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Annual changes in seminal variables of golden lanchead pitvipers (*Bothrops insularis*) maintained in captivity

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ABSTRACT

Bothrops insularis is an endemic and critically endangered snake with an estimated population of 2000 individuals restricted to Queimada Grande Island, in southeastern Brazil. Brazilian researchers established a captive breeding program for the species that includes the application of assisted reproductive technologies. The present study, therefore, aimed to evaluate semen samples from captive *B. insularis* throughout the year to ascertain seasonal differences in semen traits as well as correlations with body size and weight. Eighteen males with snout-vent length (SVL) ranging from 43.5 to 73.7 cm were collected at quarterly basis between August 2012 and May 2013. Macroscopic analysis revealed semen volumes ranging from 0.5 to 6.0 μL with samples featuring whitish to yellowish color and creamy and thick consistency. Viable sperm was obtained from all males indicating that individuals with SVL equal to or greater than 43.5 cm are sexually developed. However, adult and immature males (estimated by SVL) exhibited different seasonal profiles for motility and progressive motility. Adult males had a decrease in sperm motility and progressive motility during summer and spring, respectively, whereas the same variables did not vary throughout the year in immature snakes. Sperm concentration in all individuals was less (0.5×10^9 μL) during the winter, but no seasonal fluctuations were detected in semen volume. These findings are of particular importance to the development of reproductive tools such as male selection, artificial insemination and sperm freezing for the genetic management of this critically endangered snake.

1. Introduction

The golden lancehead (*Bothrops insularis*) is a venomous pitviper only found in Queimada Grande Island, a small landmass (43 ha) located 40 miles off the coast of southeastern Brazil (Marques et al., 2002). Due to its small population (about 2000 individuals) and highly restricted range with a continuing decrease in habitat quality and possible illegal trading, *B. insularis* is currently listed as critically endangered in the IUCN Red List (Marques et al., 2004; Martins et al., 2001, 2008). As a consequence, efforts have been directed towards the development of a captive breeding program that involves both natural and assisted reproduction. Nonetheless, successful application of assisted reproductive technologies such as artificial insemination and semen cryopreservation in *B. insularis* requires a greater understanding of the reproductive

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physiology of this species, particularly seasonal aspects of sperm quality and production.

The available information on the reproductive cycle of *B. insularis* indicates this species has a seasonal breeding pattern and other reproductive patterns similar to those observed in its continental relative, the jararaca pitviper (*B. jararaca*; Kasperoviczus, 2009; Marques et al., 2013). Mating activity occurs from March through August, but males pursue females more vigorously during June and July (winter; Kasperoviczus, 2009; Marques et al., 2013). Females exhibiting vitellogenesis (ovarian follicles ≥10 mm) were observed between March to November (late summer and late spring), while ovulations and births were recorded during September (spring) and February to April (late summer/mid autumn), respectively (Kasperoviczus, 2009; Marques et al., 2013). There is also evidence that golden lancehead females have the capacity for oviductal sperm storage (Almeida-Santos and Salomão, 2002). Similarly to other *Bothrops* species, females are larger than males, and sexually mature individuals appear to have a snout-vent length (SVL) varying from 50.5 and 55.5 cm, respectively (Kasperoviczus, 2009; Marques et al., 2013).

In males, spermatogenesis is thought to occur from April through October (autumn to mid spring) when germ cells cease proliferating and enter mitotic arrest until late summer (Kasperoviczus, 2009). However, to our knowledge, no studies have addressed the influence of season on sperm output or characteristics in *B. insularis*. Thus, the objectives of the present study were to: (1) examine whether seminal variables fluctuate throughout the year; (2) describe sperm morphology in golden lanceheads; and (3) assess possible relationships between seminal values and SVL or weight.

2. Material and methods

2.1. Animals

The breeding colony of golden lancehead pitvipers was housed at the Ecology and Evolution Lab–Butantan Institute (23°S Latitude). Ten of 18 males were classified as immature (SVL < 55 cm), with the other individuals being considered adults (SVL ≥ 55 cm). All adults were wild-caught and had been in the laboratory collection since 2009, whereas immature snakes were born in captivity in 2010. Individuals were isolated in clear plastic boxes (56.4 × 38.5 × 37.1 cm) with small holes on the sides and floors lined with cardboard. Water was provided *ad libitum* and mice as a food source were povided once a month (15% of the live weight). Animals were exposed to natural fluctuations in temperature (22 to 27 °C) and photoperiod (10 to 13 h—calculated for 23°S Latitude). Snout–vent length and body mass were recorded immediately after semen collection.

2.2. Semen collection and evaluation

Semen samples from golden lanceheads were collected quarterly (2012 to 2013) using a technique described by Zacariotti et al. (2007). A second collection was performed (after 1 week) whenever males failed to ejaculate at the time of the first attempt of semen collection. Individuals were physically restrained using clear plastic tubes and the lower abdomen was gently squeezed to empty the cloaca of feces or urine. Subsequently, pericloacal region was washed with saline solution and locally anesthetized injecting 1% lidocaine (15 mg/kg, subcutaneous/Bravet–Rio de Janeiro). Cloacal relaxation and exposure of the urogenital papilla was achieved between 7 and 15 min after lidocaine injection. Subsequently, a gentle massage was applied on the ventral wall of the body (moving the hand backward) followed by placing a small amount of pressure on either side of the cloaca until ejaculation. Semen was collected using an adjustable-volume pipette (0.5–10.0 μL, Eppendorf, Hamburg) and placed in 0.5 mL microtubes at room temperature for analysis.

The color and volume of the neat ejaculate was evaluated immediately after collection by aspirating it into an adjustable-volume pipette. Sperm concentration was assessed using Neubauer hemocytometer and a 400× magnification microscope. Due to the great concentration and viscosity, ejaculates were initially diluted in Ham's F-10 medium (Sigma Chemical Co., St. Louis, MO—1:1000) and then subjectively examined for motility and progressive motility using a 100× magnification microscope. Progressive motility of the sperm was graded on a 5-point scale (where 0 = no motility; 1 = slight side-to-side, no forward progress; 2 = moderate side-to-side, forward progress in spurts; 3 = slow, steady, forward progress; 4 = moderate, steady, forward progress; 5 = rapid, steady, forward progress).

Approximately 100 sperm per ejaculate were analyzed under 1000× magnification for sperm morphology using phase-contrast and wet-mounts with bright field microscopy (semen smears stained with eosin-negrosin). Morphological results were classified as total morphologic defects and sperm head, midpiece and tail defects. Microphotographs of sperm were taken using a digital camera coupled to a microscope, and the lengths and widths (μm) of the sperm head, midpiece and tail were measured using the software Image J version 1.47t (Schneider et al., 2012). The evaluation of sperm morphology was performed only in August 2012.

2.3. Statistical analysis

Statistical analysis was performed using SAS System for Windows (SAS Institute Inc., Cary, NC, USA), and a probability of $P \leq 0.05$ was considered to be significant. The effects of season, age and their interactions on sperm traits were determined using repeated measures. Comparisons between season and age groups were performed using parametric and non-parametric tests, according to the residue normality (Gaussian distribution) and variance homogeneity of each variable. Transformations were performed whenever necessary, but results are reported as untransformed means ± SEM.

3. Results

Between August 2012 and May 2013 a total of 72 attempts were made to collect semen from 18 males, and of