Received: 7 November 2018 | Revised: 18 September 2019 | Accepted: 30 September 2019

DOI: 10.1002/zoo.21519

ZOOBIOLOGY WILEY

RESEARCH ARTICLE

Time from injection of luteinizing hormone-releasing hormone analog affects sperm quality in the critically endangered Mississippi gopher frog (*Lithobates sevosus*)

Ashley Michelle Watt[1] | Ruth Marcec-Greaves[2] | Trevor Edgar Pitcher[1,3]

[1]Department of Biological Sciences, University of Windsor, Windsor, Ontario, Canada

[2]National Amphibian Conservation Center, Detroit Zoological Society, Royal Oak, Michigan

[3]Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario, Canada

Correspondence
Ashley Michelle Watt, Department of Biological Sciences, University of Windsor, 401 Sunset Avenue, Windsor, ON N9B 3P4, Canada.
Email: watta@uwindsor.ca

Funding information
Natural Sciences and Engineering Research Council of Canada (NSERC), Grant/Award Number: Discovery and CREATE

Abstract

The objective of this study was to investigate variation in sperm quality metrics (motility, velocity, and concentration) in the critically endangered Mississippi gopher frog (*Lithobates sevosus*) over three sampling time points after a luteinizing hormone-releasing hormone analog (LHRHa) induction injection. Sperm was repeatedly collected from 11 individuals over three sampling times (30, 60, and 120 min) after injection. Variation in sperm quality was investigated using a repeated-measures mixed model approach. Repeated measures analyses of variance showed a significant effect of sampling time for percent motility and velocity. Concentration was found to be marginally related to sampling time, while progressive motility was not significantly related to time after injection. Our findings are important for optimizing assisted reproduction-related fertilization success and increasing the successful propagation of endangered species of imperiled frogs in captive breeding programs.

KEYWORDS

amphibian, captive breeding, exogenous hormones, induction, sperm quality

1 | INTRODUCTION

For half a century, exogenous hormones have been used in captive breeding programs to stimulate the production and release of spermatozoa in a variety of anurans (frogs and toads; Kouba, delBarco-Trillo, Vance, Milam, & Carr, 2012). Historically, pituitary extracts and homogenates were used to initiate spermiation; the process by which mature spermatids are released from the supporting somatic Sertoli cells into the lumen of the seminiferous tubule (O'Donnell, Nicholls, O'Brien, McLachlan, & Stanton, 2011). However, these techniques are now rarely used due to concerns over disease transmission and euthanasia of the donor animal (Byrne & Silla, 2010; Kouba, Vance, & Willis, 2009). Alternatively, the administration of synthetic hormones, such as luteinizing hormone-releasing hormone analog (LHRHa) and human chorionic gonadotropin (hCG) have been demonstrated as effective approaches for inducing spermiation (Goncharov, Shubravy, Serbinova, & Uteshev, 1989; Kouba, Vance, et al., 2012; Roth & Obringer, 2003).

Exogenous LHRHa acts at the level of the anuran brain to stimulate the anterior pituitary to produce and release gonadotropin (i.e., luteinizing hormone; LH), which stimulates the gonads (Byrne & Silla, 2010). In contrast, hCG has a luteinizing-hormone like bioactivity, which acts at the level of the gonads to initiate sperm production (Byrne & Silla, 2010; Kouba, Vance, et al., 2012). Evidence that LHRHa and hCG can induce spermiation has been documented in many anurans (reviewed in Kouba, Vance, et al., 2012). Trends, which characterize the timing of sperm release after a hormone injection have been described across genera, while peak sperm production tends to vary between species. In the genus *Anaxyrus*, sperm release typically occurs between 3 to 7 hours after hormone injection and can last up to 24 hr (Kouba et al., 2009). This trend has been observed for *Anaxyrus baxteri* (Browne, Seratt, Vance, & Kouba, 2006), *Anaxyrus fowleri* (Kouba & Vance, 2009), *Anaxyrus americanus* (Obringer et al., 2000), and *Anaxyrus boreas boreas* (Kouba & Vance, 2009). Studies in the genus *Lithobates* have demonstrated that sperm release occurs within 30 min following an exogenous hormone, while

peak sperm production can occur between 30-60 min (Kouba & Vance, 2009; Kouba, Vance, et al., 2012). For example, in *Lithobates pipens*, peak sperm concentration occurred at 30–60 min following a combined treatment of LHRHa and hCG (Kouba & Vance, 2009).

While hCG has proven to successfully induce spermiation for a broad diversity of anurans, LHRHa has grown in popularity and is preferred by zoos and aquariums for its ability to release the animals' own endogenous hormones (Kouba, Vance, et al., 2012). However, despite several reviews (e.g., Goncharov et al., 1989; Kouba & Vance, 2009; Kouba et al., 2009), there remains a general lack of knowledge regarding the relative efficacy of LHRHa across species (but see Goncharov et al., 1989). Furthermore, there is little information available on the potential variation in sperm quality in response to time after hormone administration as studies tend to focus solely on identifying peak sperm concentration. To date, only a handful of studies have fully characterized sperm quality in response to sampling time after hormone injection (Byrne & Silla, 2010; Della Togna et al., 2017; Obringer et al., 2000). For instance, in the critically endangered Panamanian golden frog (*Atelopus zeteki*) sperm concentration, percentage of motile cells, and morphology were found to vary significantly across sampling time and hormone dosage (Della Togna et al., 2017).

Sperm quality measures, including motility, velocity, and concentration are major determinants of fertilization success (Dziminski, Roberts, Beveridge, & Simmons, 2009; Johnson, Butts, Wilson, & Pitcher, 2013). In the spotted grass frog (*Limnodynastes tasmaniensis*) sperm concentration was positively related to fertilization success and had a significant effect on fertilization rate when sperm concentration was greater than 10^4 sperm/ml (Edwards, Mahony, & Clulow, 2004). Ideally, the evaluation of sperm concentration and other sperm quality metrics can be used to optimize captive breeding protocols, which have become critical to zoos experiencing high rates of reproductive failure (Obringer et al., 2000). By characterizing sperm quality in response to sampling time, specific information on when to collect sperm can be provided to zoos, which may achieve higher fertilization success for individual species. Ultimately, this information becomes especially valuable for the many endangered anurans being bred in captivity today.

The Mississippi gopher frog (MGF, *Lithobates sevosus*) was historically found along the southern Gulf Coastal Plain of Louisiana, Mississippi, and Alabama (Hammerson, Richter, Siegel, LaClaire, & Mann, 2004). By 2012, the MGF was listed as critically endangered and only two populations were known to exist in Harrison and Jackson Counties, Mississippi, with an estimated 100 individuals (Hammerson et al., 2004). Concerned for the future of the MGF, the United States Fish and Wildlife Service (USFWS) established partnerships with a variety of zoological institutions dedicated to the recovery of this species (Richter, Crother, & Broughton, 2009). Today, the MGF has a species survival plan (SSP), which is a program developed by the Association of Zoos and Aquariums that aims to ensure the survival of selected species in captivity (Association of Zoos and Aquariums, 2018; Conway, 2011). The SSP oversees the MGF recovery plan that is currently focused on captive breeding and the reintroduction of froglets into their historic range.

One of the SSP's main concerns for the future success of the MGF are the challenges faced in captive breeding. Captive populations of the MGF often experience high rates of reproductive dysfunction (e.g., Richter, Young, Johnson, & Seigel, 2003). The exact cause of reproductive dysfunction is unknown; though it is suspected to be due to an inability to replicate natural environmental stimuli that lead to a reproductive event (Kouba et al., 2009). In males, reproductive dysfunction can manifest as a lack of breeding behaviors (i.e., calling or amplexus), or an inability to produce sperm. To overcome reproductive dysfunction, exogenous hormones for assisted reproductions are often provided to induce a spermiation response (Poole & Grow, 2012). In light of these reproductive issues, targeted research efforts are needed to investigate hormone induction and sperm quality for the survival of this species.

In this study, we examined three time points following administration of LHRHa to evaluate sperm quality in the MGF. A study by Kuba, Vance, et al. (2012) previously described the timing of sperm release in the MGF through analysis of one sperm trait (i.e., concentration) in response to postinjection sampling time. Building off this study, only three time points were chosen to decrease the stress on the animals and to allow time for spermic urine to accumulate between collection times. The objectives of our study were (a) to thoroughly investigate sperm quality in response to sampling time after hormone injection using LHRHa and (b) provide a specific time point to collect the highest quality sperm. Here, we aim to provide valuable information on sperm quality to captive breeding programs that can be extrapolated to other species within the genus *Lithobates*.

2 | METHODS

A total of 14 male MGF mean ± SE snout-vent length (66.68 mm ± 2.05 mm; range: 52.7–75.6 mm), mass (37.66 g ± 3.81 g; range: 18–58 g), age (5.55 ± 3.59 years old; range: 1–9 years) housed at the National Amphibian Conservation Center (Royal Oak, MI) were used in this study. All animals were kept on a natural light cycle operated by a timer which turned on at 7 am and turned off at 8 pm daily. Housing conditions consisted of standard plastic polycarbonate tanks (560 mm L × 380 mm W × 355 mm H) fitted with sliding lids tilted at a 30° angle. Each lid was cut on the inside perimeter to allow light to penetrate the tank. Lighting was provided by EIKO track light bulbs that were modified with removed glass to allow UV to access each tank. Approximately half of each tank bottom was covered with shag moss and cork bark, and all tanks were fitted with either a plastic hide or a cork bark cave to provide refugia. The other half of each tank bottom was filled with 21°C aged water at an approximate depth of 76.2 mm to create a pond at the front of each tank. Aged water was considered to be water that sat for a 48-hr period to allow free chlorine (Cl_2) to dissipate as gas (Poole & Grow, 2012). Tanks were cleaned once per week, though fresh moss and aged water were provided as needed throughout the week. Adult Mississippi gopher frogs were provided prey items: gutload crickets (*Gryllidae*), Dubia roaches (*Blaptica dubia*), soldier flies (*Stratiomyidae*) twice a week. Mealworms (*Tenebrio molitor*)

and wax worms (*Pyralidae*) were provided 1 week prior to the start of the study and were not provided during the study. Both mealworms and wax worms were gut loaded prior to feeding using Repashy supplement and all feed was dusted with Nekton vitamin supplement. Data were collected in accordance with the Animal Care and Ethics Certificate provided by the University of Windsor (AUPP #18-12) and with the Animal Welfare and Management Committee at the Detroit Zoo (Royal Oak, MI).

2.1 | Hormone treatment

Prior to hormone administration, urine samples were collected from each male to ensure that there were no spermatozoa present at "time zero." Once confirmed, each male received an intraperitoneal injection of 0.5 μg/g body weight of a luteinizing hormone-releasing hormone analog (cat#: L4513; Sigma-Aldrich, St. Louis, MO) to produce spermic urine (see Poole & Grow, 2012).

2.2 | Sperm sampling

Immediately after hormone injection, male MGFs were placed into holding containers (460 mm L × 311 mm W × 177 mm H) fitted with shag moss. Each container was filled with approximately 25.4 mm of aged water to cover the bottom of the container. This allowed frogs to replenish their bladders between collection times. Spermic urine samples were collected at three time points: 30, 60, and 120 min after injection. Spermic urine was also collected at time zero to make sure there was no sperm present. Before collecting urine, the posterior end of each animal was patted dry using a paper towel to prevent excess water from diluting the sample. Animals were held over a wide petri dish (100 mm D × 15 mm H) and a soft piece of catheter tubing (#BB31785-V/5; Scientific Commodities Inc., Lake Havasu City, AZ) was gently inserted into the cloaca to draw spermic urine into the petri dish. Immediately after urination, the sample was pipetted into a 1.5-ml Eppendorf tube (#05-408-129; Fisher Scientific, Pittsburgh, PA) and placed in a chilling block (#IC22; Torrey Pines Scientific, Carlsbad, CA) set at 4°C until sperm analysis could take place (see below). All spermic urine samples were analyzed within a 5-min period at each collection time to avoid artifacts caused by a time difference between analyses.

2.3 | Sperm quality

Sperm was recorded at three different sampling times (30, 60, and 120 min after injection) for each male. Before analysis, samples were gently pipetted several times using a wide-bore transfer pipette (to avoid breaking off of tails). For each male, 2 μl of spermic urine was pipetted onto a 2×-CEL glass slide (Hamilton Thorne Biosciences, Beverly, MA), covered with a glass coverslip (22 × 22 mm) and activated with 18 μl of 21°C aged water directly from the male's holding container. When released in urine, sperm motility is motile and active, however, ideal activation occurs at osmolarities below approximately 100 mOsmol/L and is considered fully active at 55 mOsmol/L (Browne et al., 2015). Before analysis, the osmolarity of each spermic urine sample was measured using a Vapro® Vapor Pressure Osmometer. The osmolarity of spermic urine samples ranged from 112 to 131 mOsmol/L and samples were diluted to fully activate sperm. Sperm were recorded using a CCD B/W video camera module (XC-ST50, Sony, Japan) at 50 Hz vertical frequency, mounted on a microscope (CX41 Olympus, Melville, NY), equipped with a 10× negative-phase objective. Videos were converted into uncompressed AVI files using VirtualDubMod 1.5.10.2 (https://virtualdubmod.en.uptodown.com/windows), an open-source video capture and processing tool. Videos were analyzed using a java-based image processing program, ImageJ (Schneider, Rasband, & Eliceiri, 2012). To set a fixed scale, one video was selected at random and a still image was captured and opened into a Microsoft word document (Version 15.40). In Microsoft word, gridlines were overlaid across the image and set to 1 mm × 1 mm. The altered image was used to set a fixed scale in ImageJ. For each video, sperm straight line velocity (μm/sec) was analyzed at 1-minute after activation. Sperm velocity was calculated in microns/second based on the time-average velocity of a sperm head along the straight line between its first and last detected position. Sperm motility and progressive motility were measured using a generalized progressive motility scale (Kouba, Vance, et al., 2012). A total of 100 sperm cells were counted and the percentage of progressive sperm (sperm with moving flagella that were swimming in a rapid forward progression), motile sperm (sperm with moving flagella that were swimming in a slow forward progression), twitching sperm (sperm with nonmoving flagella with side to side head movement), and nonmotile sperm (sperm with nonmoving flagella with no head movement). The percentage of progressive motility and motility was calculated as the number of sperm exhibiting either progressive forward movement or slow forward movement on the progressive motility scale divided by 100 cells.

2.4 | Sperm concentration

Sperm concentration was estimated by adding 10 μl of spermic urine to 190 μl of aged water. Each aliquot was gently pipetted using a wide-bore transfer pipette, and 10 μl was placed onto a Neubauer haemocytometer and examined under ×400 magnification. Sperm cells were counted in five squares (1 mm^2), four corner squares, and the center square. Concentration was estimated by counting the mean number of sperm cells in the five squares, multiplying by 25 and then by 10 (chamber depth in μm; Pitcher, Doucet, Beausoleil, & Hanley, 2009). This number was then multiplied by the initial volume of the sample divided by the volume of the original mixture in the sample. Sperm concentration was estimated as the total number of sperms per mL (×10^6/ml).

2.5 | Statistical analysis

Time after hormone injection was examined with respect to motility (%), progressive motility (%), velocity (μm/sec), and concentration (x10^6/mL) using a repeated measures mixed-model analysis of variance. This

approach was able to examine whether there were significant differences in sperm quality metrics between the three sampling times after injection (30, 60, and 120 min) at 1-min after activation. This time point after activation was chosen as an arbitrary value before the egg's jelly coat hardening, which occurs approximately 5 min after egg release (Poole & Grow, 2012). Data were tested for normality using a Shapiro–Wilk test and non-normal data were log transformed. Akaike's and Bayesian information criteria were used to assess which model was most appropriate. Sampling time after injection was considered a fixed factor, whereas male identity and male age were considered as random factors. Tukey posthoc analyses were used to compare least square means between times after injection. Data were analyzed using R, a programming language for statistical computing (Version 3.5.1; package lsmeans, package lme4).

3 | RESULTS

Postinjection sampling time was found to have a significant effect on sperm motility ($F_{2,20} = 6.84$; $P = .005$; Figure 1a), however progressive motility was not found to be related to sampling time ($F_{2,20} = 6.79$, $P = .86$; Figure 1b). Velocity was found to be significant affect by sampling time ($F_{2,20} = 3.80$, $P = .03$; Figure 1c). Concentration was found to be marginally related to sampling time ($F_{2,20} = 3.45$, $P = .05$; Figure 1d).

4 | DISCUSSION

In this study, we provide information on the peak in sperm quality (i.e., motility, velocity, and concentration) at three time points after injection. This study was designed to enhance our understanding of amphibian induction using LHRHa, which may increase the efficiency of captive breeding programs. Our results demonstrate that sampling time after injection has a significant effect on sperm quality metrics in the MGF. Time since hormone injection significantly affected percent motility and velocity and had a marginal effect on sperm concentration. However, progressive motility was not related to sampling time. These results have important implications for optimizing fertilization success for anurans in captive breeding programs.

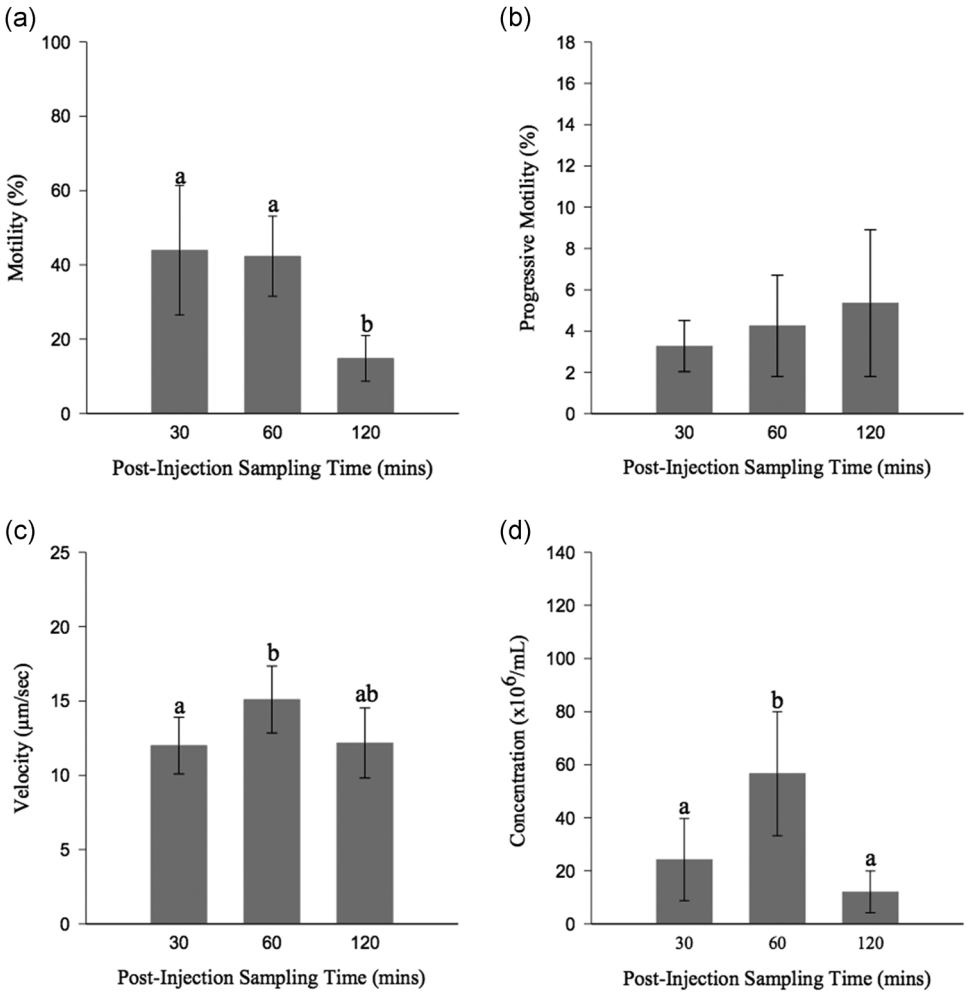

FIGURE 1 Adjusted means for (a) motility (%), (b) progressive motility (%), (c) velocity (μm/s), and (d) concentration ($\times 10^6$/ml) across postinjection sampling time (min). Means (±1 SE) with shared letters did not differ significantly from one another based on posthoc analyses

Consistent with studies on other species of endangered anurans, our results suggest that percent motility can be affected, and concentration may be affected by how long after injection time you collect the sperm sample. Variation in sperm quality across sampling time has been previously reported for a number of endangered anurans (Byrne & Silla, 2010; Della Togna et al., 2017; Obringer et al., 2000). Obringer et al. (2000) assessed spermiation and sperm quality (i.e., motility and concentration) related to several methods of LHRH administration (intraperitoneal injection, subcutaneous injection, ventral dermal absorption, and dorsal dermal absorption) and dosage levels (1.0, 0.1, and 0.01 μg) in the American toad (*Anaxyrus americanus*). Peak sperm concentration was found to differ between type of hormone administration and dosage across time in the American toad. Across all sampling times (0, 3, 7, and 12 hr) after LHRH injection, intraperitoneal-injected males reached maximum sperm production earlier than subcutaneous-injected males, with peak sperm concentration occurring 12 hr after injection (1.0 μg dosage). Motility, however, was high and not significantly different across the sampling times (>70%) before declining after 24 hr after injection (28 ± 10.2%). A more recent study by Della Togna et al. (2017) evaluated postinjection sampling time to analyze the concentration of spermatozoa in the critically endangered Panamanian golden frog following different hormones and dosages. Results showed that sampling time had a significant effect on sperm concentration, with the peak sperm concentration occurring between 2.5 to 4.5 hr after injection. Taken together, these studies show the variation in motility and concentration following a hormone injection. For our study, we found that peak motility and concentration occur at 1-hour after LHRHa injection in the MGF. It is important to recognize these differences in sperm metrics as they may increase fertilization rates during in-vitro fertilization (Browne et al., 2015).

Sperm straight line velocity was found to be significantly affected by sampling time after LHRHa injection. However, this metric has not previously been studied in anurans in the context of sampling time. Quantifying straight line velocity can be useful for estimating fertilization success as frog sperm are structurally and behaviorally different from the sperm of other external fertilizers (Dziminski et al., 2009; Hettyey & Roberts, 2006). Unique from toads, which possess a mitochondria vesicle, frog sperm must navigate through an external fertilization environment with their energy reserves to successfully fertilize an egg (Browne et al., 2015). A study by Dziminski et al. (2009) observed sperm with slower swimming velocities had a competitive advantage in fertilization in the externally fertilizing myobatrachid frog (*Crinia geogiana*). Our evaluation of sperm velocity can act as a starting place to better understand how velocity is influenced by sampling time and future studies would likely benefit from using velocity as a metric to test fertilization success in-vitro. We did not find progressive motility to be affected by sampling time.

In conclusion, our results suggest that sampling time after injection by LHRHa can have a significant impact on sperm quality in the MGF. Understanding the spermiation response to LHRHa for the MGF is key to maximizing reproductive success in captive breeding programs. Therefore, we would suggest sampling sperm at 1-hr after LHRHa injection. Globally, anuran populations are in great decline, demonstrating the importance of enhancing breeding protocols in zoological institutions that are active in in-situ and ex-situ conservation. Overall, these results could prove useful for maximizing fertilization success if sperm is sampled at optimal times after hormone injection.

ACKNOWLEDGMENTS

This research was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery and CREATE grants (TEP). This research was supported by the Detroit Zoological Society, Detroit Zoo. The authors thank Mark Vassallo for his assistance with husbandry and rearing of the Mississippi gopher frogs and Justin R.E. Barker for his assistance with the statistical analyses.

ORCID

Ashley Michelle Watt http://orcid.org/0000-0002-7835-8972

REFERENCES

Association of Zoos and Aquariums. (2018). *Population analysis and breeding and transfer plan: dusky gopher frog (Lithobates (Rana) sevosa)*. Chicago, IL: Population Management Center.

Browne, R. K., Kaurova, S. A., Uteshev, V. K., Shishova, N. V., McGinnity, D., Figiel, C. R., & Mansour, N. (2015). Sperm motility of externally fertilizing fish and amphibians. *Theriogenology*, *83*(1), 1–13.e8.

Browne, R. K., Seratt, J., Vance, C., & Kouba, A. (2006). Hormonal priming, induction of ovulation and *in vitro* fertilization of the endangered Wyoming toad (*Bufo baxteri*). *Reproductive Biology and Endocrinology*, *4*(1), 34.

Byrne, P. G., & Silla, A. J. (2010). Hormonal induction of gamete release, and in-vitro fertilisation, in the critically endangered Southern Corroboree Frog, *Pseudophryne corroboree*. *Reproductive Biology and Endocrinology*, *8*, 144.

Conway, W. G. (2011). Buying time for wild animals with zoos. *Zoo Biology*, *30*(1), 1–8.

Della Togna, G., Trudeau, V. L., Gratwicke, B., Evans, M., Augustine, L., Chia, H., & Bronikowski, E. J. (2017). Effects of hormonal stimulation on the concentration and quality of excreted spermatozoa in the critically endangered Panamanian golden frog (*Atelopus zeteki*). *Theriogenology*, *91*, 27–35.

Dziminski, M. A., Roberts, J. D., Beveridge, M., & Simmons, L. W. (2009). Sperm competitiveness in frogs: Slow and steady wins the race. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1675), 3955–3961.

Edwards, D. L., Mahony, M. J., & Clulow, J. (2004). Effect of sperm concentration, medium osmolality and oocyte storage on artificial fertilisation success in a myobatrachid frog (*Limnodynastes tasmaniensis*). *Reproduction, Fertility, and Development*, *16*(3), 347–354.

Goncharov, B. F., Shubravy, O. I., Serbinova, I. A., & Uteshev, V. K. (1989). The USSR programme for breeding amphibians, including rare and endangered species. *International Zoo Yearbook*, *28*, 10–21.

Hammerson, G., Richter, S., Siegel, R., LaClaire, L., & Mann, T. (2004). *Lithobates sevosus*. The IUCN red list of threatened species. Version 2014.3.ed

Hettyey, A., & Roberts, J. D. (2006). Sperm traits of the Quacking frog, *Crinia georgiana:* intra- and interpopulation variation in a species with a high risk of sperm competition. *Behavioral Ecology and Sociobiology*, *59*(3), 389–396.

Johnson, K., Butts, I. A. E., Wilson, C. C., & Pitcher, T. E. (2013). Sperm quality of hatchery-reared lake trout throughout the spawning season. *North American Journal of Aquaculture*, *75*(1), 102–108.

Kouba, A. J., delBarco-Trillo, J., Vance, C. K., Milam, C., & Carr, M. (2012). A comparison of human chorionic gonadotropin and luteinizing hormone releasing hormone on the induction of spermiation and amplexus in the American toad (*Anaxyrus americanus*). *Journal of Reproductive Biology and Endocrinology*, *10*, 59.

Kouba, A. J., & Vance, C. K. (2009). Applied reproductive technologies and genetic resource banking for amphibian conservation. *Reproduction, Fertility, and Development*, *21*(6), 719–737.

Kouba, A. J., Vance, C. K., Calatayud, N., Rowlison, T., Langhorne, C., & Willard, S. (2012). Assisted reproductive technologies (ART) for amphibians. In Poole, V. A., & Grow, S. (Eds.), *Amphibian husbandry resource Guide* (2nd). Silver Spring, MD: Association of Zoos and Aquariums.

Kouba, A. J., Vance, C. K., & Willis, E. L. (2009). Artificial fertilization for amphibian conservation: Current knowledge and future considerations. *Theriogenology*, *71*(1), 214–227.

Obringer, A. R., O'Brien, J. K., Saunders, R. L., Yamamoto, K., Kikuyama, S., & Roth, T. L. (2000). Characterization of the spermiation response, luteinizing hormone release and sperm quality in the American toad (*Bufo americanus*) and the endangered Wyoming Toad (*Bufo baxteri*). *Reproduction, Fertility, and Development*, *12*(1-2), 51–58.

O'Donnell, L., Nicholls, P. K., O'Brien, M. K., McLachlan, R. I., & Stanton, P. G. (2011). Spermiation: The process of sperm release. *Spermatogenesis*, *1*(1), 14–35.

Pitcher, T. E., Doucet, S. M., Beausoleil, J., & Hanley, D. (2009). Secondary sexual characters and sperm traits in Coho Salmon (*Oncorhynchus kisutch*). *Journal of Fish Biology*, *74*(7), 1450–1461.

Poole, V. A., & Grow, S. (2012). *Amphibian husbandry resource guide. Association of Zoos and Aquariums* (2nd.). MD: Silver Spring.

Richter, S. C., Crother, B. I., & Broughton, R. E. (2009). Genetic consequences of population reduction and geographic isolation in the critically endangered frog, *Rana sevosa*. *Copeia*, *4*, 799–806.

Richter, S. C., Young, J. E., Johnson, G. N., & Seigel, R. A. (2003). Stochastic variation in reproductive success of a rare frog, *Rana sevosa*: Implications for conservation and for monitoring amphibian populations. *Biological Conservation*, *111*(2), 171–177.

Roth, T., & Obringer, A. (2003). Amphibian reproduction. In W. V. Holt, A. R. Pickard, J. C. Rodger, & D. E. Wildt (Eds.), *Reproductive Science and Integrated Conservation* (pp. 359–374). Cambridge, UK: Cambridge University Press.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*, 671–675.

How to cite this article: Watt AM, Marcec-Greaves R, Pitcher TE. Time from injection of luteinizing hormone-releasing hormone analog affects sperm quality in the critically endangered Mississippi gopher frog (*Lithobates sevosus*). *Zoo Biology*. 2020;39:23–28. https://doi.org/10.1002/zoo.21519