

Collection and characterization of semen from green iguanas (*Iguana iguana*)

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Objective—To determine an efficient method for the collection of semen samples by means of electroejaculation, characterize spermatozoa quality and quantity, and determine the effect of refrigerated storage on motility of spermatozoa obtained from green iguanas (*Iguana iguana*).

Animals—18 adult green iguanas.

Procedures—Green iguanas were anesthetized, and semen samples were obtained by means of electroejaculation. Up to 3 series of electrostimulations were performed; the procedure was stopped after a semen sample was obtained. Various semen sample variables were evaluated.

Results—Semen samples were obtained from 16 iguanas; most (n = 10) iguanas produced a semen sample after the second series of electrostimulations. Median semen sample volume was 0.05 mL. Mean spermatozoa concentration was 269.0×10^6 spermatozoa/mL. Median percentage of motile spermatozoa was 78%. The only morphological abnormality of spermatozoa was bent tails (mean percentage in a semen sample, 5.7%). Spermatozoa motility decreased significantly during refrigeration (4°C); median percentage motility after 24, 48, and 72 hours of refrigeration was 60%, 33%, and 0%, respectively.

Conclusions and Clinical Relevance—Results of this study suggested electroejaculation can be performed to collect semen samples from green iguanas, characteristics of iguana semen samples are similar to those for semen samples obtained from other reptiles, and motility of iguana spermatozoa decreases during refrigeration within 48 to 72 hours. (*Am J Vet Res* 2013;74:1536–1541)

Gamete collection, gamete preservation, and artificial insemination have become an integral part of in situ and ex situ conservation programs for threatened and endangered species. Although assisted reproductive technologies are typically investigated and used for higher-taxa animals, few studies have been conducted to investigate such techniques for reptiles, particularly lizards. Such studies are warranted because almost 300 species of reptiles are in need of conservation efforts, yet the conservation needs of < 20% of reptile species have been evaluated.^{1,2}

The phylogenetic suborder Lacertilia includes many threatened or endangered species that could ben-

efit from reproductive management, such as the Komodo dragon (*Varanus komodoensis*), Mona ground iguana (*Cyclura stejnegeri*), Fiji banded iguana (*Brachylophus fasciatus*), Fiji crested iguana (*Brachylophus vitiensis*), and La Gomera giant lizard (*Gallotia bravoana*), among others.^{3,4} Many captive populations of such animals are not self-sustaining because of geographic isolation from potential mates or environments different from their native environment, resulting in abnormalities in physiologic and behavioral cues necessary to stimulate copulation.^{2,5,6} Assisted reproductive technologies such as semen collection and preservation can increase the genetic diversity and reproductive competence of such species and, thereby, their long-term survival.

A large amount of information is known regarding the reproductive biology of reptiles^{7–9}; however, few studies^{1,10,11} have been conducted to evaluate collection methods and characterize semen in reptiles, despite the high number of reptiles in the world. Semen collection has only been reported^{2,a} for 2 species of lizard: the common house gecko (*Hemidactylus frenatus*) and the McCann's skink (*Oligosoma maccanni*). Although the spermatozoal morphology of various lizard species have been described in several reports^{12–18} in an attempt to determine phylogenetic traits, no studies have been conducted to determine collection methods and characteristics of semen in iguanids, to the authors' knowledge. The development of safe, consistent semen col-

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lection protocols is warranted for the development of reproductive assistance programs for such reptiles.

The purpose of the study reported here was to determine a protocol for the collection and evaluation of semen samples from green iguanas (*Iguana iguana*). This species was selected because such animals are readily available, and findings for this species may be applicable for other threatened or endangered iguanids and possibly other lizard species (eg, varanids). The objectives were to determine an efficient method for the collection of semen samples by means of electroejaculation, characterize spermatozoa quality and quantity, and determine the effect of refrigerated storage on motility of spermatozoa of captive green iguanas. Hypotheses were that semen samples could be collected from green iguanas by means of electroejaculation, spermatozoa concentrations would differ significantly among ejaculates of different colors, and spermatozoa would survive < 72 hours during storage at a standard refrigeration temperature.

Materials and Methods

Animals—This study was conducted with approval of the University of Illinois Institutional Animal Care and Use Committee. Eighteen male green iguanas (age, > 3 years) were used in the study; the lizards were from a captive breeding population in El Salvador. Iguanas were determined to be healthy on the basis of results of thorough physical examinations. Weight was measured for each animal.

Semen sample collection and analysis—Because semen samples were collected by use of an electroejaculator, the iguanas were anesthetized with propofol^b (10 mg/kg, IV) to minimize discomfort. Electroejaculation was used rather than manual massage because that method was considered to be potentially more successful for collection of semen samples because of the size of the animals. The cloaca was lavaged with 20 mL of saline (0.9% NaCl) solution prior to semen sample collection to avoid contamination of the samples. The electroejaculator was comprised of a variable voltage power source and a plastic rectal probe (diameter, 2.6 cm) with 3 electrodes (3.0 mm) arrayed longitudinally. Animals were electroejaculated by performance of 15 cloacal intromissions at 4 V, followed by 15 cloacal intromissions at 6 V (Figure 1). A period of 3 minutes was allowed between each series of electroejaculations. Iguanas were electroejaculated up to 3 times by use of this protocol. After collection of a semen sample was successful, electroejaculation was discontinued. The number of times electroejaculation was required for each animal was recorded. Semen samples were collected with a 1-mL syringe without a needle.

After collection, semen samples from each iguana were evaluated to determine sample volume and color and spermatozoa concentration, motility, and morphology. Volume was measured with the 1-mL collection syringes. Each fresh semen sample was extended by 1:10 dilution in modified Ham's F-10 nutrient mixture with albumin.^c Motility of spermatozoa in extended semen samples was estimated by placing a drop of diluted semen on a slide under a coverslip at ambient tempera-

ture and estimating the percentage of progressively motile spermatozoa to the nearest 5% in 5 hpfs (magnification, 400X). To evaluate concentration of spermatozoa, extended semen samples were diluted 1:10 in formal saline solution (total dilution, 1:100) and spermatozoa were counted in a hemocytometer chamber by means of phase contrast microscopy (magnification, 400X). Spermatozoa concentration (cell count) in each sample was calculated by use of the hemocytometer and a conversion factor that accounted for dilutions. Morphology of spermatozoa was evaluated by observing 100 spermatozoa by means of oil-immersion microscopy (magnification, 1,000X). The percentage of each spermatozoal morphological type was determined for each semen sample.

The semen samples were evaluated to determine spermatozoa viability following refrigerated storage. Diluted semen samples were kept at ambient temperature for approximately 10 minutes during the initial semen evaluation. Then, extended semen samples were diluted 1:1 with ambient temperature refrigeration medium—test yolk buffer.^c A 0.3-mL aliquot of each diluted semen sample was pipetted into 2-mL tubes.^d Motility of spermatozoa was estimated before sample cooling (time, 0 hours). The rate of forward progression of spermatozoa was not evaluated. Tubes were placed in a standard refrigerator (4°C). To determine longevity of spermatozoa motility during refrigeration, motility for each semen sample was estimated every 24 hours until motility was 0%.

Statistical analysis—The distribution of data for semen sample volume and spermatozoa motility and concentration were evaluated separately by use of the Shapiro-Wilk test and Kolmogorov-Smirnov test. For normally distributed data, mean \pm SD and range were calculated. For nonnormally distributed data, median, range, and the 10th and 90th percentiles were calculated. The 95% confidence intervals were calculated for binomial proportions. The Pearson correlation test was used to determine whether body weight was correlated with spermatozoa concentration and whether spermatozoa motility percentage was correlated with semen sample volume or spermatozoa concentration. The Levene test for equality of variances was used to determine whether the data were homogeneous. One-way



Figure 1—Representative photograph of a male iguana depicting an electroejaculation procedure used to obtain semen samples.

ANOVA was used to assess differences among semen sample color types for semen sample volume, spermatozoa motility, and spermatozoa concentration for normally distributed data; for data that were nonnormally distributed, a Kruskal-Wallis test was used for such analyses. A Friedman test for nonparametric analysis of repeated data was used to compare spermatozoa motility in refrigerated semen samples over time. For data with significant differences, a Wilcoxon paired rank test was used to compare spermatozoa motility at the time of collection (0 hours) with that in semen samples after 24, 48, and 72 hours of refrigeration. Values of $P \leq 0.05$ were considered significant. Statistical analyses were performed with software.^{e,f}

Results

The ambient daytime temperature range during the times of semen sample collection in the present study was 29° to 32°C. The mean \pm SD weight of the iguanas was 2.5 \pm 0.31 kg (range, 2.0 to 3.2 kg). The median time to achieve anesthesia after administration of propofol was 2 minutes (10th to 90th percentile, 2.0 to 4.1 minutes; range, 2 to 17 minutes). Semen samples were successfully collected from 16 of 18 (95% confidence interval, 13 to 18) iguanas. A semen sample was obtained from most (10/16) iguanas after the second series of electrostimulations; a semen sample was obtained from 5 iguanas after the first series of electrostimulations and from 1 iguana after the third series of electrostimulations. Hemipenal eversion (complete, partial, or unilateral) was observed in 13 of 16 iguanas from which a semen sample was obtained. The median volume of the semen samples was 0.05 mL (10th to 90th percentile, 0.01 to 0.10 mL; range, 0.01 to 0.37 mL). The mean \pm SD spermatozoa concentration in semen samples was 269.0 $\times 10^6$ spermatozoa/mL \pm 311.64 $\times 10^6$ spermatozoa/mL (range, 12.0 $\times 10^6$ spermatozoa/mL to 1,030.2 $\times 10^6$ spermatozoa/mL). No significant correlation was detected between spermatozoa concentration and body weight ($r = -0.12$; $P = 0.65$) or semen sample volume ($r = 0.10$; $P = 0.71$).

Three color types of semen samples were identified: clear, tan, and white. Most (9/16) semen samples were tan; 5 semen samples were white, and 2 were clear. Spermatozoa concentrations were significantly ($P = 0.04$) different among semen sample colors. White semen samples had significantly higher spermatozoa concentrations (mean \pm SD, 573.2 $\times 10^6$ spermatozoa/mL \pm 326.4 $\times 10^6$ spermatozoa/mL; range, 258.0 $\times 10^6$ spermatozoa/mL to 1,030.2 $\times 10^6$ spermatozoa/mL) versus tan (mean \pm SD, 190.7 $\times 10^6$ spermatozoa/mL \pm 245.7 $\times 10^6$ spermatozoa/mL; range, 23.2 $\times 10^6$ spermatozoa/mL to 823.0 $\times 10^6$ spermatozoa/mL) and clear (mean \pm SD, 12.9 $\times 10^6$ spermatozoa/mL \pm 1.2 $\times 10^6$ spermatozoa/mL; range, 12.0 $\times 10^6$ spermatozoa/mL to 13.7 $\times 10^6$ spermatozoa/mL) semen samples. No contamination of semen samples with urates was observed. No significant ($P = 0.35$) difference in semen sample volume was detected among sample color types.

The median percentage of motile spermatozoa at the time of semen sample collection was 78% (10th to 90th percentile, 48.2% to 95.0%; range, 44% to 95%). No significant ($P = 0.7$) difference was detected in sper-

matozoa motility among semen sample colors. Motility was not correlated with spermatozoa concentration ($r = 0.29$; $P = 0.29$) or semen sample volume ($r = 0.14$; $P = 0.59$). Most (mean, 94.2%; SD, 3.8%; range, 87% to 100%) spermatozoa in semen samples had a normal appearance. The only morphological abnormality of spermatozoa detected in semen samples of the iguanas was bent tails (mean \pm SD, 5.7% \pm 3.8%; range, 0% to 13%).

A significant ($P = 0.001$) reduction in the percentage of spermatozoa that were motile was detected during refrigeration. Compared with motility at the time of semen sample collection (78%), the percentage of spermatozoa that were motile in semen samples was significantly ($P = 0.001$) lower after 24 (median, 60%; 10th to 90th percentile, 3.5% to 81.5%; range, 0% to 85%), 48 (median, 33.0%; 10th to 90th percentile, 0% to 69.5%; range, 0% to 80%), and 72 (median, 0%; 10th to 90th percentile, 0% to 43.3%; range, 0% to 51.0%) hours of refrigeration. Overall, the median percentage of motile spermatozoa in semen samples decreased by 23.1%, 57.7%, and 100% after 24, 48, and 72 hours of refrigeration, respectively.

Discussion

Unlike for mammals, the neuronal pathway that controls ejaculation in reptiles is unknown, to the authors' knowledge. However, anatomic similarities between urogenital systems of mammals and those of reptiles suggest that they have similar innervation. Green iguanas have paired testicles located dorsally and oriented longitudinally in the coelom. The epididymis arises from the caudal aspect of the testis, courses in a caudal direction to become the ductus deferens alongside the kidneys, and eventually terminates at the hemipenes.¹⁹ The paired hemipenes are sac-like structures located lateral to the cloaca that invert into the base of the tail, held in place by a retractor muscle.²⁰ They lack erectile tissue and are not associated with the urinary tract.²¹ During copulation, one of the hemipenes is everted and sperm travels through the mesonephric ducts to the sulcus spermaticus, a dorsal groove on the outside of the base of each hemipenis, which forms a passage for sperm to be deposited in the cloaca of a female.^{22,23} Unlike mammals, green iguanas do not have accessory sex organs.

Electroejaculation has been attempted in reptiles of many species. That technique has been successful for Madagascar ploughshare tortoises (*Astrochelys yniphora*), green sea turtles (*Chelonia mydas*), black marsh turtles (*Siebenrockiella crassicollis*), olive ridley turtles (*Lepidochelys olivacea*), hawksbill turtles (*Eretmochelys imbricata*), alligators, Galapagos tortoises (*Chelonoidis nigra*), red-eared pond turtles (*Trachemys scripta elegans*), and checkered garter snakes (*Thamnophis marciatus*); techniques used for successful electroejaculation of such animals have been similar to those used for mammals,²⁴⁻²⁷ suggesting neuronal innervation of the genital tracts in reptiles is similar to that in mammals. In another study,²⁸ animals were manually restrained with or without the use of sedatives, a rectal probe was inserted into the cloaca, and massage of the ventrolateral aspect was typically performed in a caudal direction from the area of the testes to the cloaca.

Slow withdrawal of a rectal probe leads to contraction of the ductus deferens that moves semen to the tip of the hemipenis.²⁹ In some reptiles, maximum electrical stimulation is indicated by observation of extension of the hind limbs.²⁹ Such procedures have typically been adequate for semen sample collection, and viable motile spermatozoa have been obtained.

Results of the present study suggested electroejaculation was an effective method for collection of semen samples from green iguanas; samples were collected from 16 of 18 animals in this study. This is the first report of electroejaculation and semen sample collection in a species of lizard, to the authors' knowledge. We could not identify a reason that semen samples were not obtained from 2 of the iguanas because the protocol used was consistent for all 18 animals; however, a semen sample may not have been obtained from those iguanas because they may have required a deeper plane of anesthesia than the other animals,³⁰ had recent breeding activity, or had physiologic or anatomic differences from the other iguanas that could not be detected by means of gross examination. Electroejaculation in cattle is frequently performed without anesthesia; however, because we expected the procedure may cause discomfort, iguanas in this study were anesthetized. All iguanas received the same dose of propofol and were considered to be anesthetized, although effects of that anesthetic drug can be affected by method of administration, tissue distribution (eg, variability of fat content among animals), and time. Also, because this study was conducted during the breeding season for green iguanas, they may have recently produced an ejaculate and were not capable of producing a semen sample at the time the study was conducted. To minimize the likelihood of this, the animals included in the study were collected from a pen that housed only males. However, that would not have completely eliminated the possibility of recent ejaculation because male iguanas may attempt to breed other males during a breeding season. It is also possible that the anatomy or body conformation of those 2 iguanas affected our ability to collect semen samples from them; however, their weights and sizes were similar to those of the other iguanas in the study, so those factors were not expected to be important. In addition, pathological abnormalities of the reproductive tracts of those animals could not be ruled out because follow-up diagnostic testing was not performed.

In mammals, ejaculation of semen (including by means of electroejaculation) is associated with engorgement of the penis and discharge of ejaculate through the urethra. However, in the green iguanas in the present study, ejaculation of semen did not seem to require eversion of a hemipenis because semen was collected from 3 of 18 iguanas without hemipenis eversion. This observation was important because collection of semen samples may have been missed if the investigator expected hemipenis eversion was required for ejaculation, especially considering the small median semen sample volume (0.05 mL). In addition, after hemipenis eversion, semen could typically be collected from the iguanas by means of movement of the probe without further electrical stimulation.

Macroscopic examination of a semen sample is typically the first step in characterizing the quality of

the sample. For the green iguanas in the present study, semen sample color was useful for characterizing sample quality but sample volume was not. Although tan semen color was most commonly observed, white semen samples were found to have a higher spermatozoa concentration. These results were similar to those determined for semen samples of corn snakes (*Elaphe guttata*)³¹ for which white or opaque coloration of semen samples is associated with a higher number of spermatozoa in a semen sample versus samples of other colors. Similar results have also been determined for semen samples of mammals.³² Results of the present study indicated semen sample volume was not correlated with spermatozoa concentrations, suggesting the volume of a semen sample may not be predictive of quality (eg, spermatozoa concentration). This finding suggested semen samples, regardless of volume, should be evaluated microscopically to determine quality. Although the semen sample volumes for the iguanas in this study were small, compared with volumes of semen samples typically obtained from mammals, they were similar to those reported for other squamates: Angolan pythons (*Python anchietae*) and Timor pythons (*Python timoriensis*; range, 0.1 to 0.4 mL [obtained by means of manual collection]),¹⁰ Sinaloan milk snakes (*Lampropeltis triangulum sinaloae*; range, 0.25 to 0.50 mL [obtained by means of manual collection]),¹¹ Brazilian rattlesnakes (*Crotalus durissus terrificus*; mean, 0.02 mL),¹ checkered garter snakes (range, 0.05 to 0.10 mL [obtained by means of electroejaculation]),²⁸ and corn snakes (mean, 0.01 mL [obtained by means of manual collection]).³¹ Such small semen sample volumes are likely attributable to the lack of accessory sex glands in reptiles, which produce a large amount of the semen volume in mammals.

The spermatozoa concentrations (mean, 269.0×10^6 spermatozoa/mL) in semen samples obtained from green iguanas in this study were 18% to 57% of the values reported for other reptiles, including Angolan and Timor pythons ($1,500 \times 10^6$ spermatozoa/mL),¹⁰ Brazilian rattlesnakes ($1,380 \times 10^6$ spermatozoa/mL),¹ corn snakes (852×10^6 spermatozoa/mL),³¹ and green sea turtles (470×10^6 spermatozoa/mL).³³ The low concentration of spermatozoa in semen samples obtained from iguanas in the present study may have been attributable to differences between lizards and other reptiles. It is also possible that spermatozoa concentrations were low for iguanas because of the time of year during which semen samples were collected or the technique used to collect the samples. Semen samples were collected from these iguanas during the breeding season, so spermatozoa concentrations may have been affected by the number of times the animals had recently produced semen. Also, spermatozoa concentrations and semen sample volumes may have been affected by the electroejaculation method, considering that electroejaculation is an unnatural method of ejaculate production for iguanas.

The median percentage of motile spermatozoa at the time of semen sample collection from green iguanas in this study was 78%, which was similar to the value for snakes (Brazilian rattlesnake, 64%¹; Argentine boa constrictor [*Boa constrictor occidentalis*], 63%³⁴; checkered garter snake, 50% to 70%²⁸; corn snake, 92.5%³¹)

but substantially higher than the value for green sea turtles (36%).³³ Spermatozoa motility is likely associated with reproductive anatomy and physiology. For example, other authors³⁵ have theorized that the lower spermatozoa motility in chelonian semen, compared with that for semen of snakes and lizards, may be attributable to the short distance between the epididymis and penile groove in such animals, suggesting a low need for postepididymal spermatozoa activation. That distance is longer in iguanas, which may lead to a need for higher spermatozoa motility. Furthermore, some lizards (eg, *Lacerta vivipara*) have synchronized maturation of male and female gametes, and fertilization occurs shortly after copulation in such animals, which is different from the process in chelonians³⁵ and a possible reason why there is a speculated trade-off between motility and spermatozoa longevity in chelonian species. Postcollection semen sample handling, including the use of diluents, can also affect motility. For McCann's skinks, the percentage of motile spermatozoa in semen samples is highest (70%) following dilution in a medium developed for turtle semen,³⁵ and motility is maintained during 5 days of storage at 4°C.² In the present study, a single type of diluent was used (Ham's F-10 nutrient mixture with albumin), so it was not possible to determine whether diluent type had a similar effect on iguana semen. Although spermatozoa motility in semen samples of iguanas was higher at the time of collection than it is for skinks, motility decreased during 72 hours of refrigeration; these findings suggested that evaluation of other semen diluents may be warranted to increase the storage time during which spermatozoa are motile in iguana semen samples. Environmental temperature can also affect spermatozoa motility. Motility of spermatozoa was higher at low temperatures than at high temperatures for 2 of 3 turtle species examined in another study,³⁵ suggesting that fertility may be highest during autumn and winter matings. In the present study, semen samples were collected during the same time of year during which turtle spermatozoa is highest. During a preliminary study conducted in the summer (July) of the year during which the present study was conducted, we could not collect semen from the iguanas. Therefore, ambient temperature may have affected iguana semen production.

Few morphological abnormalities were observed for spermatozoa of green iguanas in the present study; approximately 94% of spermatozoa in semen samples were characterized as morphologically normal. Typically, morphological abnormalities of spermatozoa have not been evaluated in studies of reptile semen collection methods. However, in a study³¹ in which corn snake spermatozoa were evaluated, only 75% of the spermatozoa were considered morphologically normal; that percentage is comparable to the percentage determined for spermatozoa of domestic mammals.³⁶ The most common morphological abnormality of spermatozoa of corn snakes in that other study³¹ was bent tails (24.3%), which was the only morphological abnormality detected for spermatozoa of green iguanas in the present study (5.7%). Bent tails are typically considered an iatrogenic morphological change that develops during postcollection handling because of exposure to

a hypotonic solution or rapid cooling during sample buffering or dilution.³⁶ Modified Ham's F-10 nutrient mixture was used to dilute the semen samples obtained from corn snakes in that other study³¹ and green iguanas in the present study, so it was unlikely that the diluent caused the differences in spermatozoa morphology. Likewise, the environmental temperatures during that other study³¹ and those in the present study were similar, and cooling of semen samples was not performed until after spermatozoa were evaluated. A reason for the difference in spermatozoa morphology between corn snakes in that other study³¹ and green iguanas in the present study was not identified. However, because the percentage of morphologically normal spermatozoa for green iguanas was high, further experiments to determine causes of iatrogenic bent tail formation were not conducted.

Results of the present study indicated the quality of green iguana semen decreased during refrigeration; the percentage of motile spermatozoa significantly decreased from the time of collection to 24, 48, and 72 hours after collection. Although other cooling methods for semen samples should be evaluated, results of this study suggested that green iguana semen samples stored in a refrigerator at 4°C may have viable spermatozoa only long enough for use in assisted reproduction up to 24 hours after collection (median spermatozoa motility at that time was 60%). Successful artificial insemination has been performed by use of stored semen samples for corn snakes (30% motility)⁶ and checkered garter snakes (50% motility)²⁸; however, a semen sample with < 50% spermatozoa motility is not typically considered satisfactory for assisted reproduction in domestic mammals. Cryopreservation may prolong viability of spermatozoa in iguana semen samples after collection. Although we did not evaluate cryopreservation methods in the present study because of the small semen sample volumes, further studies may be warranted to determine the effects of cryoprotective additives on the motility of spermatozoa in thawed semen samples of green iguanas.

Reptilian reproduction differs in many ways from mammalian reproduction. For reptiles, sperm may be stored in the reproductive tract of females for months to years after breeding, sex determination is temperature dependent, and some species reproduce by means of parthenogenesis. Therefore, it is not surprising that variables for semen samples of reptiles (eg, spermatozoa volume, concentration, and motility) are different from those for mammals. For example, although semen sample volumes for reptiles are lower than those for mammals, spermatozoa concentrations in such samples are higher.³² Low semen sample volumes for reptiles, which may be caused by a lack of accessory sex glands (which produce seminal fluid in mammals), may be attributable to the short distance spermatozoa travel to reach the reproductive tracts of females during mating; such small ejaculate volumes may be compensated by high spermatozoa concentrations. Few data are published regarding the physiology of fertilization techniques and spermatozoa competition in reptiles that could help explain some of these differences among animals.² Further studies to determine such physi-

ologic factors may aid development of semen sample cryopreservation methods and refinement of artificial insemination techniques for reptiles.

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