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Effects of wild, semi-captive, and captive management on male Chiricahua leopard frog sperm quality with implications for conservation breeding programs

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Abstract

The Chiricahua leopard frog (*Lithobates chiricahuensis*) is a threatened species endemic to the southwestern United States and Northern Mexico. Captive breeding programs were established to support reintroduction efforts, yet reproductive output has been lower than needed for recovery of the species. This study aimed to evaluate the effects of captivity on amphibian reproduction by (1) determining if captive, semi-captive, and wild male *L. chiricahuensis* produce sperm at similar rates and concentration in response to hormone treatment; and (2) evaluating the quality of sperm obtained over time from these populations. Males from captive, semi-captive, and wild locations were administered a combination of human chorionic gonadotropin and gonadotropin-releasing hormone to stimulate sperm production and release. A high percentage of males in the captive (60%), semi-captive (100%), and wild (95.3%) populations produced sperm following treatment. Sperm quality (forward progressive motility and total sperm motility) did not differ between groups. However, sperm quantity (sperm/ml) differed ($p < .05$) between populations, with semi-captive and wild males producing higher concentrations of sperm than captive males. These results suggest that Chiricahua leopard frog sperm quantity, but not quality, may become negatively impacted by long-term captivity in indoor, controlled settings.

KEYWORDS

amphibian, assisted reproduction, captive-breeding, conservation, exogenous hormones, fertility, frog, spermiation

1 | INTRODUCTION

The Chiricahua leopard frog (*Lithobates chiricahuensis*) is a federally listed ranid species endemic to the southwestern United States and parts of Mexico and breeds from March through September in small ponds, cienagas and streams (Stebbins, 2003). Because of the species' dependence on water in arid environments, factors such as water pollution, invasive aquatic species, and drought have had a substantial deleterious effect on wild populations (Rosen et al., 1994). The accessibility to viable water sources for breeding has been further hampered by the reduction of historical habitat for farming, leaving streams and marshes polluted, destroyed, or converted to cattle ponds (Southwest Endangered Species Act Team, 2008). The largest threat to *L. chiricahuensis* in New Mexico is chytridiomycosis, a disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which has a reported mortality rate of 90–100% in various anuran species (Berger et al., 2005; Longcore et al., 1999). Outbreaks of chytridiomycosis have been found in several populations of wild *L. chiricahuensis* and have contributed heavily to the species' decline (Berger et al., 1999; Christman & Jennings, 2018; Sigafus et al., 2014). In an attempt to salvage the remaining habitat and circumvent the effects of *Bd* on wild populations, the federal government issued a recommendation for protection and captive breeding efforts following federal listing of the species in 2002 by the U.S. Fish and Wildlife Service department under the Endangered Species Act (Brennen & Holycross, 2009). Unfortunately, continuing loss of critical habitat, spread of invasive species, and the continued threat of chytridiomycosis impede the reestablishment of the species into historic habitats (Southwest Endangered Species Act Team, 2008).

To augment wild populations of *L. chiricahuensis*, captive breeding programs have been established across several institutions including Turner Enterprise's Ladder Ranch, the Fort Worth Zoo, and the Phoenix Zoo. These institutions have spearheaded *L. chiricahuensis* conservation efforts through the reintroduction of egg masses, tadpoles, and head-started metamorphic froglets into native and historic locations across the southwestern United States. Since 1995, the Phoenix Zoo has released 26 egg masses; 15,562 tadpoles; 11,119 juveniles; and 140 adult frogs (T. Harris, Personal Communication, 2020) while the Fort Worth Zoo has released 1600 captive-bred tadpoles since 2015. The Ladder Ranch has released 69 egg masses; 94,000 tadpoles; and 493 metamorphic froglets from semi-captive breeding since 2011 (D. Barber, Personal Communication, 22 Sept. 2020). Despite the reported reproductive success, natural breeding of *L. chiricahuensis* within these captive programs has been sporadic, resulting in offspring numbers far below sustainable reintroduction numbers needed for recovery efforts. One strategy for bolstering low reproductive output for amphibian assurance colonies is the use of assisted reproductive technologies (ARTs), such as hormone therapy and in vitro fertilization. For example, increases in gamete production can be achieved through the application of exogenous hormones such as human chorionic gonadotropin (hCG) or gonadotropin-releasing hormone (GnRH), which stimulate a natural hormone cascade that results in breeding behaviors followed by gamete production and release (Kouba et al., 2009; Kouba et al., 2012; Vu & Trudeau, 2016). Exogenous hormone treatment has been successful at eliciting reproduction and gamete production in a variety of threatened and endangered amphibian species such as *Anaxyrus baxteri* (Browne et al., 2006), *Atelopus zeteki* (Della Togna et al., 2017), *Lithobates sevosa* (Kouba et al., 2011), *Litoria booroolongis* (Silla et al., 2019), and several newt species (Guy et al., 2020), but has yet to be applied to *L. chiricahuensis*.

When establishing breeding recommendations and repopulation efforts for threatened species, it is important to determine the reproductive potential of animals that have been kept in captivity for extended periods and how the captive environment may have impacted reproductive output compared to their wild counterparts; for example, the extent of differences in fecundity between wild and captive amphibians, and whether reduced fecundity in captivity is due to reduced gamete release and quality. Premature or early onset senescence is another potential concern regarding maintaining captive breeding colonies. Premature senescence has been observed most commonly in large, long-lived mammals such as rhinoceroses and elephants (Hermes et al., 2004; Tidière et al., 2016). There have been several studies in various species examining the effects of captivity on physiological and reproductive parameters; however, the definitions of captivity have varied widely, creating uncertainty in what specific features of a captive environment affect physiology. For example, captive conditions for large megafauna are commonly indoor paddocks linked to outdoor enclosures with exposure to natural environmental cues, but which are limited in space (Bobe & Labbé, 2010). In comparison, non-farmed fish and herpetofauna are more often housed in entirely man-made indoor enclosures away from natural environmental cues required for reproduction. In addition, it is often unclear in published studies whether captive animals were born in captivity or born in the wild and translocated to a captive environment (Farquharson et al., 2018). While the issue is complex, it has been suggested that lack of frequent, natural breeding opportunities contributes to poor reproductive fitness (Hermes et al., 2004). Many captive amphibians are housed in separate sex-biased groupings and only exposed to opposite-sex conspecifics during short breeding events based on recommended pairings. Thus, many animals

within captive collections can go several years without being paired for breeding until their genetic value becomes of greater import for recruitment or retention. A lack of exposure to social, physiological, and environmental cues necessary for annual reproductive cyclicity may impact natural reproductive hormone cascades and gamete development. In the absence of annual environmental cues, captive animals are at a higher risk of reproductive failure, possibly due to premature senescence. Studying reproductive senescence can be challenging, as captive animals tend to have longer lifespans compared to their wild counterparts, making it difficult to determine exactly when natural age-related senescence occurs and whether or not it is impacted by conditions in captivity (Tidière et al., 2016).

In studies that have differentiated between animals born in captivity and those born in the wild and taken into captivity, wild-born captive animals have significantly greater reproductive success as measured by higher gamete quality, more successful breeding events, and more healthy offspring than those born in captivity, particularly in aquaculture settings (Farquharson et al., 2018). A direct comparison of gamete quality between captive, semi-captive, and wild populations of threatened amphibians has not been conducted and would help elucidate whether low reproductive output in captive amphibian populations is due to suboptimal gamete quality or quantity compared to their wild counterparts. Furthermore, determining if male frogs held in captivity for many years can still produce gametes at a comparable quantity and quality as their wild counterparts provides assurance that they can be relied upon for breeding efforts without necessitating frequent capture and replenishment with wild animals.

Due to the critical importance of bolstering *L. chiricahuensis* reproductive output in the captive population to support recovery efforts, it is imperative that hormone therapy strategies be developed for long-term sustainability management. It is likewise critical to determine whether captivity has a deleterious effect on gamete quality or quantity in threatened amphibians. Thus, we investigated the effects of exogenous hormone treatments on male *L. chiricahuensis* sperm production across three populations: captive, semi-captive, and wild. Captive males refer to those under human care in small indoor enclosures, with temperature and light-controlled environments while semi-captive males are under human care but housed in large, outdoor enclosures. The objectives of this study were to determine if captive, semi-captive, and wild male *L. chiricahuensis* spermiate in response to hormone treatment, and if these three management scenarios affect sperm quantity and quality.

2 | METHODS

2.1 | Animals

Captive males were either wild caught at sexual maturity ($n = 16$) or born in captivity ($n = 4$) and housed at the Fort Worth Zoo in Fort Worth, TX in an indoor artificial habitat for at least 1 year. Briefly, animals were housed in 66 × 46 × 23 cm polycarbonate tanks set at a tilt to provide a dry level and an aquatic level. All tanks included hides on the dry portion and artificial plants in the aquatic side. UV lighting was provided to all tanks via fluorescent bulbs over each enclosure. Temperature and lighting were controlled to mimic the natural environmental fluctuations across seasons with April and May temperatures maintained between 26 and 28°C and day lengths set to 14 h daylight/10 h of night. Frogs received a primary diet of crickets four times a week, and an addition of wax worms and earthworms weekly. Insects were gut-loaded with Repashy Superload with an additional vitamin supplement of NektonRep and calcium carbonate. Captive males had an average weight of 42.7 ± 3.6 g and had spent an average of 3.4 ± 0.3 years in captivity (Table 1). Husbandry guidelines and research protocols were approved by the Institutional Animal Care and Use Committee (IACUC) for both the Ladder Ranch and Fort Worth Zoo (#17-H001).

Semi-captive males ($n = 26$) were housed in 2.4 m^3 outdoor ranariums equipped with aviary netting at the Ladder Ranch in Caballo, NM for a minimum of 1 year. Each enclosure consisted of a 416 L water tank for breeding with an adjacent land access containing native vegetation. Housing groups consisted of two to three male conspecifics with two females and diets were foraged native insects with a supplement of crickets. For sperm collection, males were held in plastic tubs with 2 cm of water inside the ranch building for approximately 4–5 h before being returned to their outdoor enclosures. Males had been held under semi-captive conditions for an average of 3.3 ± 0.3 years with an average weight of 41.6 ± 1.0 g (Table 1). During collections, ambient temperatures ranged from 32 to 35°C. Day length for Caballo, NM in May are near 14 h.

Wild males ($n = 43$) were collected from several field sites in southwestern New Mexico during the breeding season. Males were located at night and captured by hand or dip net. Males were then placed in buckets of shallow water obtained from streams where they were caught, held until morning, and separated into individual plastic tubs with stream water for the duration of sperm collection. Approximate air temperatures during sperm collections ranged from 27 to 33°C. Day length was similar to semi-captive male conditions. Males were held no more

TABLE 1 Male *Lithobates chiricahuensis* sperm response and quantity or quality parameters based on population management groups. Parameters collected following hormone induction include percent of responders, forward progressive sperm motility, total motility, sperm concentration, and total sperm. Letters denote significant differences ($p < .05$) within a column and data are shown as mean ± SEM

Population	Animals	Years in captivity	Weight (g)	Responders (%)	Forward progressive motility (%)	Total motility (%)	Sperm/ml ($\times 10^6$)	Total sperm ($\times 10^6$)
Captive	20	3.4 ± 0.3	42.7 ± 3.6	60	14.6 ± 2.2	56.6 ± 3.4	0.51 ± 0.22[a]	0.06 ± 0.03[a]
Semi-captive	26	3.3 ± 0.3	41.6 ± 1.0	100	22.4 ± 2.8	66.9 ± 3.3	4.11 ± 0.77[b]	0.84 ± 0.28[b]
Wild	43	0.0 ± 0.0	28.8 ± 1.5	95.3	24.9 ± 2.9	70.3 ± 2.8	5.54 ± 1.00[b]	0.98 ± 0.39[ab]

than 16 h from time of capture, with all frogs returned to the locations from which they were obtained following sperm collections. Wild male frogs averaged 28.8 ± 1.5 g (Table 1). Males were sexually mature, as determined by the presence of vocal sacs and enlarged thumb pads. All collections were approved under U.S. Fish and Wildlife permit #TE43754A-2 through the Turner Endangered Species Fund at the Ladder Ranch.

2.2 | Exogenous hormone, sperm collection, and analysis

Sperm collections were conducted from 2017 to 2022, during the months of April and May, within the natural breeding season of *L. chiricahuensis*. Males from all three population groups ($n = 89$) were given a combination of hCG (Millipore Sigma, CG5) and a GnRH agonist (GnRHa; Millipore Sigma, L1898). All males were treated with an average of 7.7 ± 0.2 IU hCG/g body weight (BW) and an average of 0.5 ± 0.03 μg/g BW GnRHa in phosphate buffered saline delivered intraperitoneally.

A baseline urine sample was taken from each male prior to hormone treatment to determine the background level of sperm production. No animals were producing spermic urine prior to hormone administration. Spermic urine samples were collected at 1, 2, and 3 h following hormone treatment. Urine samples were obtained using a 0.76 mm × 1.22 mm sterile vinyl catheter (Scientific Commodities Inc. #BB31785-V/4) to drain the cloacal contents into a petri dish and then analyzed for the presence of sperm. Frogs were handled for sperm collection no longer than 2 minutes to reduce handling stress. Each male was sampled only once. Sperm concentration was measured using a hemacytometer (Hausser Scientific #3200) and manual cell counter. Sperm motility parameters are reported as the percentage of 100 spermatozoa that were subclassified as: (1) forward progressively motile (FPM), defined as spermatozoa moving forward through flagellar movement; (2) motile (M), defined as spermatozoa exhibiting flagellar movement but not progressing forward; and (3) nonmotile (NM), defined as spermatozoa exhibiting no observable movement. Sperm sample total motility (TM) is the sum of FPM and M. All sperm samples were analyzed using an Olympus CX43 phase-contrast microscope.

2.3 | Statistical analysis

A generalized linear mixed model with fit by maximum likelihood (package lmerTest) was used to detect differences among populations (captive, semi-captive, and wild) in sperm concentration and motility parameters with relation to sperm collection timepoints. The GLMM was generated with captive, semi-captive and wild status, animal weight, years spent under each condition, and collection time points set as the fixed effects and male ID set as a random effect. A binomial family with a logit link was used to analyze proportional (TM and FPM) data. For count data (sperm/ml and total sperm), data were log-transformed and analyzed under a Gaussian family with an identity link. If the GLMM determined a difference between groups, a multiple comparison test (package multcomp) was used to determine specific differences between individual levels. Data are reported as mean ± SEM and were considered significant at $\alpha \leq .05$. Data were tested for normality and homogeneity of variance using the Shapiro–Wilk's test and Levene's test, respectively. All data were run in RStudio with version R-4.0.5.

3 | RESULTS

Overall, 60% (95% CI 59.8–60.2), 100%, and 95% (95% CI 95.2–95.4) of captive, semi-captive, and wild males, respectively, produced sperm during the experimental period following hormone treatment. Significantly fewer ($F = 13.9$, df = 2, $p < .01$; Table 1) captive males produced sperm than both semi-captive and wild males. At 1-h post-hormone treatment, males from each group had begun producing sperm (Figure 1). The highest

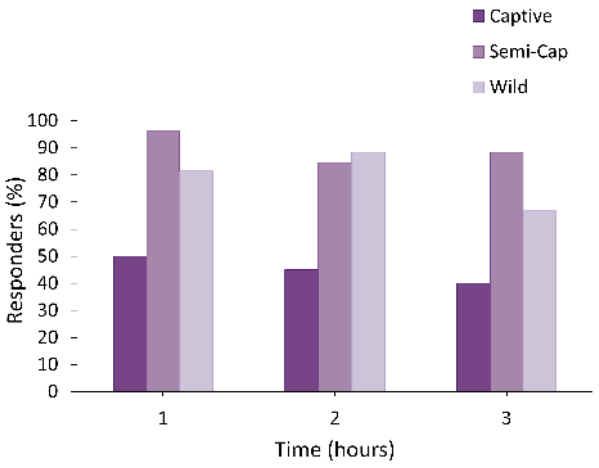

FIGURE 1 Percentage of males responding to hormone treatments over time between wild, semi-captive and captive males

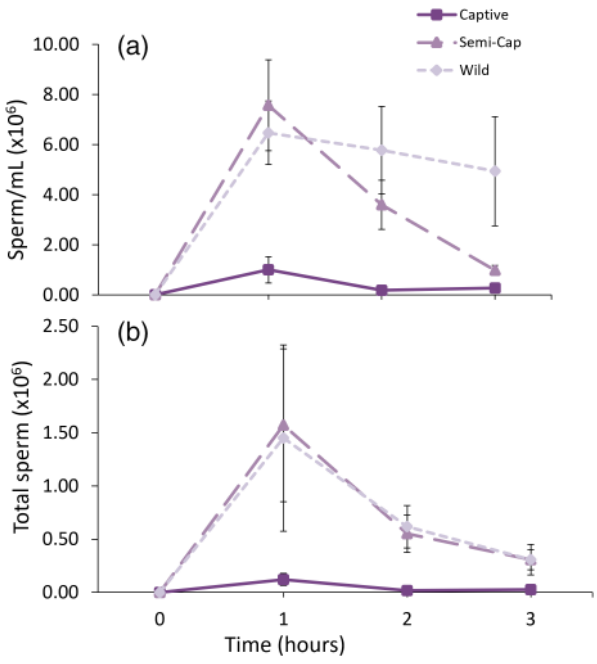

FIGURE 2 Average sperm concentration/ml (panel a) and total sperm collected (panel B) across the three population groups, captive, semi-captive, and wild, over time. Data are shown as mean ± SEM

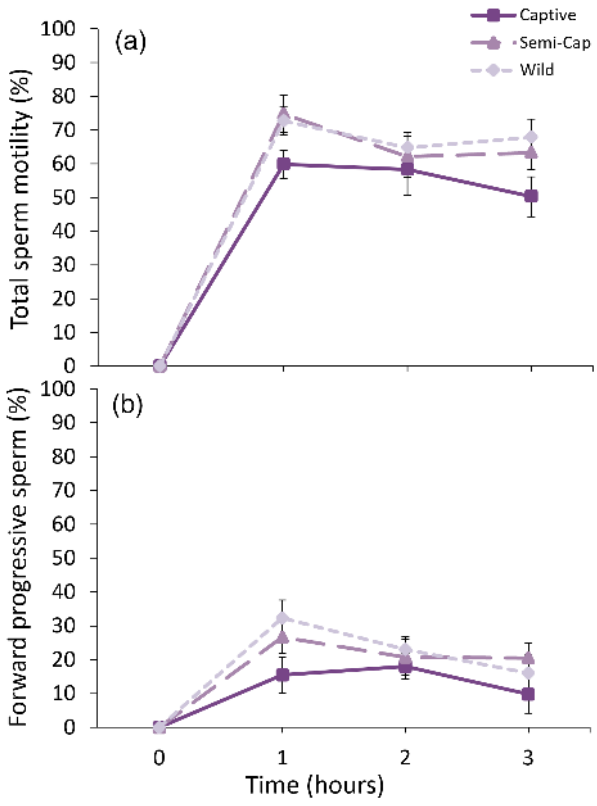

FIGURE 3 Average total sperm motility (panel a) and forward progressive sperm (panel b) across the three population groups, captive, semi-captive, and wild, over time. Data are shown as mean ± SEM

percentage of captive (50%; 95% CI 49.8–50.2%) and semi-captive (96.2%; 95% CI 96.1–96.2%) males producing sperm was at 1-h posttreatment, and the highest percentage of responding wild (88.4%; 95% CI 88.3–88.5%) males occurred at 2 h. The percentage of captive (40%; 95% CI 39.8–40.2%) and wild (66.7%; 95% CI 66.5–66.8%) frogs producing sperm began to decline at 3-h posttreatment, while the percentage of semi-captive (88.5%; 95% CI 88.3–88.6%) males producing sperm demonstrated a slight increase between hours 2 and 3 posttreatment (Figure 1). These time series data suggest that the majority of male *L. chiricahuensis* will reliably produce sperm following hormone administration within the first 3 h of treatment, which should be considered when using ARTs for sperm collection in this species.

Sperm concentration parameters (sperm/ml and total sperm) differed between populations (Table 1). While semi-captive and wild males produced similar ($p > .05$) concentrations of sperm/ml (semi-captive mean = 4.11×10^6, 95% CI 2.57–5.66×10^6; wild mean = 5.54×10^6, 95% CI 3.54–7.54×10^6), captive males released significantly lower ($F = 8.66$, df = 2, $p < .01$) concentrations of sperm/ml (0.51×10^6, 95% CI 0.04–0.98×10^6) than both wild and semi-captive males. Across population groups, sperm/ml was significantly impacted ($F = 15.15$, df = 2, $p < .001$) by collection time (Figure 2a). The highest average concentration of sperm/ml occurred at 1-h posttreatment (mean = 1.39×10^6, 95% CI 0.28–2.51×10^6). At hours 2 (mean = 0.57×10^6, 95% CI 0.30–0.84×10^6) and 3 (mean = 0.28×10^6, 95% CI 0.13–0.43×10^6), sperm continued to significantly decline in a time-dependent manner. Total sperm only differed significantly ($F = 4.46$, df = 2, $p = .01$) between semi-captive (mean = 0.84×10^6, 95% CI 0.28–1.39×10^6) and captive males (mean = 0.06×10^6, 95% CI 0.01–0.11×10^6); total sperm from wild males

(mean = 0.98 × 10^6, 95% CI 0.2–2.76 × 10^6) did not differ ($p > .05$) from either group (Table 1). Time did not significantly impact total sperm release ($p > .05$; Figure 2b).

The percentage of total motile sperm was similar ($F = 1.40$, df = 2, $p = .24$) across the three populations (captive mean = 57%; 95% CI 50–64%; semi-captive mean = 67%, CI 60–73%; wild mean = 70%; 95% CI 65–76%) and remained high across all time points; moreover, we found no effect of time ($F = 1.92$, df = 2, $p = .16$) on total sperm motility (1-h mean = 72%, 95% CI 66–78%; 2-h mean = 64%; CI 67–71%, wild mean = 64%, 95% CI 58–70%). However, while not significant, the highest percentage of total sperm motility was released at 1-h post-hormone treatment (72%; Figure 3a). Captive males saw a time-dependent decrease in total sperm motility at each subsequent hour. Conversely, semi-captive and wild male sperm motilities decline between hours 1 and 2 but see a slight increase from hour 2 to 3. Forward progressive sperm motility was also not affected by population ($F = 1.16$, df = 2, $p > .05$; Figure 3b). While not significant, peak forward progressive motility occurred at 1-h posttreatment in all groups (captive = 15.5%, semi-captive = 26.6%, and wild = 31.5%) and subsequently declined across all groups at hour 3. Taken together with responder and sperm concentration data over time, these results support that the first hour following hormone treatment results in the highest number of responding males and the best average sperm quality.

While wild males (28.8 ± 1.5 g), on average, weighed less than semi-captive (41.6 ± 1.0 g) and captive males (42.7 ± 3.6 g), weight did not have a significant effect on concentration of sperm/ml ($F = 0.95$, df = 1, $p = .33$); total sperm ($F = 0.36$, df = 1, $p = .55$); or on total sperm motility ($F = 2.83$, df = 1, $p = .34$). However, weight did have a significant effect on forward progressive sperm motility ($F = 2.72$, df = 1, $p = .01$) with males having higher weights exhibiting a negative correlation with forward progressive sperm. Finally, the number of years animals spent in each population (captivity and semi-captivity) did not significantly impact any of the parameters pertaining to sperm quality ($p > .05$; Table 1).

4 | DISCUSSION

Here, we provide the first assessment of sperm traits from wild, semi-captive, and captive *L. chiricahuensis*. Moreover, we show that all three populations produce sperm at high rates in response to an intraperitoneal injection of the combined reproductive hormones hCG and gonadotropin-releasing hormone (GnRH). This is the first time that a direct comparison of sperm parameters has been made in a threatened amphibian species across three levels of management conditions. We found that, while sperm motility parameters did not differ across populations, sperm/ml differed significantly, with wild and semi-captive animals releasing sperm in higher concentrations than captive animals. Across captive, semi-captive, and wild populations, males responded to treatment at a rate of 60, 100, and 95%, respectively. This is consistent with reports for several other amphibian species when administered exogenous hormones. For example, *L. sevosa* respond to the same treatment of hCG and GnRHa at an average rate of 83% (Kouba et al., 2011). Moreover, in response to hCG alone, 80% of *A. baxteri* (Browne et al., 2006), between 83 and 100% of male *Rhaebo guttatus* (Hinkson et al., 2019) and 100% of *Litoria booroolongensis* (Silla et al., 2019) produced sperm. When GnRHa has been given alone the hormone elicited a spermiation response rate of 82% in *L. booroolongensis* (Silla et al., 2019) but only 20% in *R. guttatus* (Hinkson et al., 2019). Surprisingly, while wild males weighed significantly less than both semi-captive and captive males, weight only affected the quality of sperm in terms of the amount of sperm exhibiting forward progression and vigor; as such, heavier males tended to release sperm with lower forward progression. Weight may be correlated with age, and thus larger animals may have been older than their smaller counterparts, but without exact ages for animals in the wild population this is an undeterminable effect. It is also possible that the weight and thus quality of sperm is related to animal fitness.

Captive amphibians are often kept in indoor enclosures, and are missing environmental cues required for the natural initiation of reproductive hormone cascades, gamete maturation, and subsequent stimulation of breeding behaviors (Kouba et al., 2009). The lack of environmental cues has also been previously cited as a factor in premature senescence in captive animals (Hermes et al., 2004). It is possible that one or more of these factors exhibit negative influences in sperm quantity, while lacking an effect on sperm quality. Nearly all (80%) of the animals classified as "captive" in the current study were originally wild born. It has been previously reported that wild-born animals across varying aquatic taxa are more reproductively successful in captivity than captive-born animals in aquaculture systems, from breeding behaviors to gamete quality (Farquharson et al., 2018). While this trend was strongest in aquaculture, it was also observed in both laboratory research and conservation program settings (Farquharson et al., 2018). Wild-born animals may have already established epigenetic cyclical patterns in hormone expression due to previous exposure to environmental factors during development and as wild adults. These patterns may not be expressed in captive animals, thus reducing their ability to reach the levels of

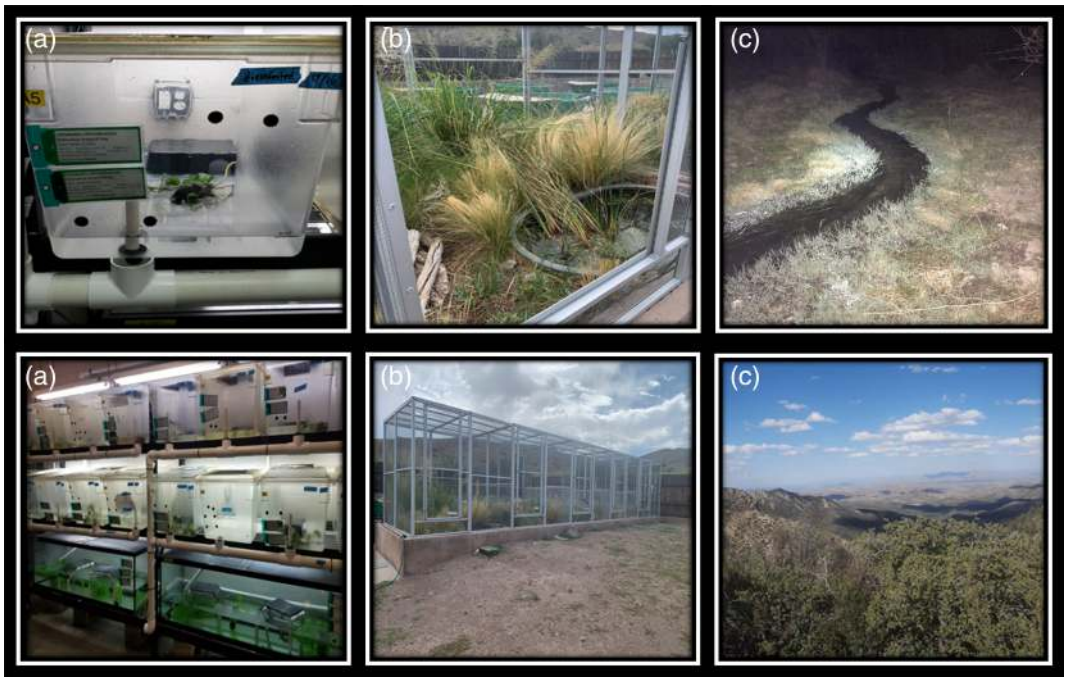

FIGURE 4 Images of population management conditions. (a) Captive, the Fort Worth Zoo; (b) semi-captive, the Ladder Ranch; and (c) wild, New Mexico field site

fecundity of wild conspecifics. The majority of the captive and semi-captive males in this study was caught in the wild at sexual maturity and had spent an average of 3.4 and 5.1 years at the Fort Worth Zoo and Ladder Ranch, respectively. While both institutions housed captive individuals, those at Ladder Ranch (semi-captive) were housed in large outdoor ranariums while those at Fort Worth Zoo (captive) were housed indoors. Because males housed in an indoor captive environment exhibited the lowest concentrations of sperm/ml, while semi-captive and wild males did not differ in sperm/ml concentrations, these results suggest that indoor captivity is capable of negatively influencing production of sperm after an average of only 3.4 years in a captive setting (Figure 4).

While investigations into the factors affecting sperm quality in amphibians are limited, several components influencing sperm production and quality in fish have been proposed over the years. These include the impacts of photoperiod, temperature, nutrition, age, and breeding season (Rurangwa et al., 2004). Due to the highly seasonal nature of reproduction for many amphibian species, challenges with meeting nutritional needs in captivity, and aging captive populations, these same factors may also impact amphibian sperm quality and subsequently contribute to the decline in captive amphibian fertility. Here, we found that sperm concentrations were lowest in males housed in an indoor captive setting and were outperformed by males housed in an outdoor captive setting. In captive greater amberjack, males raised in captivity were found to have smaller seminiferous lobes, lower testosterone levels, and reduced spermatogenesis (Zupa et al., 2017). As spermatogenesis is mediated by testosterone levels, reduced testosterone would likely result in lower sperm production. In captive male frogs, lack of seasonal cues in captive males housed indoors may influence seasonality, reduce circulating testosterone, and subsequently reduce sperm production. Additionally, stress levels may decrease testosterone production, as has been reported in certain fish species (Kubokawa et al., 1999). Deleterious effects of stress on circulating steroid hormones and gonadotropins have been found in captive female Indian skipper frogs compared to their wild counterparts (Pancharatna & Saidapur, 1992), leading to a marked difference in follicular development rates and reduced oocyte production. Reductions in follicular development and breeding behaviors were also found in captive female Indian bullfrogs when compared to wild females (Hoque & Saidapur, 1994).

Despite the production of captive and semi-captive offspring across breeding programs for the species, the difficulty remains that natural breeding attempts of captive *L. chiricahuensis* are below sustainability levels, are not reflective of wild fecundity, and do not produce enough animals to establish a successful reintroduction program. Here, we report that sperm concentration in captive frogs is lower than semi-captive and wild frogs. It is possible that the lower sperm concentrations seen here may reduce a male's ability to fertilize egg clutches in breeding programs and could be a contributor to the

lower fertility observed in the captive species management group. Interestingly, there was no significant variation in total sperm between wild and captive males despite wild individuals having the highest average of total sperm of all three groups. However, wild males also had the highest variation in total sperm, ranging from 500 sperm to 24.2 $\times$ 10^6 sperm per sample. Regardless of this variation, the average total sperm of wild males was almost 17$\times$ higher than that of captive males, and approximately 72% of wild individuals had a total sperm value greater than the average captive male.

It is important to note that semi-captive males performed comparably to wild males, but better than captive males; thus, it may be possible to increase male fertility by housing male *L. chiricahuensis* outdoors at their respective zoological facilities where they have access to environmental cues that indoor-housed animals do not. Additionally, the results presented here are from hormonally induced males. ARTs have been hailed as a solution to reduced fertility in captive animals; however, we show that male sperm concentrations in captive individuals remain low following a single treatment of exogenous hormones. If captive male frogs exhibit chronically low levels of circulating testosterone, acute exogenous hormone therapies may not be sufficient to compensate for long-term androgen deficiencies. Because spermatogenesis is not an instantaneous process and is driven by androgen fluctuations (Rastogi et al., 2011), increasing sperm concentrations may require repeated hormone supplementation to increase circulating testosterone levels and allow for spermatogenic development that is more consistent with cycles found in wild males.

The effects of captivity on other taxa, primarily large mammals, have been studied at length to inform captive breeding program management, yet little is known about the effects of captivity on amphibian fecundity. However, it is apparent that mimicking environmental cues and more naturalistic housing is necessary for eliciting natural breeding behaviors in many captive animals, especially amphibians (Kouba et al., 2009). Moreover, when favorable breeding conditions or nutritional needs are not met, anurans may not undergo proper gametogenesis or attempt to breed in future breeding years (Cayuela et al., 2014). In order to artificially stimulate reproductive cyclicity in captive amphibians, many zoological institutions and breeding facilities put their animals through a period of artificial hibernation (Calatayud et al., 2015; Kouba et al., 2021; Roth et al., 2010). Unfortunately, this process can pose a risk to animal health and its success is variable with high chance of mortality (Kouba et al., 2009). In addition, despite the attempted replication of environmental cues, many species still do not readily or sufficiently reproduce in captivity (e.g., *L. sevosa* or *A. zeteki*) or breeding attempts are minimally effective, as reported for *L. chiricahuensis*. For these species, the use of ART may be necessary for reproduction to bypass the need for environmental cues. This study demonstrates that hormone therapies are a successful tool for eliciting sperm from *L. chiricahuensis* and provides insight into the long-term effects of captivity on sperm output. Due to their dwindling populations and low natural breeding success in captivity, the use of ART and alternative semi-captive housing for this species may be important strategies for increasing offspring numbers and enhancing conservation efforts for *L. chiricahuensis*.

AUTHOR CONTRIBUTIONS

Allison R. Julien—Conceptualization, methodology, investigation, data curation, formal analysis, visualization, validation, writing original draft, review and editing. Kristen R. Counsell—Investigation. Isabella J. Burger—Data collection, data modeling and analysis, and manuscript editing. Andrew J. Kouba—Project administration, acquisition of funding and resources, writing review and editing. Diane Barber—Project conceptualization, acquisition of funding, supervision and resources, coordination of data collection and study animals at The Fort Worth Zoo. Cassidi Cobos—Coordinated study animals at the Ladder Ranch. Randy D. Jennings and Bruce L. Christman—Facilitation of animal collection in the field and provided geographic and species expertise. Carrie K. Kouba—Project conceptualization and administration, acquisition of funding and resources, supervision, investigation, data collection, writing review and editing.

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CONFLICT OF INTEREST

The authors declare that there were no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All animal collections and handling were approved by both Fish and Wildlife Service permits and IACUC committees at each intuition. These reference numbers can be found in Section 2.

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