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CHARACTERIZATION AND COOLED STORAGE OF SEMEN FROM CORN SNAKES (*ELAPHE GUTTATA*)

Brooke M. Fahrig, M.S., Mark A. Mitchell, D.V.M., M.S., Ph.D., Bruce E. Eilts, D.V.M., M.S., Dipl. A.C.T., and Dale L. Paccamonti, D.V.M., M.S., Dipl. A.C.T.

Abstract: The phylogenetic order Squamata has many representatives that could benefit from the use of semen preservation as a tool for assisting conservation. To date, few studies have been made evaluating the potential for collecting and preserving semen from snakes. The objectives of this study were to characterize semen parameters of the corn snake (*Elaphe guttata*), including appearance, volume, concentration, sperm motility, and sperm morphology, and to determine the longevity of corn snake sperm motility stored at 4°C. Single semen samples were collected from 22 adult corn snakes. The appearance of the corn snake semen was generally cloudy, and the color was white to tan. Corn snake spermatozoa initially exhibited a median motility of 92.5%. Corn snakes were found to produce small-volume ejaculates (median 0.01 ml). However, the overall concentration of the snake ejaculate was high ($\bar{x} = 852 \times 10^6 \pm 585 \times 10^6$ spermatozoa/ml). Morphologically, a mean of 75.7 ± 9.3% of the sperm cells in an ejaculate were normal. Snake ejaculate with a white appearance had significantly higher sperm concentrations ($\bar{x} = 1{,}859 \times 10^6 \pm 1{,}008 \times 10^6$ sperm cells/ml; $F = 15.74$, $P = 0.001$) than tan ejaculates ($\bar{x} = 601 \times 10^6 \pm 439 \times 10^6$ sperm cells/ml). Sperm motility decreased significantly in samples that were stored at 4°C for greater than 48 hr in a refrigerator or Equitainer I. This is the first study to characterize semen volume, appearance, and concentration; sperm motility; and sperm morphology in captive corn snakes. The information derived from this study can be used to develop a model for a collection, cooled storage, and shipping program for semen from endangered or threatened captive and wild snakes.

Key words: Corn snake, *Elaphe guttata*, semen, spermatozoa.

INTRODUCTION

The preservation of spermatozoa is an important reproductive assistance tool used in conservation programs to increase the genetic diversity of threatened and endangered species. Although routinely used to manage conservation programs for higher vertebrates, no attempts have been made to establish reproductive assistance programs for reptiles. The phylogenetic order Squamata has many representatives that could benefit from the use of semen preservation as a tool for assisting conservation. At present, the Maria Island snake (*Liophus ornatus*) and the San Francisco garter snake (*Thamnophis sirtalis tetrataenia*) are listed as endangered species, whereas the Aruba Island rattlesnake (*Crotalus unicolor*), Copperbelly water snake (*Nerodia erythrogaster neglecta*), and many other species of snakes are listed as threatened.[10] The long-term survival of these species could depend on the development of successful assisted reproduction programs, which could include artificial insemination with cooled or cryopreserved spermatozoa.

The reproductive biology of snakes has been described.[8,9] However, few studies describe the collection and characterization of snake semen.[4,6,7,11] The first report evaluating snake semen described a manual semen collection method but did not describe the semen characteristics.[4] A later study both described a manual semen collection method and characterized semen appearance and volume as well as sperm motility, concentration, and morphology for eight species of snakes.[6] This manual semen collection method has also been modified and used to study Brazilian rattlesnake (*Crotalus durissus terrificus*). In that study, the appearance, volume, and concentration of the semen collected and the motility of the spermatozoa were described.[11] Quinn et al.[7] collected semen from garter snakes by electroejaculation and reported semen volume and concentration and sperm motility. Variability in the semen samples among these 10 snake species suggests that individual reference values are needed for most species. To date, no studies have been published on the collection and characterization of spermatozoa from corn snakes (*Elaphe guttata*).

Only one study has evaluated the effect of cooled storage on snake spermatozoa. The authors had success in storing diluted snake semen at 5°C or in an ice bath for up to 96 hr, but percent motility was not reported.[6] Successful cooled storage of diluted snake semen makes cooperative efforts to artifi-

From the Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University, Skip Bertman Drive, Baton Rouge, Louisiana 70803, USA. Correspondence should be directed to Dr. Mark A. Mitchell.

cially inseminate female snakes across the United States possible.[6] The development of safe, consistent semen collection, evaluation, and storage protocols is needed to establish reproductive assistance programs for snakes.

The immediate goals of this study were to characterize semen parameters of the corn snake, including semen appearance, volume, and concentration and sperm motility and morphology and to determine the longevity of corn snake spermatozoa motility stored at 4°C. The long-term goal of this project is to use these techniques to develop a collection, cooled storage, and shipping program for captive and wild snakes. Currently, the genetic diversity of snakes maintained in U.S. zoological institutions is limited. The techniques developed in this study could be used to collect and store sperm from wild threatened or endangered snakes. This genetic material could then be transported worldwide by cooled shipping methods and be used to artificially inseminate female snakes.

MATERIALS AND METHODS

Twenty-two adult male corn snakes ranging in age from 3 to 10 yr were obtained for semen collection from a private reptile collector located in Louisiana. The samples were collected between May and June 2005. All of the snakes used in this study had successfully reproduced in 2004. The snakes were housed in plastic containers (58 × 43 × 13 cm; 23 × 17 × 5 inches) on aspen bedding. The environmental temperature and humidity were maintained between 26°C and 27°C and 50% and 60%, respectively. The snakes were offered whole mouse prey weekly and had access to chlorinated tap water in a bowl. A thorough physical examination was performed on the animals to determine that they were in good health. Debris around the cloaca was removed manually to minimize contamination. The cloaca was lavaged with physiologic saline before sample collection. Semen was collected by gentle craniocaudal digital massage of the caudoventral one third of the snake. Semen expelled from the vent was collected with a tuberculin syringe.[6] A single semen sample was collected from each snake.

The ejaculates from each animal were immediately evaluated for appearance, volume, concentration, sperm motility, and sperm morphology. Semen sample volume was approximated to the nearest 0.005 ml. Each fresh semen sample was evaluated for appearance and then extended by adding 1 ml of modified Ham's F10 with albumin (Irvine Scientific, Santa Ana, California 92705, USA) to the sample regardless of initial sample volume. Motility of the spermatozoa in the extended semen was estimated by placing a drop of diluted semen on a slide under a coverslip at ambient temperature (26–27°C) and estimating the percentage of progressively motile sperm cells to the nearest 5% in five different microscopic fields under ×400 magnification. To determine the concentration, a portion of the extended semen was further diluted 1:10 in formol-buffered saline, and the sperm cells were counted in both chambers of a hemacytometer under phase contrast microscopy (×400). Concentration of the semen sample was calculated with the hemacytometer sperm cell count and a conversion factor, taking into account both dilutions. Morphology was evaluated by observing 100 sperm cells under phase-contrast microscopy at ×1,000 magnification; the percentage of each different morphologic type was determined for each sample.

The semen collected from 11 of the snakes was prepared for refrigerated storage. The diluted semen was held at room temperature for approximately 10 min while the initial semen evaluation was performed. The extended semen was then diluted 1:1 with Refrigeration Test Yolk Buffer (Irvine Scientific, Santa Ana, California 92705, USA) at ambient temperature. Two 0.3-ml aliquots of the diluted sample were pipetted into separate 2-ml cryotubes (Nunc, Rochester, New York 14625, USA). Motility of the sperm (0 hr) was then estimated before cooling. One of the vials was placed in a room temperature Styrofoam rack and placed in a standard refrigerator (4°C), and the other vial was placed in the cup-style chamber of an Equitainer I cooled semen transporter (Hamilton Research Inc., South Hamilton, Massachusetts 01982, USA) for storage. For storage in the Equitainer I, only one set of two ice packs was used inside the container beginning at 0 hr to simulate the shipping process, during which ice packs would not be replaced. The Equitainer I cooled semen transporter with two ice packs was determined by the manufacturer to keep semen cool for up to 70 hr. To determine sperm longevity, motility was estimated every 24 hr until motility dropped to 0%.

The distribution of snake age, ejaculate volume, motility, and spermatozoa concentration were evaluated separately by the Shapiro–Wilk test statistic. For normally distributed values, the mean, standard deviation, 95% confidence interval (CI), and minimum and maximum (min/max) were calculated. For nonnormally distributed values, the median, 25–75% quartiles, and min/max were calculated. The Levene test for equality of variances was used to determine whether the data were homogeneous. One-way analysis of variance (ANOVA) was used

Table 1. Abnormal morphologic findings in corn snake semen (n = 22).

Abnormal morphologic types	Median (%)	25–75% quartile	Min/max
Folded tail	18.5	11.0–21.3	6–40
Detached head	1.0	0.0–3.0	0–10
Proximal droplet	0.5	0.0–5.3	0–14
Distal droplet	0.5	0.0–3.0	0–7
Coiled tail	0.0	0.0–1.3	0–4
Abnormal head shape	0.0	0.0–0.0	0–1

to assess between-group differences for ejaculate volume, spermatozoa motility and concentration by age, and ejaculate appearance for normally distributed data. For data that were not normally distributed, a Kruskal–Wallis one-way ANOVA was used to assess differences between and within groups, respectively. Motility in the two different cooling methods was evaluated over time with the use of Friedman's nonparametric analysis of repeated data. Where differences were apparent, Rhyne and Steele's method for comparison of related samples to a control time (0 hr) was used. Paired t-tests were used to assess differences in spermatozoa motility between the two cooling methods at 24, 28, 72, 96, and 120 hr. Values of $P < 0.05$ were considered to reflect statistical differences. Statistical analyses were performed with SPSS 8.0 (SPSS Inc., Chicago, Illinois 60606, USA).

RESULTS

The median age of the snakes in this study was 5 yr (25–75% quartile 4–6 yr; min/max 3/10 yr). Age had no significant effect on semen volume (F = 0.3, P = 0.6), concentration (F = 0.3, P = 0.9), or motility (F = 0.002, P = 0.9). The mean time to stimulate the snakes to ejaculate was 9.1 ± 2.9 min (95% CI 7.7–10.4 min; min/max 4/15 min). Appearance of the semen was generally cloudy, and the color was white to tan. Ejaculate volume (F = 0.6, P = 0.5) and sperm motility (F = 1.7, P =0.2) were not significantly affected by semen appearance. The spermatozoa initially exhibited a median percentage of progressive motility of 92.5% (25–75% quartile 83.0–96.3%; min/max 43/99%). Corn snakes were found to produce ejaculates with a median volume of 0.01 ml (25–75% quartile 0.005–0.010 ml; min/max 0.005/0.030 ml). The mean concentration of the corn snake ejaculates was 852 × 10^6 ± 585 × 10^6 sperm cells/ml (95% CI 578–1,126 cells/ml; min/max 33/2,161 cells/ml). The ejaculates that had a white appearance had significantly higher sperm concentrations ($\bar{x}$ = 1,859 × 10^6 ± 1,008 × 10^6 sperm cells/ml; F = 15.74, P = 0.001) than those with a tan appearance ($\bar{x}$ = 601 × 10^6 ± 439 × 10^6 sperm cells/ml). Morphologically, 75.7% (95% CI 71.6–79.9%; min/max 60/89%) of the sperm cells in an ejaculate were normal, whereas approximately 24.3% (95% CI 20.1–28.4%; min/max 11.0/40.0%) of the spermatozoa exhibited folded tails and other abnormal morphologies (Table 1).

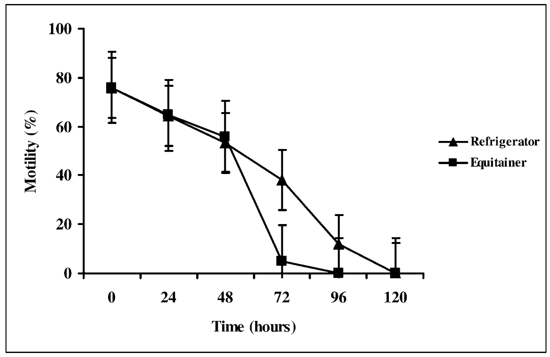

Figure 1. Percent motility (mean ± SE) at 24-hr intervals of corn snake spermatozoa stored at 4°C (n = 11).

Mean motility of the spermatozoa stored at 4°C in either a standard refrigerator or an Equitainer I was greater than 50% at 48 hr, but motility of spermatozoa from all snakes in both the refrigerator and Equitainer I dropped to 0% by 120 hr (Fig. 1). Motility of the spermatozoa stored in either the refrigerator or Equitainer I between 0 and 24 hr was not significantly different. Motility was not significantly reduced at 48, 72, 96, and 120 hr compared with 0 and 24 hr for samples stored in the refrigerator or Equitainer I (Fig. 1). Spermatozoa motility in samples that were cooled in the refrigerator was significantly higher than for those stored in the Equitainer I at 72 hr (P = 0.02; refrigerator 38.1 ± 6.8%, Equitainer I 14.5 ± 5.9%) and 96 hr (P = 0.003; refrigerator 11.7 ± 3.0%, Equitainer I 0.2 ± 0.2%). Spermatozoa motility between the refrigerated or Equitainer I samples at 24 hr (P = 0.9), 48 hr (P = 0.3), or 120 hr (P = 0.9) was not significantly different (Fig. 1).

DISCUSSION

This is the first study to characterize semen appearance, volume, and concentration and sperm motility and morphology in captive corn snakes. In this study, the corn snake semen had a cloudy, white to tan appearance. In comparison, semen collected manually from the Brazilian rattlesnake had a white, milky appearance.[11] Corn snake ejaculates that had a white appearance had significantly higher sperm counts than tan ejaculates. More concentrated semen might appear to have a whiter appearance than less concentrated semen because of the increased number of sperm cells, as evidenced by greater opacity of more concentrated semen samples in other vertebrates. Individuals collecting semen samples from corn snakes might be able to predict the potential value of certain samples on the basis of their appearance. Whether this difference occurs in other species of snakes has not been tested but should be pursued.

Corn snake spermatozoa initially exhibited motility comparable to that of domestic mammals.[1] However, compared with domestic mammals, corn snakes were found to produce low-volume, highly concentrated ejaculates.[1] The low volume and high concentration of snake semen might be necessary because of the sperm transport mechanism from male to female. During copulation, a hemipenis evaginates into the cloaca of a receptive female. Sperm is carried by the Wolffian ducts to the base of the hemipenis and then travels by the sulcus spermaticus on the outside of the hemipenis into the female.[5] A higher concentration of sperm might be expected in snakes because of the distance the sperm must travel and the expected losses that might be incurred along the way. Although it might be expected that a higher volume of fluid would also be associated with the ejaculate for the same reason, snakes do not possess the accessory organs found in mammals and, therefore, probably must rely on higher concentrations of sperm. The reproductive tract of the male snake is composed of smooth muscle. Because of the small volume of ejaculate, snakes might rely more on muscle contractions to propel the semen through the reproductive tract than on hydrostatic pressure.

Semen collected manually from an Angolan python (*Python anchietae*) and Timor python (*Python timoriensis*) had a volume of 0.1–0.4 ml and a concentration of approximately $1{,}500 \times 10^6$ sperm cells/ml,[6] whereas semen collected manually from the Brazilian rattlesnake had a volume, motility, and concentration of 0.015 ml, 70%, and $1{,}522 \times 10^6$ sperm cells/ml, respectively.[11] Checkered garter snake semen collected via electroejaculation was found to have a volume range of 0.05–0.10 ml and an initial motility of 50–70%.[7] In this study, the volume of semen produced by corn snakes was similar to the garter snakes and rattlesnakes, but lower than the pythons. The lower semen volumes observed in the corn, garter, and rattlesnakes might be associated with the smaller body size of these snakes when compared with that of the pythons. The lower concentration of sperm in the corn snakes could be attributed to the reproductive behavior of these snakes. Corn snakes will copulate with multiple females in a given reproductive season. Whereas many snakes have a relatively short reproductive window of 2–3 mo, the breeding season for corn snakes can as long as 6–7 mo (March–September) under appropriate conditions. An extended reproductive window combined with high spermatozoa motility (92.5%) would allow corn snakes to disseminate their genetic material while maintaining lower overall semen concentrations.

Morphologically, 75% of the corn snake sperm cells in an ejaculate were normal. The percent normal morphology in these snakes is comparable to that of the ram, bull, boar, stallion, and buck.[1] In mammals, a small number of morphologic abnormalities is considered normal in a healthy animal. Of the commonly measured semen parameters, morphologic abnormalities have the greatest negative correlation to fertility of farm animals, and heat stress is the main cause of sperm abnormalities in these animals.[1] The effect of heat stress on reptile spermatozoa has not been evaluated. Reptiles are ectotherms and depend on their environmental temperature to regulate their core temperature. It would not be unexpected for a corn snake to experience a 2.7–5.5°C (10–15°F) difference in body temperature in a given day. Although it has not been evaluated, one might expect that semen production would diminish and sperm abnormalities would increase in snakes that experience a large drop in body temperature. Genetics and exogenous drugs have also been associated with sperm cell abnormalities in higher vertebrates. To date, no studies have been made evaluating the abnormal morphologies of snake semen in comparison to that of mammals. Mengden et al.[6] speculated that abnormalities in snake semen morphology could be an indication of either the condition of the donor or problems in collection and storage techniques.

Folded tails were the most common abnormality observed in this study, and are also the most commonly observed abnormality in bull semen.[3] A folded tail, also known as distal midpiece reflex, can be induced in vivo or in vitro. In vivo, distal midpiece reflex occurs during epididymal passage and is recognized microscopically by observing cytoplasmic droplet material being trapped in the bend of the tail.[3] Bovine epididymal malfunction associated with distal midpiece reflex can be induced by low testosterone because of a variety of adverse stimuli, such as estradiol treatment, low thyroid activity, ephemeral fever, and scrotal insulation.[3] In vitro, exposure of bovine spermatozoa to a hypotonic solution or rapid cooling in a phosphate buffer or physiologic saline can cause distal midpiece reflex, which is recognized by observation of folded tails with no cytoplasmic droplet material trapped in the bend.[3] The pH and temperature of the samples could not be tested in this study because of the small size of the ejaculate. The pH of the diluents used in this study was 7.2–7.4, the physiologic range for semen of most higher vertebrates. Because the temperature of the ejaculate was presumed to be similar to the snake's body temperature (environmental temperature) and because the diluent was held at room temperature, we did not expect a large fluctuation in the temperature of the ejaculate to occur in this study. However, because the known causes of in vivo distal midpiece reflex in the bovine are not likely to affect the snake and because no cytoplasmic droplet material was observed in the majority of snake sperm tail bends, the folded tails were most likely iatrogenically induced. Proximal droplets and detached heads were also observed in corn snake spermatozoa. In bovine, proximal droplets are a sign of abnormal spermiogenesis and are commonly associated with a variety of other sperm defects.[2] A high number of detached heads is associated with testicular hypoplasia in bulls.[2] In snakes, detached heads could suggest that the donor snake is under stress and that proximal droplets along the head and tail are indicators of immature sperm cells.[6] Both of these changes were rare in this snake population.

Corn snake sperm motility decreased significantly in samples that were cooled for greater than 48 hr. However, corn snake spermatozoa maintained greater than 50% motility after up to 48 hr of cooled storage. At 72 and 96 hr, the samples stored in the Equitainer I had a dramatically lower motility than those stored in the refrigerator. As reported by the manufacturer, the Equitainer I cooled semen transporter is designed to cool and maintain semen for up to 70 hr. By 72 hr, the Equitainer I would be expected to lose its ability to cool and maintain the sperm motility. Although the motility of the refrigerated sperm was significantly higher than that found in the Equitainer I, it was not considered sufficient for reproductive assistance.

In domestic mammals, 50% motility is generally not considered satisfactory for use in assisted reproductive techniques, such as artificial insemination. However, in checkered garter snakes, fertilization resulted from artificial insemination of 0.05 ml of semen with 50% motility. These were the minimum values for volume and motility recorded for these snakes and, therefore, might not be the absolute minimum required for successful fertilization.[7] Because corn snake semen had greater than 50% motility at 48 hr when stored at 4°C in both the refrigerator and Equitainer I, it seems feasible to store samples at 4°C for up to 48 hr for use in artificial insemination and other reproductive assistance techniques. Cooled semen samples may be shipped in an Equitainer I container over a 48-hr period to any location where artificial insemination will take place. Samples can also be stored locally in a standard refrigerator for up to 48 hr until the sample can be used for artificial insemination.

For cases in which 48 hr is insufficient, cryopreservation of semen may be attempted. A single report claims some success in cryopreserving snake sperm.[6] However, we could not reproduce the results with corn snake semen by the same methods. Further studies should be performed to determine the feasibility of long-term cryopreservation of snake semen. The information derived from this study can be used to develop a model for a collection, cooled storage, and shipping program for semen from endangered or threatened captive and wild snakes.

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