1). Age categories (1–2, 3–4, and 8–9 years old) were determined according to life history information available for the Mississippi gopher frog. At 1–2 years of age, male gopher frogs will have likely undergone their first reproductive event (Richter & Seigel, 2002). Ages 3–4 are middle-aged, and at 8–9 years old, males are at the latter end of their natural life expectancy, which is estimated at 7 years old in the wild (Richter & Seigel, 2002). Intermittent reproductive events in captivity produced gaps in these age classes, which prevented examination of males aged 5–6 years old. Males also varied in terms of where they were sourced; some were "wild-caught" individuals, collected as tadpoles from Glen's pond in the DeSoto National Forest (Harrison County, MS, USA) and subsequently captive-reared, or some were sourced as "captive-bred", the first generation of frogs produced successfully in captivity. All males were housed in standard plastic polycarbonate tanks or glass tanks fitted with sliding lids. Enclosures were fitted with cover, aged water, and a moss substrate. Tanks were cleaned once per week, though fresh moss and aged water were added as needed throughout the week. Data were collected in accordance with the Animal Care and Ethics Certificate Provided by the University of Windsor (AUPP #18-12).

2.2 | Induction and spermic urine collection

To induce spermiation, males at the Dallas Zoo and the Memphis Zoo were administered 10 IU/g body weight of hCG (cat#: C1063, human chorionic gonadotropin; Sigma-Aldrich) and 0.4 μg/g body weight of GnRH (cat#: L4513, des-Gly^{10}, D-Ala^{6}; Sigma-Aldrich). Males at the Detroit Zoo were given a single dose of 0.5 μg/g body weight of GnRH. All hormones were administered by intraperitoneal injection (IP; Poole & Grow, 2012). IP injections were administered as previous studies have shown IP injections produce increased levels of sperm compared to animals receiving ventral/dorsal absorption or subcutaneous injections (Obringer et al., 2000; Rowson et al., 2001). Immediately following injection, males were placed into separate holding containers containing aged water to cover the bottom of the container. This allowed males to replenish their bladders between collection times. To capture peak sperm production, spermic urine was collected from each male at 1-h posthormone injection (Kouba et al., 2012a; Watt et al., 2019). To collect spermic urine, the posterior end of each male was patted dry using a paper towel to prevent excess water from diluting the sample. Males were then held over a wide petri dish (100 × 15 mm) and a piece of catheter tubing (cat#: BB31785-V/5; Scientific Commodities Inc.) was inserted into the cloaca of each male drawing spermic urine into the petri dish.

TABLE 1 Mean (±*SE*) snout-vent length (mm), mass (g), age categories (years), source population (wild-origin or captive-origin), and induction hormones used to collect sperm of male Mississippi gopher frogs (*Lithobates sevosus*) across three institutions within their respective captive breeding programs

	Dallas Zoo	Detroit Zoo	Memphis Zoo
Snout-vent length (mm)	63.48 ± 1.60	66.33 ± 2.22	69 ± 1.97
Mass (g)	34.44 ± 2.05	37.82 ± 4.17	38 ± 3.87
Age categories (years)	*N* = 15; 1–2	*N* = 4; 1–2 *N* = 3; 3–4 *N* = 4; 8–9	N = 3; 1–2 N = 1; 8–9
Source population origin	Wild-origin (*N* = 15)	Wild-origin (*N* = 7) Captive-origin (*N* = 4)	Wild-origin (*N* = 3) Captive-origin (*N* = 1)
Human chorionic gonadotrophin (hCG)	10 IU/g body weight	N/A	10 IU/g body weight
Luteinizing hormone releasing hormone (LHRHa)	0.4 μg/g body weight	0.5 μg/g body weight	0.4 μg/g body weight

Immediately following urination, the sample was pipetted into a 1.5 ml Eppendorf tube (Thermo Fisher Scientific) and spermic urine volume (µl) was recorded using an adjustable-volume pipette. Samples were placed in a chilling block (cat#: IC22; Torrey Pines Scientific) set at 4°C until analysis. All spermic urine samples were analyzed within a 5-min period using a blind observer approach.

2.3 | Sperm motility

Sperm samples were analyzed using a microscope (CX41 Olympus), equipped with a 10× negative-phase objective (Byrne et al., 2015; Watt et al., 2019). For each male, 2 µl of spermic urine was pipetted onto a 2X-CEL glass slide (Hamilton Throne Biosciences) and covered with a glass coverslip (22 × 22 mm). Percent motility was measured using a generalized progressive motility scale (Kouba et al., 2012b; Watt et al., 2019). A total of 100 sperm cells were counted and the number of sperm that were motile (sperm exhibiting forward movement), twitching (sperm with flagellar or side to side head movement, but not forward movement), and no movement (sperm with nonmoving flagella and no head movement) were recorded. Total motility was calculated by adding the number of sperm that were motile and twitching together.

2.4 | Sperm concentration

Sperm concentration was evaluated by counting the number of sperm cells in each of the four large corner squares (64 smaller squares; 1 mm^2) using a Neubauer hemocytometer under ×400 magnification (Kouba et al., 2012b; Watt et al., 2019). The average number of spermatozoa present on both sides of the hemocytometer was multiplied by the dilution factor and then multiplied by 2500 (the standard conversion factor). Sperm concentration was calculated as the total number of spermatozoa per ml of spermic urine.

2.5 | Sperm morphology

For sperm morphology measurements, an aliquot of 10 µl was removed from the spermic urine samples and fixed with 10 µl of 8% glutaraldehyde (cat#: G7526-10Ml; Sigm-Adrich). Each sample was stored in an Eppendorf tube and was gently pipetted up and down ten times using a wide bore tip to mix the sperm with the fixative and to ensure the sperms' flagella remained intact. Fixed samples were stored in the refrigerator (~4°C) and were stained within 1-month following fixation. To prepare for staining, sperm samples were gently pipetted ten times using a wide bore tip to ensure proper mixing of sperm that may have settled at the bottom. From each sample, 5 µl was pipetted onto a glass microscope slide (25 × 75 × 1 mm; cat#: 1301; Globe Scientific Inc.) and was evenly smeared across the surface of a slide. Smeared slides were placed onto a slide warmer (cat#: 3377038; Lab-Line) and left to dry for 1 h. Once dry, slides were removed and stained using a Shandon Kwik-Diff Stain Kit (cat#: 9990700; Thermo Fisher Scientific). This procedure involved immersing each slide into three different reagents (1) fixative, (2) eosin, (3) methylene blue to stain sperm cells. Slides were then placed onto the slide warmer and left for 2 h until dry. Sperm morphology was analyzed at ×400 using an Olympus BX51 microscope fitted with an Olympus DP72 camera. Sperm were measured for head length (µm), flagella length (µm), and total sperm length (µm) using imaging software provided with the Olympus DP2-BSW imaging software. Head length (including the midpiece) was measured from the apex of the sperm head to the junction of the flagellum across the midline and flagellum length was measured from the same junction of the sperm head to the end of the terminal filament (Byrne et al., 2003). Sperm total length was recorded as the sum of the head length and flagellum length. Twenty sperm per male were measured based on a diminishing returns curve of accuracy (see Hosken & Blanckenhorn, 1999) for sperm morphology for this species, which showed that approximately twenty spermatozoa gave ample accuracy (Watt & Pitcher, unpublished data).

Sperm collected (see above for sperm fixation and analysis method) from a subsample of Mississippi gopher frogs (n = 10; spanning all age categories) housed at the National Amphibian Conservation Center (Detroit Zoo, Royal Oak, MI, US) were evaluated for potential differences in additional sperm morphological metrics potentially associated with sexual maturation and ageing. Sperm were classified as atypical if the head was bent or broken and/or the flagellum was coiled, broken, or had a cytoplasmic droplet present (Della Togna et al., 2017; Della Togna et al., 2018). Our definition allowed sperm to be classified as atypical with the understanding that older males may exhibit reproductive senescence or younger males may be experiencing reproductive immaturity (Richter & Seigel, 2002). In addition, the use of exogenous hormones may result in the expulsion of a range of sperm with different developmental stages (e.g., immature to fully mature; Wilson et al., 1998). Slides were examined by scanning the entire smear and only sperm positioned one millimeter from the slide's edge were analyzed to avoid confounding edge effects (Byrne et al., 2003). A maximum of 100 (mean = 94; range: 40–100) spermatozoa per male were classified if all morphological parameters (e.g., head, midpiece and flagellum; N = 940) were present (Della Togna et al., 2018).

2.6 | Statistical analyses

All data were analyzed using RStudio, a development environment for R programming language (Version 1.1.463; packages; car, multcompView, lsmeans, rcompanion; RStudio Team, 2015). Assumptions for normality were tested before data analyses using a Shapiro–Wilk test and data that were not distributed normally were log-transformed to meet the assumption. Next, to account for the unbalanced sample size across ages and institutions (see Table 1) we used a two-way ordinal analysis of variance (Akritas et al., 1997) to examine the main effects and interactions for each factor in relation

to sperm motility (%), concentration (10^6 cells/ml), morphology (μm). We considered age and source population origin and then age and induction hormone protocol as main factors in our analysis as well as their respective interactive effects. Then, we used a one-way ordinal analysis of variance to examine the main effect of age in relationship to atypical sperm (%) to account for the subsample of gopher frogs which were housed at one institution (Detroit Zoo) only. Tukey post hoc analyses were used to determine where significant differences occurred amongst categories. The data that support the findings of this study are available from the corresponding author upon reasonable request.

3 | RESULTS

Our first set of models examined age and source population origin as main factors in relation to sperm related metrics (as well as their interaction terms). We found that the age category factor, source population origin factors and their respective interaction terms were not significant as they related to sperm motility or concentration (see Tables 2 and 3). We found that total sperm length did not vary with source population origin but did vary significantly with age, (Figure 1; Tables 2 and 3); the oldest males (8–9 years old) having significantly longer sperm than males 1–2 years old ($p < .002$), but not males 3–4 years old ($p = .46$). Likewise, sperm head length and flagellum length were not significantly different among source population origin but both sperm head length and flagellum length were significantly related to age, as males in the 8–9-year-old age category also exhibited significantly longer sperm heads ($p < .01$) and flagellums ($p < .006$) than males 1–2 years old, but not males 3–4 years old (head: $p = .37$; flagellum: $p = .67$; see Tables 2 and 3). There was no significant difference in sperm head length ($p = .99$) or flagellum length ($p = .78$) between males 1–2 years old and males 3–4 years old. We did not find any significant interaction effects between age and source population origin in sperm total length, head length, or flagellum length.

Our second set of models examined age and induction hormone protocol as main factors in relation to sperm related metrics (as well as their interaction terms). Similar to our first set of models (above), we found the age category factor, induction hormone factors and their respective interaction terms were not significant as they related to sperm motility or concentration (see Tables 2 and 3). We found that total sperm length did not vary with induction hormone protocol but did vary significantly with age, (Figure 1; Tables 2 and 3); the oldest males (8–9 years old) having significantly longer sperm than males 1–2 years old ($p < .02$), but not males 3–4 years old ($p = .11$). Sperm head length and flagellum length were not significantly different among induction hormone protocol but both sperm head length and flagellum length were significantly related to age, as males in the 8–9 years old age category also exhibited significantly longer sperm heads ($p < .04$) and flagellums ($p < .049$) than males 1–2 years old, but not males 3–4 years old (head: $p = .09$; flagellum: $p = .29$; see Tables 2 and 3). There was no significant difference in sperm head length ($p = .96$) or flagellum length ($p = .90$) between males 1–2 years old and 3–4 years old. We did not find any significant interaction effects between age and induction hormone protocol in sperm total length, head length or flagellum length. In both models, we found that age was a significant factor for morphology, regardless of source population origin or hormone induction protocol.

For the subset of males ($n = 10$) from the Detroit Zoo there was a significant relationship between male age and the proportion of atypical sperm present in a sample ($F = 7.85$; $df = 2$; $p = .02$). Males aged 1–2 years old (59.8 ± 2.9%) had significantly more atypical sperm than males 3–4 years old (49.5 ± 1.5%; $p = .04$), and males 8–9 years old (50.0 ± 4.0%; $p = .04$). There was no significant difference found between males 3–4 years old and males 8–9 years old ($p = .99$).

4 | DISCUSSION

In this study we examined different sperm quality metrics between three different age categories of gopher frogs from three captive breeding zoo populations to better understand the potential deleterious effects of senescence on reproduction. We also examined the potential effects of differences in induction hormone protocols and source population origins of frogs among the various zoos and any

TABLE 2 Mean (±*SE*) sperm motility, concentration, sperm total length, sperm head length, and sperm flagellum length across male age categories (1–2, 3–4, and 8–9 years old), source population origins (wild-caught or captive-bred), and induction hormone protocols (hCG + GnRH or GnRH)

	Age category			Source population origin		Induction hormone protocol	
	1–2	3–4	8–9	Wild-caught	Captive-bred	hCG + GnRH	GnRH
Sperm metric	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*
Motility (%)	36.4 ± 6.8	62.3 ± 7.2	43.6 ± 16.2	36.2 ± 6.2	66.0 ± 6.3	35.6 ± 6.8	48.0 ± 9.9
Concentration (×10^6 cells/ml)	0.58 ± 0.2	1.25 ± 1.2	1.88 ± 0.9	0.72 ± 0.2	1.81 ± 1.0	0.58 ± 0.2	1.37 ± 0.5
Total length (μm)	51.1 ± 3.4	63.7 ± 9.6	91.8 ± 6.3	57.2 ± 4.2	71.2 ± 9.1	50.6 ± 4.3	73.8 ± 5.3
Head length (μm)	21.9 ± 1.6	24.7 ± 5.8	36.8 ± 2.1	24.2 ± 1.7	28.1 ± 5.3	21.8 ± 1.8	29.2 ± 2.6
Flagellum length (μm)	29.1 ± 2.3	39.0 ± 3.8	54.9 ± 6.9	33.1 ± 2.6	43.1 ± 4.9	28.8 ± 2.8	44.1 ± 3.2

TABLE 3 Results for two-way ordinal analyses of variance (*F* value and *p* value, see Section 2 for details) that examined (i) age category (1–2, 3–4, and 8–9 years old; *n* = 30 males total) and source population origin (captive-born or wild-origin) and (ii) age category and induction hormone protocol (hCG + GnRH or GnRH only) as main factors, (including their respective interactions) in relation to sperm motility, concentration, sperm total length, head length and flagellum length

Sperm metric	Age category		Source population origin		Age category × source population origin		Age		Induction hormone protocol		Age category × induction hormone protocol	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Motility (%)	0.15	.87	0.40	.54	0.39	.53	0.47	.63	0.12	.73	0.75	.39
Concentration (×10^6 cells/ml)	1.63	.21	1.87	.18	1.87	.18	0.73	.49	0.37	.55	3.42	.07
Total length (μm)	7.85	**.002**	0.01	.91	0.01	.91	4.64	**.02**	3.34	.08	1.07	.31
Head length (μm)	5.99	**.007**	0.02	.89	0.02	.88	3.81	**.03**	1.61	.22	0.62	.44
Flagellum length (μm)	6.14	**.006**	0.01	.92	0.01	.91	3.26	**.05**	2.89	.11	0.92	.35

Note: p Values that are equal to or less than .05 are reported in bold.

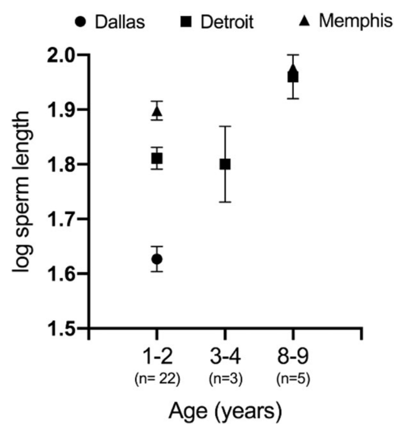

FIGURE 1 Log-transformed means (±1 *SE*) for sperm total length (μm) across males of different age categories (years) and institutions (Dallas Zoo, Detroit Zoo, Memphis Zoo) in the Mississippi gopher frog (*Lithobates sevosus*)

interaction effect between age and these factors to broadly explore potential variation in sperm quality. Male age had no significant effect on most sperm quality metrics except for morphology. Induction hormones and source populations did not have any significant effect on any of the sperm quality metrics. Moreover, there was no significant interaction effects (between age and induction hormones and/or source populations) found for any of the sperm quality metrics examined.

There was no significant variation in most of the sperm quality metrics (i.e., motility, concentration) between males across young and old age categories. This finding was contrary to expectations as senescence theory predicts an age-associated decline in the quality of sperm (Saino et al., 2002). Though, it is possible that the "soft" captive setting (i.e., males are provided an ample diet and ideal temperature and humidity with no predators) masked the relationship between male age and sperm traits because of high condition factors (or lack of life history trade-offs) allowing all males to devote sufficient energy to gametogenesis (Tidière et al., 2016). This finding is critical for zoological institutions as there is often little choice but to use older males for breeding as acquiring new wild stock is rarely possible. However, as fertilization success would not likely be impacted by using sperm from older males, our measure of sperm quality does not include factors, such as DNA and mitochondrial quality, which are important for cellular processing (Della Togna et al., 2020). Furthermore, any downstream consequences on offspring quality due to epigenetic effects as found in male mice (*Mus musculus*; Smith et al., 2009) remains under studied (Curley et al., 2011).

We found that sperm morphology size metrics were positively related to age. Our study found that as males age their sperm elongate. Males 8–9 years old had approximately 11% longer sperm than males aged 3–4 years old, and approximately 19% longer sperm than males aged 1–2 years old. Growth in amphibians is indeterminant (Lardner & Loman, 2003), thus, as males age their body size should increase as well as the absolute size of their testes (Liu et al., 2012). One explanation for our results may be that longer sperm may occur with age because larger testes can produce and store longer sperm (Byrne et al., 2003; Zeng et al., 2014). Zeng et al. (2014) found a consistent positive correlation between sperm length and relative testis size across 67 species of anurans. At a proximate level, older males in good condition may invest more into testis development, likely due to past reproductive experience (Byrne et al., 2003). However, theoretical models suggest that investment into longer spermatozoa can occur at the cost of sperm quantity (see Parker, 1993). Interestingly, in our study, we did not find any trade-off in quantity and quality metrics, instead we found a positive correlation between total sperm length and sperm concentration (Pearson correlation, $r = .58$; $p < .001$) in a post hoc analysis we conducted. However, the mechanism and potential adaptive significance of greater sperm length remains unclear for our focal species. In amphibian mating systems with competitive fertilization, sperm flagellum length has often been associated with greater velocity and is often used as an indicator of competitive fertilization

success (Byrne et al., 2003; Humphries et al., 2008; Simpson et al., 2014; but see Dziminski et al., 2009). However, there is little information on sperm form and function in amphibians which possess noncompetitive mating systems (as is the case in our focal species, where they form amplectic pairings). We also found that the percentage of atypical sperm a male produced was negatively related to age. Our study found that younger males (1–2 years old) had a significantly higher percentage of atypical sperm present in their ejaculate. Males 1–2 years old had approximately approximately 21% more atypical sperm than males aged 3–4 years old, and approximately approximately 20% more atypical sperm than males aged 8–9 years old. This result likely arises because sperm were not fully developed in younger frogs, who were at near the age of first reproduction in this species (Richter & Seigel, 2002; Roth et al., 2010). Moreover, across all age categories, the percentage of atypical sperm was found to be very high. A recent paper by Hinkson and Poo (2020) explored the effect of inbreeding on sperm quality in Mississippi gopher frogs and found sperm quality (forward progressive motility, total motility, concentration and viability) were significantly reduced compared to an outbred sister species, the northern leopard frog (*Lithobates pipiens*). The potential reproductive consequences of inbreeding depression found in this study may explain the overall high percentage of atypical sperm across all age categories.

Although our findings show clear differences in sperm morphology metrics between age categories, we wanted to further explore the possible role differences in induction hormone protocols and source population origins of frogs may play in affecting these sperm quality metrics. In captivity, the Mississippi gopher frog has rarely been observed to breed naturally and, therefore, requires exogenous hormones to induce spermiation (Poole & Grow, 2012). Males at the Dallas and Memphis Zoo were administered a hormone cocktail of GnRH and hCG, while due to logistical constraints Detroit Zoo males received GnRH only. Differences in sperm quality and quantity have been found to be influenced by the type of hormone administered (Hinkson et al., 2019; Kouba et al., 2009, 2012b; Silla & Roberts, 2012). For example, Kouba et al. (2011) showed that sperm concentration was highest in Mississippi gopher frogs that received a combination of GnRH and hCG compared to either hormone individually. Similarly, Kouba et al. (2009) found that spermic urine production in the northern leopard frog (*Lithobates pipiens*) occurred only when a cocktail of GnRH and hCG was administered and the combination produced higher sperm concentrations than either GnRH or hCG alone. Mechanistically, GnRH works directly at the level of the brain which stimulates the anterior pituitary to produce and release gonadotropin, which then stimulates the testes (Byrne & Silla, 2010). Sperm production may improve with both hormones as they effectively bind and target different areas of the reproductive hormone cascade (Clulow et al., 2014). Interestingly, our study showed found that both hormone treatments were equally effective at producing high concentrations of sperm. In addition to differences in the induction hormones between zoos, we also hypothesized that differences between source population origins (wild-caught vs. captive-born offspring) among zoos could have had an influence on sperm quality metrics. Although inbreeding coefficients were presumably high for all individuals examined, it is unlikely that significant differences exist in levels of inbreeding between wild-caught and captive-bred frogs as wild-caught individuals are regularly brought into captivity (Reichling & Sullivan, 2016). Additionally, a study by Hinkson et al. (2016) found that the original founders of the captive population were genetically representative of the population as a whole, with similar levels of relatedness and genetic diversity. Thus, while it is likely that all frogs used in this study were inbred to some degree, they are presumed to be genetically similar in terms of inbreeding levels across our different categories. To this end, we did not find a significant difference in any sperm quality metrics between wild-caught and captive-bred individuals.

Overall, this study demonstrated that sperm morphology changed with age, while sperm motility and concentration were not affected by the ageing process. Our findings suggest that younger males may be relatively poor to breed due to higher levels of atypical sperm, while older males (3–4 and 8–9 years old) retain their reproductive capacity. While senescence theory generally predicts a decline in sperm quality with age, we found no evidence that age reduces sperm quality or quantity in the Mississippi gopher frog. By providing a comprehensive overview of age on sperm quality metrics, our data is directly relevant for captive breeding programs that are attempting to maximize fertilization success and increases our understanding of the relationship between age and sperm quality in amphibians. Overall, this study can be used to address some of the potential factors (induction hormones and source populations) which may cause underlying variation in sperm quality and we encourage further investigation into those factors to aid programs working towards producing offspring for species recovery and reintroduction. Currently, amphibian populations worldwide are experiencing declines at unprecedented rates and captive breeding programs are challenged by low reproductive output due to poor gamete quality and fertilization rates (Browne et al., 2006). These results demonstrate the need for future research into the downstream relationship between age, fertilization success, and offspring fitness to determine if breeding older males poses a risk outside of sperm quality and what this may mean for reintroduction success.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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