Zoo Biology 26:155–160 (2007)

Technical Report

Semen Collection and Evaluation in Free-Ranging Brazilian Rattlesnakes (*Crotalus durissus terrificus*)

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Two hundred-ninety species of reptiles are estimated to need urgent action for conservation, with at least 113 threatened species worldwide. The International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species includes 80 species of snakes, with six native Brazilian species, a number likely to be an underestimation. Some authors believe that assisted reproduction would be an important tool to improve reproduction in captivity of some reptiles. An efficient technique for semen collection and evaluation is an important step in development of protocols for cryopreservation of semen or artificial insemination in snakes, contributing to the conservation of endangered species. Although these techniques are important, some basic semen parameters are described for four of the ~2,900 snake species in the world. The Brazilian rattlesnake (*Crotalus durissus terrificus*) was chosen as a model for semen collection in snakes because it is found quite often in Sao Paulo State. Semen was collected once from each animal by the same investigator during the mating season of this species in Brazil. After antiseptic cleansing of the skin around the cloaca, the snakes were injected subcutaneously with a dose of 15 mg/kg of 1% solution of lidocaine around the cloaca. Semen was collected with ventral massages after cloacal relaxation and directly from genital papilla inside the cloaca. A total of 28 ejaculates from 39 animals were obtained, representing

Grant sponsors: Conselho Nacional de Pesquisa (CNPQ); Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP); Grant number: 02/13947-1.

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Received 11 April 2006; Revised 28 December 2006; Accepted 5 January 2007

DOI 10.1002/zoo.20126
Published online 5 April 2007 in Wiley InterScience (www.interscience.wiley.com).

collection efficiency of 71.80%. Semen volume and concentration in Brazilian rattlesnakes ranged from 3–70 μl and from $0.94–2.23 \times 10^9$ spermatozoa/ml, respectively. Zoo Biol 26:155–160, 2007.

Keywords: spermatozoa; conservation; reptiles; serpentes

INTRODUCTION

Two hundred-ninety species of reptiles are estimated to need urgent action for conservation, with at least 113 threatened species worldwide [Ebenhard, 1995]. Brazil alone has 650 species of reptiles, with 330 species of snakes [Rodrigues, 2005]. The International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species [International Union for Conservation of Nature and Natural Resources, 2004] includes 80 species of snakes, with six native Brazilian species, a number likely to be an underestimation.

The captive propagation of endangered species plays an important role in conservation. But because most captive populations of snakes are not self-sustaining, some researchers believe that artificial insemination could be an important tool in snake conservation [Mengden et al., 1980; Quinn et al., 1989; Langlada et al., 1994]. Although semen collection and evaluation is an important step toward artificial insemination, some basic semen parameters are described for just four of the world's ~2,900 snake species [Mengden et al., 1980; Samour, 1986; Quinn et al., 1989; Langlada et al., 1991].

Previous researchers obtained semen from snakes, usually contaminated with feces and urates, by a 'stroking' method [Fitch, 1960]. Later, an improved collection technique resulted in viable and uncontaminated semen by exposing the genital papilla inside the cloaca and massaging the ventral portion of the snake toward the cloaca [Mengden et al., 1980; Samour, 1986]. Electro-ejaculation associated with ventral massages was attempted with variable results [Mengden et al., 1980; Quinn et al., 1989]. Another method required euthanasia of the snake, removal of the vas deferens and manual extrusion of semen into a Petri dish [Langlada et al., 1991], a technique that it is not acceptable in a conservation program.

In our study, the massage technique described by Mengden [1980] was modified by using a local anesthetic around the cloaca. We obtained and evaluated semen from Brazilian rattlesnakes (*Crotalus durissus terrificus*) as a model for the development of assisted reproduction techniques in snakes.

MATERIALS AND METHODS

Animals

The Brazilian rattlesnake (*Crotalus durissus terrificus*) was chosen for this study because it is found quite often in Sao Paulo State. Almeida and Santos et al., 2004 documented previously the presence of mature spermatozoa in males of this species with total length ≥ 56 cm by histology. All 39 males in the present study were considered sexually mature because they measured between 77–121 cm total length. The snakes were wild-caught in Sao Paulo State by farmers, ranchers, animal control personnel, or firemen, and brought to the Laboratory of Herpetology at the

Butantan Institute. Semen collection was attempted within 48 hr of the snake's arrival to avoid the influence of prolonged captivity. All housing and procedures were reviewed and approved by the Butantan Institute and the University of Sao Paulo Institutional Animal Care and Use Committees and were carried out according to the Guiding Principles for the Care and Use of Laboratory Animals.

Semen Collection and Evaluation

Semen was collected once from each animal by the same investigator in the fall of 2003, the mating season for this species in Brazil [Almeida-Santos et al., 2004]. The snake was restrained using a snake hook and a plastic tube [Murphy and Armstrong, 1978]. Each animal was weighed, measured, and examined for general body condition. After antiseptic cleansing of the skin around the cloaca, the snakes were injected subcutaneously (s.c.) around the cloaca using a 1% solution of lidocaine local anesthetic (Cristália, Sao Paulo, Brazil). The dose of 15 mg/kg was diluted to a total volume of 1.0 ml in normal saline. The total volume of anesthetic was divided between four injection sites (0.25 ml/site) anterior to the cloaca (Fig. 1). The snake was released and assessed for cloacal relaxation every 5 min. The anesthesia allowed access to the genital papilla inside the relaxed cloaca, usually within 15 min. Semen was collected directly from the papilla with a needleless 1-cc syringe (Fig. 2), by massaging the ventral region of the snake toward the cloaca (Fig. 3) as described by Mengden et al. [1980].

Seminal volume, motility, and progressive motility were assessed immediately after collection. The ejaculate volume was determined by aspirating the semen into a graduated micropipette. Motility was expressed as the percentage of motile sperm.

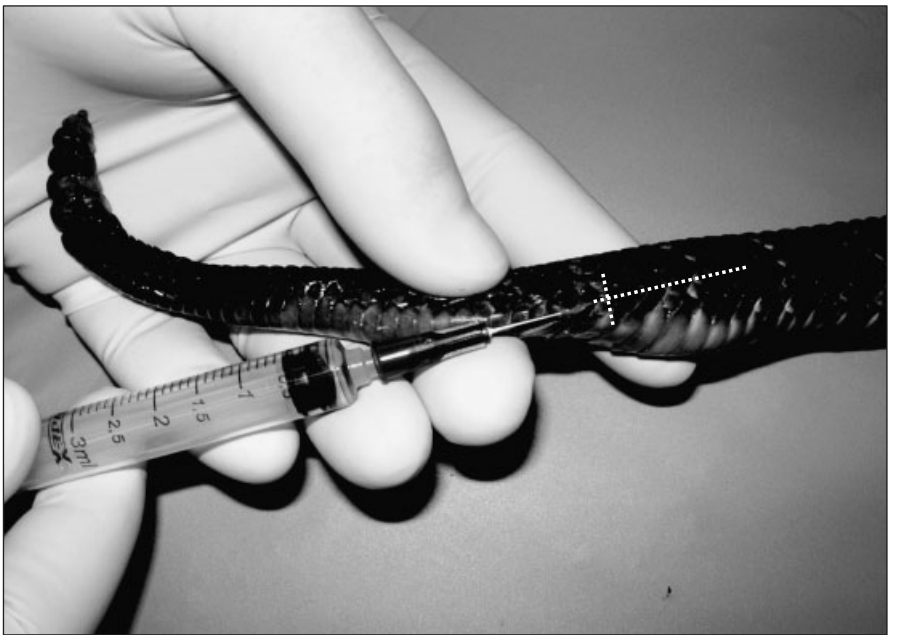

Fig. 1. Local anesthetic was injected at four different sites anterior to the cloaca. The dashed line represents the two injection sites on the right side of snake.

Fig. 2. Semen was collected directly from the genital papilla (arrow) inside the cloaca, with a needleless 1-cc syringe.

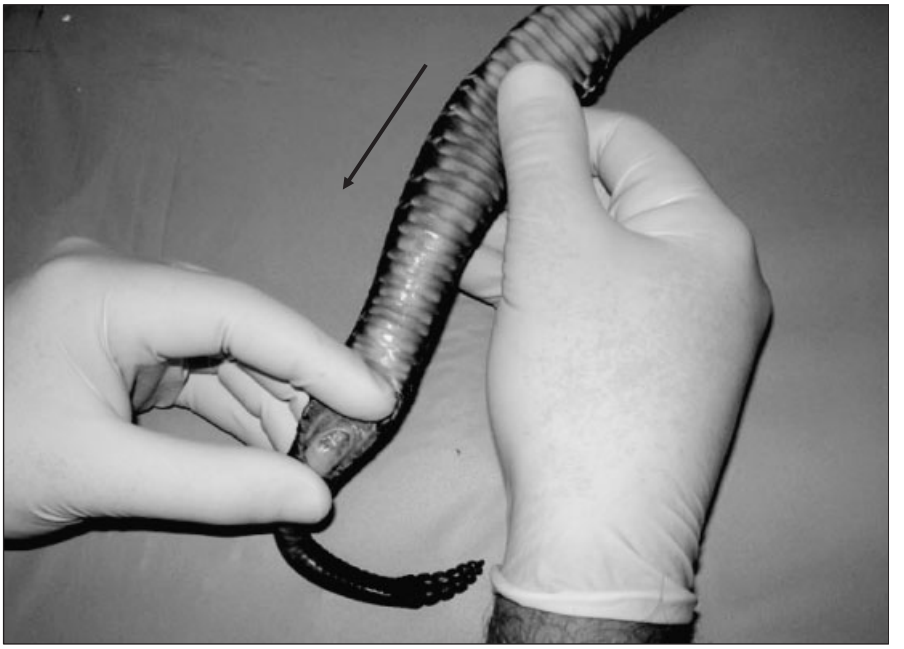

Fig. 3. Semen was collected by ventral massage (arrow represents the direction of movement toward the cloaca).

TABLE 1. Seminal characteristics of 28 wild caught Brazilian rattlesnakes (*Crotalus durissus terrificus*) collected during fall 2003 in Sao Paulo State

Parameter	Mean ± SEM	Range
Seminal volume (μl)	18.52 ± 5.61	3–70
Sperm concentration ($\times 10^9$/ml)	1.38 ± 0.13	0.94–2.23
Motility (%)	63.88 ± 4.43	5–90
Progressive motility	2.96 ± 0.17	1–4

Spermatozoa' speed of progression was graded on a 5-point scale (0 = no motility, 1 = non-progressive motility, 2 = slow progressive motility, 3 = lateral head movement accompanied by slow progressive motility, 4 = fast progressive motility, and 5 = very fast progressive motility). Sperm concentration was assessed after dilution of 2 μl of semen to 998 μl of 10% buffered formalin solution. The concentration was counted in duplicate using a hematocytometer chamber (improved Neubauer, 0.10 mm depth).

Statistical Analysis

The results are reported as means ± SEM, with *n* representing the number of animals.

RESULTS

Collection and Characteristics of Semen

A total of 28 ejaculates from 39 animals were obtained, representing collection efficiency of 71.80%. Semen parameters are depicted in Table 1.

DISCUSSION

This study represents an important step in the development of assisted reproduction in snakes. The addition of local anesthesia helps the semen collection procedure giving a better control over the cloaca and avoiding contamination.

In our experiment, we obtained semen samples in approximately 72% of the snakes on the first attempt. Mengden et al. [1980] collected semen from eight different species of snakes and obtained samples in 50% of the animals on the first attempt. Interspecies variation is an important factor that could explain the comparatively lower collection efficiency in their study. Individual variations can contribute to this difference, particularly when comparing wild-caught animals vs. captive ones.

The volume and concentration of semen obtained in our experiment were within ranges of results obtained for other species using similar methods [Mengden et al., 1980; Samour, 1986; Quinn et al., 1989]. However, the average number of 0.0036×10^9 spermatozoa/ejaculate described for the Brazilian rattlesnake [Langlada et al., 1991] is approximately seven times smaller than the results we obtained (0.02484×10^9 spermatozoa/ejaculate). These results are probably due to differences in collection techniques. The results described by Langlada (1991) are the average for

an entire year whereas our data is just for fall (mating season). Total motility and spermatozoa' speed of progression in the present study were within range described for the Brazilian rattlesnake and other species [Mengden et al., 1980; Samour, 1986; Quinn et al., 1989; Langlada et al., 1991].

An efficient technique for semen collection and evaluation is an important step in development of protocols for cryopreservation of semen or artificial insemination in snakes, contributing to the conservation of endangered species.

CONCLUSIONS

1. Semen collection is possible in Brazilian rattlesnakes using ventral massage associated with local anesthesia for cloacal relaxation.
2. Semen volume and concentration in Brazilian rattlesnakes ranged from 3–70 μl and from $0.94–2.23 \times 10^9$ spermatozoa/ml, respectively.

ACKNOWLEDGMENTS

We thank Dr. B. Durrant for her advice and comments on this manuscript.

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