

Annual changes in seminal variables of golden lancehead 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 ($0.5 \times 10^9 \mu\text{L}$) 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.

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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 provided 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 μ L, 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 \times 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 \times 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 \times 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 (μ m) 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 \pm SEM.

3. Results

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

Table 1

Characteristics of semen samples (mean \pm SD and range) collected from captive males of golden lancehead pitvipers (*Bothrops insularis*, $n = 18$) between August 2012 and May 2013.

Variable	Adults ($n = 8$)		Immatures ($n = 10$)	
	Mean \pm SD	Range	Mean \pm SD	Range
SVL (cm)	67.87 \pm 3.96	65–73.87	50 \pm 3.60	43.5–55
Body mass (g)	132.28 \pm 22.43	90–170	56.62 \pm 15.95	30–98
Ejaculate volume (μ L)	3.21 \pm 1.38	1–6	2.92 \pm 1.01	0.5–5
Percentage of motile sperm (%)	64.37 \pm 32.84	0–95	58.54 \pm 20.42	10–90
Progressive motility (0–5 scale)	3.14 \pm 1.37	0–4.5	3.06 \pm 0.89	2–4
Concentration ($\times 10^9$ sperm/mL)	1.26 \pm 0.87	0.2–2.8	1.49 \pm 0.64	0.5–3.5

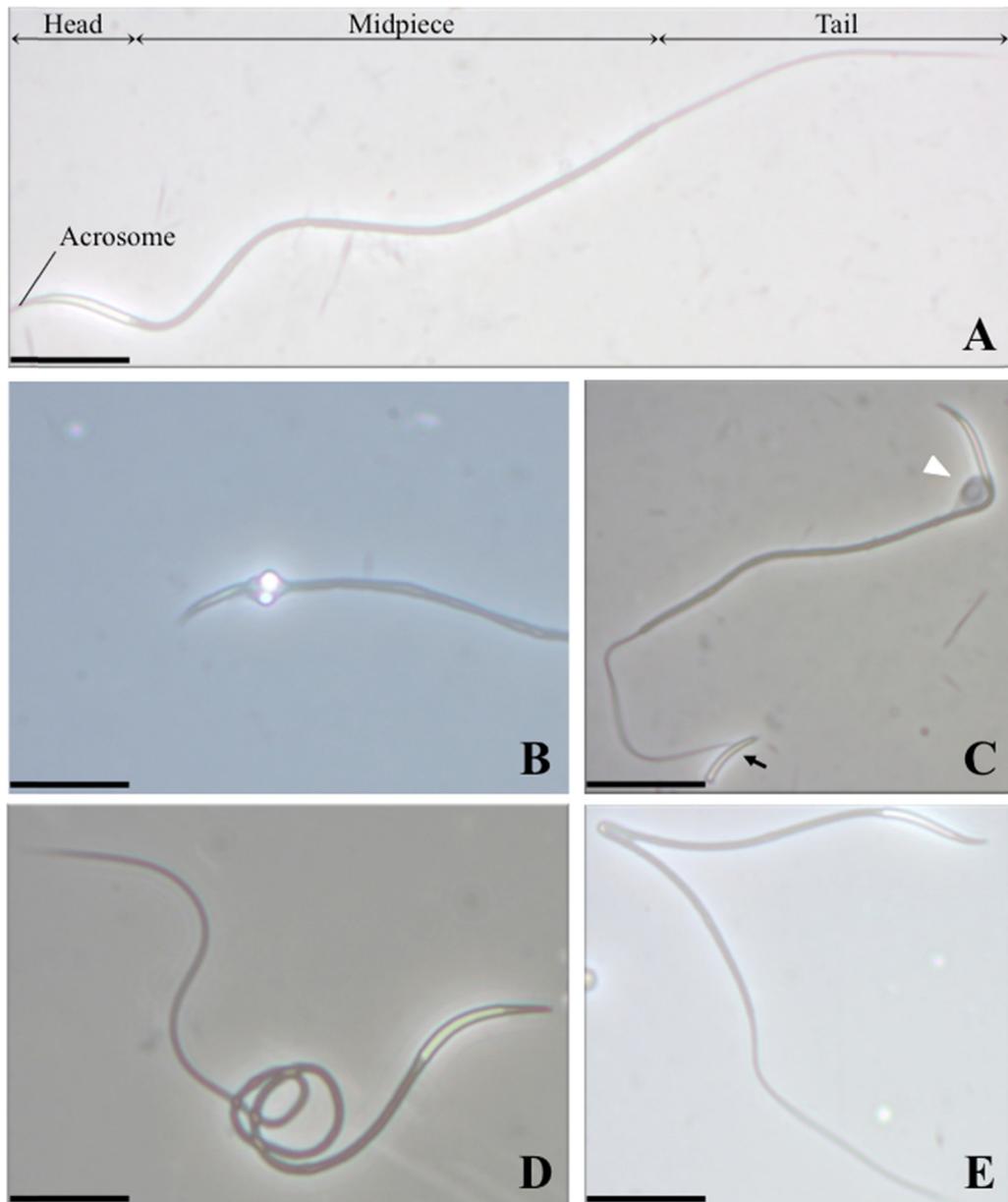


Fig. 1. Light micrographs illustrating morphologically normal sperm and various sperm defects in golden lancehead pitvipers (A) Normal sperm (B) Head swelling; (C) cytoplasmic droplet (white arrow head) and head detachment (black arrow); (D) coiled midpiece (E); bent midpiece; Magnification 1000 \times , bars = 10 μ m.

Table 2

Incidence of different sperm defects observed in captive golden lancehead pitvipers (mean \pm SD) during August 2012.

Variable*	Specific defect**
Head defects	Macrocephalic 40.21 \pm 33.32%
7.73 \pm 5.82%	Head detachment 29.25 \pm 26.95%
Midpiece defects	Cytoplasmic droplets 0.84 \pm 2.05%
78.77 \pm 10.46%	Bent midpiece 5.48 \pm 7.06%
	Coiled midpiece 77.71 \pm 20.07%
Tail defects	Coiled Tail 85.26 \pm 29.69%
12.39 \pm 7.92%	Bent tail 7.05 \pm 15.16%

* Category expressed as a percentage within total sperm defects (number of morphologically normal sperm was excluded from these calculations).

** See Fig. 1 for illustration of some of the defects; Percentages of main specific defects within each category (mean \pm SD).

these, 58 ejaculates were obtained (efficiency of 80.5%), and the data for characteristics of the ejaculates along with biometric data are summarized in Table 1. The color of semen samples varied from white to slightly yellow, and the consistency was typically creamy and thick. The mean weight and SVL of males considered adults was 132.28 \pm 3.96 g and 69.12 \pm 0.62 cm, and of immature males was 56.62 \pm 2.52 g and 50.0 \pm 0.54 cm.

The sperm were typically filiform in shape with a slender tapering head capped by a small conical acrosome (Fig. 1). The acrosome was short, measuring 3.22 \pm 0.10 μ m in length and 0.51 \pm 0.01 μ m wide at its point of termination. Normal sperm heads were slightly curved (11.45 \pm 0.70 μ m long and 0.87 \pm 0.07 μ m wide), whereas midpieces were in average 54.30 \pm 2.09 μ m long and 0.63 \pm 0.02 μ m wide. The total average length of sperm was 98.44 \pm 2.10 μ m. There was a mean value of 45.76 \pm 17.34% morphologically abnormal sperm in the samples examined. These defects were in three broad categories—head defects (7.73 \pm 5.81%), midpiece defects (78.77 \pm 10.45%), and tail defects (12.39 \pm 7.92%; Fig. 1 and Table 2). Most sperm abnormalities occurred in the midpiece region (largely bent and coiled midpieces; Table 2).

Statistical analysis indicated there were significant interactions between age and season only for sperm motility and progressive motility, and therefore seasonal effects on these variables were evaluated in adults and immature snakes concurrently to assess the effect of age within each season. The influence of season upon semen volume and sperm concentration, however, was assessed independent of age groups, and vice versa. Findings revealed that semen volume did not vary with season of year ($P > 0.05$), whereas sperm concentration in the winter was less than in other seasons ($P < 0.05$; Fig. 2). Sperm motility in mature males was less during the spring when compared with the winter and autumn ($P < 0.05$), but motility only fluctuated slightly with season in sexually immature males ($P > 0.05$; Fig. 3A). Similarly, sperm from mature males had less progressive motility in the spring ($P < 0.05$) while immature males did not have a seasonal pattern of progressive sperm motility ($P < 0.05$; Fig. 3B). Sperm from immature males generally had greater sperm concentrations than adult males ($P < 0.05$; Fig. 4).

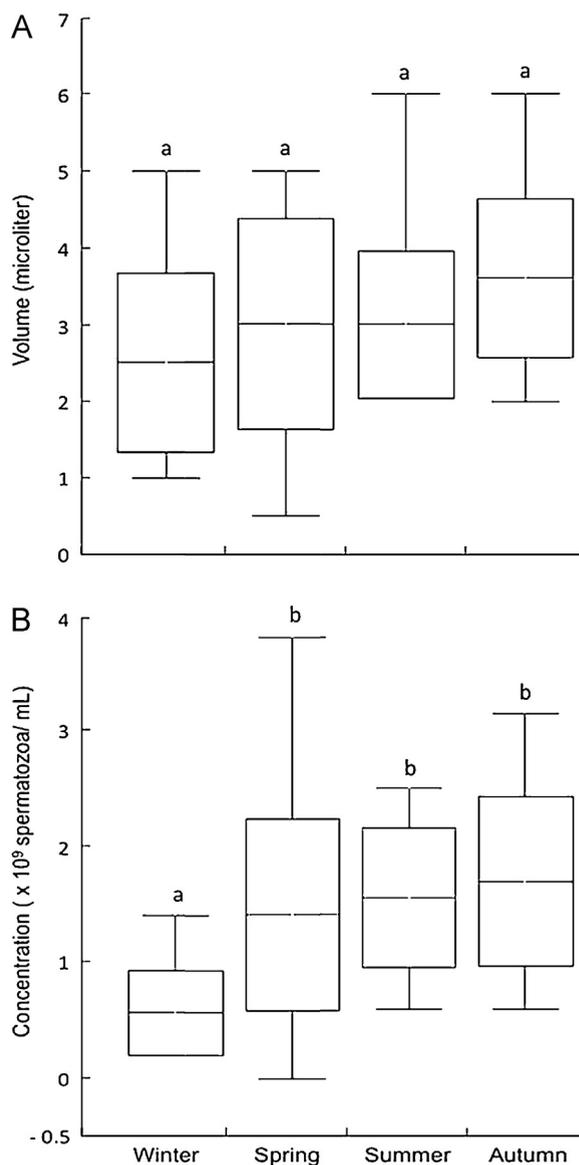


Fig. 2. Box-plots showing seasonal changes in ejaculate volume (A) and sperm concentration (B) from 18 golden lancehead pitvipers (upper/lower box boundaries indicate 75th/25th percentiles, respectively; upper/lower error bars indicate 90th/10th percentiles, respectively; solid line in box is the median); No interaction between season and age was found for both variables ($P > 0.05$), therefore, results from adult and immature snakes were analyzed together; Different letters above box-plots indicate that values are different ($P < 0.05$).

4. Discussion

Much of the knowledge concerning snake reproduction has been derived from anatomical and histological studies of specimens from zoos and museums, which allow only broad estimates for the timing of spermatogenesis in males or vitellogenesis in females (Taylor and DeNardo, 2011). However, repeated collection of ejaculates to assess sperm production and quality may provide a valuable technique to investigate reproductive characteristics, including

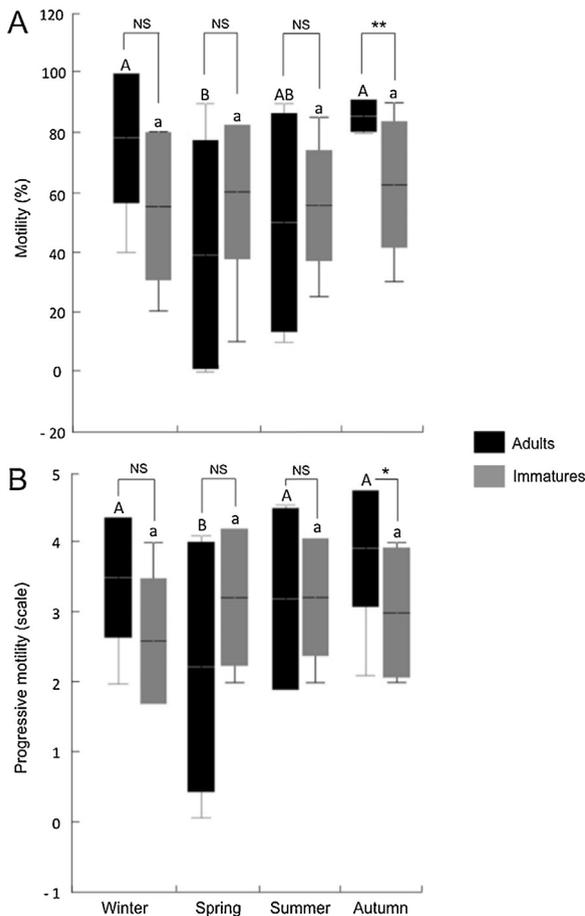


Fig. 3. Box-plots showing seasonal changes in sperm cell motility (A) and progressive motility (B) from 18 golden lancehead pitvipers (upper/lower box boundaries indicate 75th/25th percentiles, respectively; upper/lower error bars indicate 90th/10th percentiles, respectively; solid line in box is the median); Because of a significant interaction between season and age, was observed for these variables ($P < 0.05$), results from adults ($n = 8$, black bars) and immature snakes ($n = 10$, gray bars) were illustrated separately; Differences ($P < 0.05$) between seasonal means are indicated by letters above box-plots with adult (uppercase A and B) and immature snakes (lowercase a and b) considered separately; Statistical differences between adult and immature snakes within each season are shown by symbols above brackets (NS—not significant, * $P < 0.05$, and ** $P < 0.01$).

sexual maturity, seasonality and fertility in both captive and wild snakes. The ease, speed and safety of the procedure described herein demonstrate applicability of such procedures in both laboratory and field conditions. Collection efficiency was 80% in *B. insularis* and slightly less (72%) in Brazilian rattlesnakes (*Crotalus durissus terrificus*; Zacariotti et al., 2007). The efficiency of the technique used in the present study enabled for observation of seasonal changes in seminal variables, but also allowed for confirmation that males that were evaluated to be biometrically immature were capable of producing viable sperm that in some samples were comparable to the characteristics of the sperm produced by adults.

On average, the semen volume obtained in *B. insularis* of $3.05 \pm 1.19 \mu\text{L}$ was considerably less than that of the *Crotalus durissus terrificus* of $18.49 \mu\text{L}$ (Zacariotti, 2004).

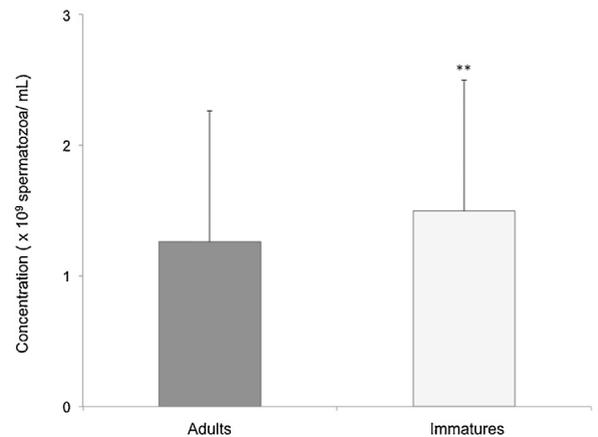


Fig. 4. Comparison of sperm concentration between adult ($n = 8$) and immature ($n = 10$) male golden lancehead pitvipers; Bars represent mean values \pm SE, and asterisks (**) indicate differences ($P < 0.01$).

Nevertheless, Schulte-Hostedde and Montgomerie (2006) described semen volumes ranging from 10 to $30 \mu\text{L}$ in northern watersnakes (*Nerodia sipedon*), a species with similar SVL and body mass as *B. insularis*, thus indicating there was no relationship between semen volume and male size within these three species. Interspecies differences in semen volume may be associated with male competition for females. The mating pattern in *N. sipedon* is characterized by aggregations in which a single female mates with multiple males. This behavioral pattern favors males with greater semen production capacity such as semen volume, sperm concentration and sperm motility in providing the genes for subsequent generations (Schulte-Hostedde and Montgomerie, 2006). In contrast, golden lanceheads do not exhibit male–male combat behaviors or mating aggregations in which several males attempt to copulate with a single female (Marques et al., 2013). Therefore, the lesser degree of male competition in *B. insularis* may have resulted in lesser sperm volumes as the species has evolved then in similar sized males of *C. durissus terrificus* or *N. sipedon*. The mating pattern in *B. insularis* might also explain the absence of significant seasonal fluctuations in semen volume between adult and immature males.

However, special care should be taken in interpreting data on ejaculate volumes, as these data might not express the sperm producing capacity from the testes in *B. insularis*. First, because samples obtained by massage represent only a portion of the semen stored in the terminal portion of the ductus deferens (Gist, 2011; Trauth and Sever, 2011), rather than the sperm output at the time of the semen collection. Second, ejaculate volume in snakes is affected by the sexual segment of the kidney (SSK—Bishop, 1959), a structure found only in squamate reptiles, which releases secretions (carbohydrates, lipids, and proteins) into the ducts and ureter contributing to the seminal fluid (Gist, 2011). Hence, in the present study it is stressed that these findings do not establish the existence of seasonal changes in sperm production of golden lanceheads, but most importantly provide information regarding seminal variables throughout the year, which may be further used in semen

cryopreservation and/or artificial insemination programs for this reptile.

Mature males had greater sperm motility and progressive motility (85.7% and 3.8%, respectively) than immature snakes (62.5% and 3.0%, respectively) during the autumn. This is the season that precedes most courtship and mating in both wild and captive populations (Kasperoviczus, 2009; Marques et al., 2013). Video recordings made in the Queimada Grande Island provided evidence that only males larger than 65 cm were actively searching and courting females, whereas males smaller than 50 cm never displayed such behaviors (Marques and Almeida-Santos, unpublished data). Furthermore, despite the lack of detailed behavioral analysis, none of the immature males in the present study performed sexual behavior when placed next to females, however, with adult males sexual behavior was expressed when males were placed in proximity to females. These findings suggest that large lancehead males might have an enhanced probability of reproductive success by producing high-quality ejaculates (in terms of motility and progressive motility) during the autumn–early winter—the seasonal period when there is greater reproductive behavior in approaching females than what occurred with the smaller males. Moreover, spermatogenesis in this species was documented at this time of the year (Kasperoviczus, 2009; Marques et al., 2013), which may partially account for the increase in sperm motility and progressive motility.

Overall, sperm concentration in *B. insularis* was less during the winter, and immature snakes had a more concentrated ejaculate than adult snakes during the winter. This reduced sperm concentration in the winter was surprising because previous information about this species indicated that testicles are entirely inactive during the summer (Kasperoviczus, 2009). A possible cause for such a decrease in sperm concentration could be the small number of immature males collected during winter (six of ten) which had greater sperm counts (Fig. 4). Although it was expected in the present study that sexually mature males would have more concentrated ejaculates than immature snakes this was not what occurred because immature snakes had greater sperm concentrations than mature snakes. Similar results have been previously described in the *N. sipedon* (Schulte-Hostedde and Montgomerie, 2006). In this previous study, small male snakes compensated for a reduced probability of copulation by investing more metabolic energy toward ejaculate production than large males, leading to more equitable reproductive success across different male body sizes (Schulte-Hostedde and Montgomerie, 2006). Nevertheless, it is believed that differences between adult and immature males may be linked to the activity of SSK. Because the SSK is hypertrophied during the mating season and is under control of androgens (Aldridge et al., 2011; Graham, 2006; Krohmer et al., 2004; Rojas et al., 2013; Schuett et al., 2002), it is likely that an increase in SSK secretions during the winter led to diluted ejaculates from adults thus decreasing sperm concentrations.

Morphologically, sperm from golden lanceheads were of the sauropsid type, long and narrow with a vermiform

appearance and a slightly curved head, resembling sperm from a close relative the crossed pit viper (*Bothrops alternatus*) and other snake species (Austin, 1965; Cunha et al., 2008; Schulte-Hostedde and Montgomerie, 2006; Tourmente et al., 2008, 2011). An acrosome, nucleus, mid-piece, and flagellum were clearly discerned with light microscopy, and overall sperm length was $98.44 \pm 2.10 \mu\text{m}$. This measure is slightly greater than those reported for crossed pit vipers and Chaco lanceheads (*Bothrops diporus*; about $92 \mu\text{m}$ and $88 \mu\text{m}$, respectively), but less than sperm lengths of some Colubridae species, such as the *N. sipedon* of $112 \mu\text{m}$ and $159 \mu\text{m}$ of wagler's snake (*Waglerophis merremii*; Schulte-Hostedde and Montgomerie, 2006; Tourmente et al., 2008, 2009, 2011). Therefore, it appears as though differences in this variable are not linked to SVL because sperm cells of both colubrids are of lesser size compared with golden lanceheads of with an average length of 56.6 and 58.9 cm, respectively. Comparative research of 25 species of snakes concluded that increases in sperm competition are correlated with sperm elongation, which in turn, is largely explained by increases in sperm midpiece length; presumably because greater midpiece length is correlated with increased mitochondrion numbers and, therefore, the supply of ATP for metabolic functions (Tourmente et al., 2009).

Unfortunately, there are few studies assessing semen quality in snakes, and none of the studies estimated the fertility potential of sperm. The present study revealed an average of 45.8% of morphologically abnormal sperm in golden lanceheads, a ratio similar to that reported for Brazilian rattlesnakes during winter (42.3%), but greater than those of corn snakes and Zanjani vipers (24.3% and 38.3%, respectively; Fahrig et al., 2007; Moshiri et al., 2014; Zacariotti, 2004). The greater prevalence of sperm abnormalities in *B. insularis* can be caused by several factors (e.g. population consanguinity, captive management, nutrition, seasonality, semen handling, etc.), however, the lack of information regarding this species makes it difficult to determine the ultimate causes of sperm abnormalities. As most defects detected in the present study were comprised of coiled/bent sperm midpieces and tails (Table 2), it is assumed that these abnormalities may have occurred as a consequence of the detrimental effects of composition of the diluent or post ejaculation protocols used in the present study (currently conducting research to test both hypotheses).

In summary, results from the present study reveal the interactions between season and age influencing seminal variables such as sperm motility and progressive motility in *B. insularis*, whereas none of these factors significantly impacted volume of the ejaculate. Despite the lack of interaction, season and age independently affected sperm concentration in this species. Results of the present study emphasize the importance of repeated collection and semen analysis to elucidate various aspects of the reproductive biology of snakes, which are difficult to be explained using only anatomical and/or histological data. The present study also served as a foundational study for future research to achieve the creation of a cryobank for golden lancehead pitvipers, and possibly other endangered snake species in Brazil.

Conflict of interest statement

None.

Acknowledgments

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