Reproductive Biology of the Brazilian Lancehead, *Bothrops moojeni* (Serpentes, Viperidae), from the State of São Paulo, Southeastern Brazil

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Abstract. *Bothrops moojeni* inhabits riparian forests and adjacent open, wet areas in the Brazilian Cerrado. We address several aspects of the reproductive biology of a population from southeastern Brazil, including sexual maturation and dimorphism, male and female reproductive cycles, and sperm storage. We also provide the first record of male–male combat behavior in the wild. We sampled specimens held in the scientific collection of the Butantan Institute, São Paulo, Brazil. After macroscopic analyses and dissections, we collected the testes of males and the posterior uterus of females showing uterine muscular twisting for microscopic analyses. Males < 54.5 cm and females < 58.0 cm were sexually immature. Mature females are larger than males, although male–male combat is reported for this species. Male–male fighting and copulation occur during late summer in the wild. Histological analysis of the testes confirmed that sperm production occurs simultaneously with those reproductive events. Females showed an extended period of vitellogenesis, from February to November (late summer to spring). We observed stored sperm in the female reproductive tract during autumn. Because mating is not synchronous with ovulation, sperm storage is an obligatory component of the reproductive cycle of *B. moojeni.* Ovulation begins in August (winter) but most females with the presence of embryos in the oviducts occurred from January to March (summer). Parturition in captivity occurred in February, confirming that births are concentrated in the summer. Given that the Cerrado is highly fragmented in southeastern Brazil, studies on the reproductive biology of additional *B. moojeni* populations might provide useful information regarding intraspecific variation in reproduction in pit vipers.

Keywords. Cerrado; Histology; Reproductive behavior; Reproductive cycles; Snakes; Sperm storage.

Resumo. *Bothrops moojeni* ocorre em matas de galeria e áreas abertas adjacentes no Cerrado brasileiro. Nós investigamos vários aspectos da biologia reprodutiva de uma população do sudeste do Brasil, incluindo maturação e dimorfismo sexual, ciclos reprodutivos de machos e fêmeas e estocagem de esperma. Registramos pela primeira vez o comportamento de combate entre os machos em vida livre. Foram amostrados espécimes depositados na Coleção Científica do Instituto Butantan, São Paulo, Brasil. Após a análise macroscópica e dissecção das serpentes, foi coletado o testículo de machos e o útero posterior de fêmeas na presença de contorção muscular uterina para análises microscópicas. Machos menores que 54,5 cm e fêmeas menores que 58 cm são sexualmente imaturos. Fêmeas sexualmente maduras são maiores que os machos, embora haja combate entre os machos desta espécie. O combate entre os machos e a cópula ocorrem durante o verão. Análises histológicas dos testículos confirmaram que a produção de esperma ocorre simultaneamente a estes eventos reprodutivos. As fêmeas apresentaram um período de vitelogênese prolongado, de Fevereiro a Novembro (final do verão até a primavera). Esperma estocado no trato reprodutivo de uma fêmea foi observado durante o outono. Como o acasalamento não ocorre em sincronia com a ovulação, a estocagem de esperma é um componente obrigatório do ciclo reprodutivo de *B. moojeni.* O período de ovulação teve início em agosto (inverno), embora a maioria das fêmeas com presença de embriões nos ovidutos tenha sido coletada entre Janeiro e Março (verão). A parturição em cativeiro ocorreu em Fevereiro, confirmando a concentração do período de nascimento durante o verão. Sabendo-se que o cerrado encontra-se altamente fragmentado no sudeste do Brasil, o estudo da reprodução de populações adicionais de *B. moojeni* pode fornecer dados importantes sobre a variação dos aspectos reprodutivos em serpentes da subfamília Crotalinae.

INTRODUCTION

Bothrops moojeni Hoge, 1966 is a large pit viper known from Brazil, Paraguay, and Argentina. It can be found in riparian forests and adjacent open, wet areas in central and southeastern Brazil, throughout the Cerrado biome (Nogueira et al., 2003; Campbell and Lamar, 2004; Wallach et al., 2014; Uetz et al., 2016), a hotspot of biodiversity. It is a venomous species that is important for public health due to the high incidence of snakebites (Rojas et al., 2007).

In the field, mating occurs in March (late summer) in southeastern Brazil (Zacariotti et al., 2011). Births are concentrated in summer (around December and January) in populations in southeastern and central Brazil (Nogueira et al., 2003). Bothropoid snakes are known to have a complex reproductive pattern. In the field, reproductive strategies include long-term sperm storage (LTSS) as a tactic to ensure synchronization of reproductive events (Almeida-Santos and Salomão, 2002). LTSS occurs by means of a uterine muscular twisting (UMT): after mating in late summer/autumn, the posterior uterus contracts, holding sperm in the posterior oviduct throughout autumn and winter. At the time of ovulation in spring, the oviduct relaxes, sperm moves anteriorly, and fertilization occurs (Almeida-Santos and Salomão, 1997, 2002).

Although the timing of reproduction in *Bothrops* Wagler, 1824 has been considered conserved throughout the lineage (Almeida-Santos and Salomão, 2002), the addition of new information could help to understand the plasticity of *B. moojeni* in response to climate variation (e.g., ovulation may occur earlier than predicted, around July, during winter and not only in spring; Nogueira et al., 2003). Analysis of populations from different climatic regions may indicate some possible differences in reproductive traits (Barros et al., 2012, 2014a). Data on reproduction in captivity showed a shift of six months in the timing of male–male combats, copulation, and subsequent parturition in *B. moojeni* (Leloup, 1975). This observation reinforces the hypothesis that this species might present the genetic potential for plasticity in some reproductive traits under different environmental conditions and highlights the importance of studying different populations separately.

Considering the major differences in environmental conditions among distant regions in Brazil we asked whether the timing of reproductive events differ in populations of *Bothrops moojeni* from distinct regions. Herein, we present new data on the reproductive biology of a population of *B. moojeni* from São Paulo, southeastern Brazil, aiming to contribute to the understanding of geographical variation in the reproductive biology of a widespread pit viper. We investigate the reproductive cycles of males and females considering sperm storage and the relationship between seasonal activity and reproductive events.

MATERIALS AND METHODS

We analyzed 178 specimens of *Bothrops moojeni* (70 females, 60 males, and 48 immature individuals) from the state of São Paulo, southeastern Brazil (20°22′–22°39′S, 46°56′–52°06′W; Fig. 1), preserved in the "Alphonse Richard Hoge" scientific collection of the Butantan Institute (IB), São Paulo, Brazil. The climate in São Paulo State is subtropical humid according to the Köppen classification. During the summer (late December–March), the mean minimum temperature is ca.17°C and the mean maximum temperature is ca.28°C. During the winter, the temperature ranges between 11–23°C (IAG-USP, 2011). We characterize the seasons as summer (late December
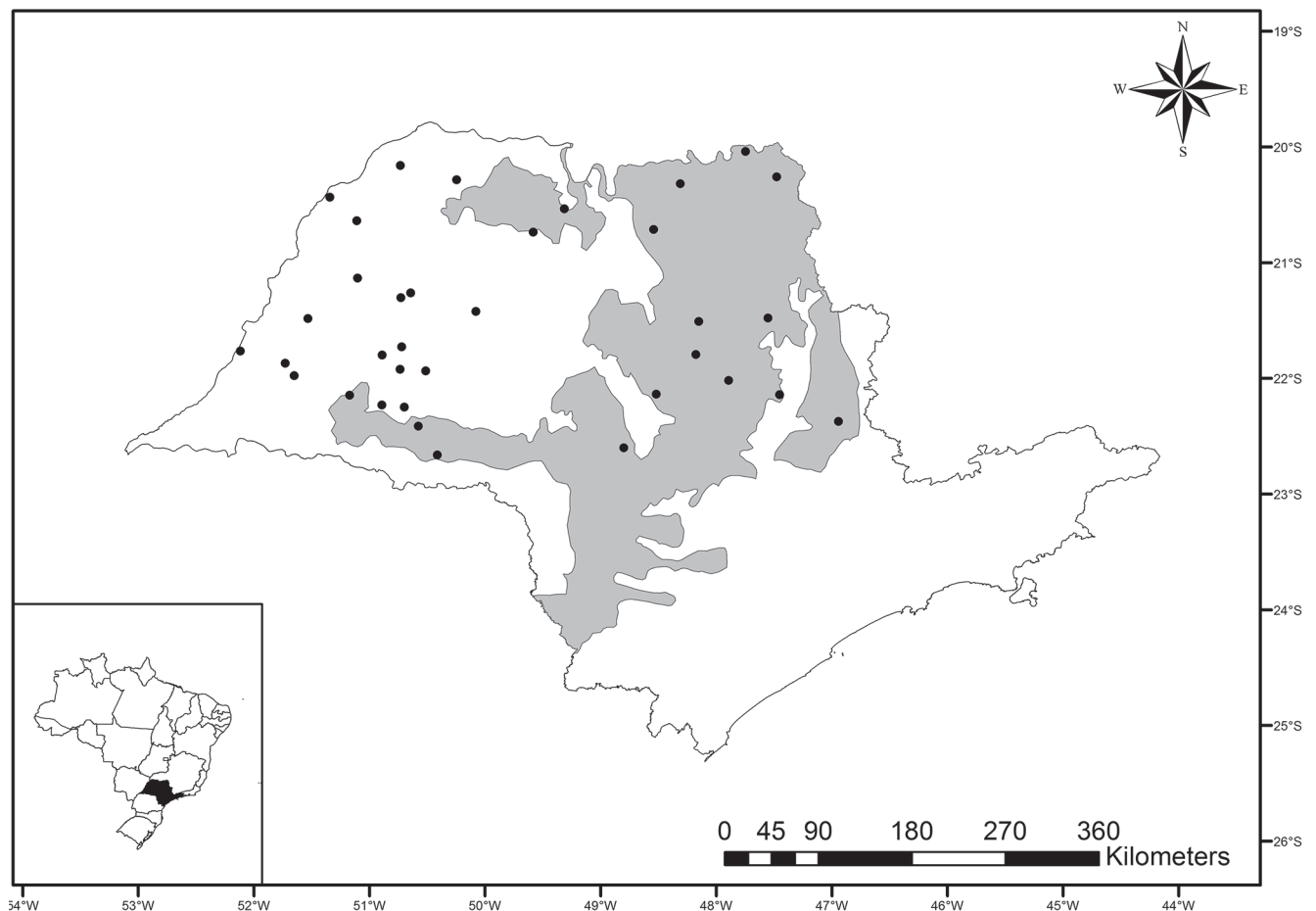
Figure 1. Geographic distribution of the sampled specimens of *Bothrops moojeni* in the state of São Paulo, Brazil. The gray area represents the Cerrado and the white area represents the Atlantic Forest.

Reproductive Biology of the Brazilian Lancehead, *Bothrops moojeni* (Serpentes, Viperidae), from the State of São Paulo, Southeastern Brazil
Selma Maria Almeida-Santos, Verônica Alberto Barros, Claudio Augusto Rojas, Letícia Ruiz Sueiro, Roberto Harutomi Corrêa Nomura

175

to late March), autumn (late March to late June), winter (late June to late September) and spring (late September to late December) to allow comparisons with other studies.

For every specimen, we recorded the snout–vent length (SVL) and sexual maturity (cf. criteria in Shine, 1977a,b). Different data were recorded for sexually mature individuals: for females, we recorded the diameter of the largest ovarian follicle or the presence and number of embryos and the presence/absence of uterine muscular twisting (UMT) in the posterior uterus, described as a sperm storage site in snakes (Almeida-Santos and Salomão, 1997, 2002; Barros et al., 2012, 2014a). For males, we recorded the length, width and thickness of the testes to determine the testicular volume (TV) using the ellipsoid formula $TV = 4/3.\pi.a.b.c$, where a = half of the length, b = half of the width, and c = half of the thickness (Pleguezuelos and Feriche, 1999). We also measured the diameter of the distal portion of the ductus deferens, close to the cloaca.

We collected the twisted uteri (UMT) from females (*n* = 9) as well as the testes of some males (*n* = 7) for histological analysis. All organs were preserved in 70% alcohol and processed for light microscopy using the paraffin method. Sections were cut 5 μm thick, and stained with hematoxylin and eosin (H/E).

The seasonal activity pattern of *B. moojeni* was inferred by comparing the number of snakes received at the Butantan Institute in each season using a chi-square test. Records from 1917 to 2002 were used (see Almeida-Santos and Salomão, 2002). A Mann-Whitney test was applied to test sexual dimorphism in SVL in mature individuals. The degree of sexual dimorphism (SSD) was calculated as 1 – (mean adult SVL of the large sex/mean adult SVL of the smaller sex) according to Gibbons and Lovich (1990) and Shine (1994). Positive and negative

values of SSD correspond to females larger than males and vice versa, respectively. Analysis of covariance (ANCOVA) was employed to compare testes volume among the seasons (post hoc comparison: Tukey's test), and SVL was used as the covariate.

Differences in the ductus deferens diameter among seasons were investigated using the Kruskal-Wallis test (post hoc test: comparison by the Dunn method). Statistical analyses were performed using Bioestat (version 5.00), assuming $P < 0.05$ as the criterion for significance. The analyses were carried out according to Zar (1999) and all variables were tested for normality and homoscedasticity prior to analysis. A map was drawn using ArcGis (ESRI, 2009).

RESULTS

Sexual maturity and dimorphism

Males smaller than 54.5 cm and females smaller than 58 cm were sexually immature. Mature males averaged 78.9 cm SVL (SD = 13.4, *n* = 63, range = 54.5–111 cm), and mature females averaged 93.7 cm SVL (SD = 18.4, *n* = 55, range = 58–140.5 cm). Mature females were significantly larger than mature males (Z = 4.05; U = 1796; *P* < 0.0001). The degree of sexual dimorphism (SSD) was 0.16.

Female reproductive cycle, timing of birth, and sperm storage

Females showed primary vitellogenesis throughout the year and an extended period of secondary vitellogenesis, from February to November (from late summer to spring). The presence of embryos was observed from August to March (Fig. 2). Uterine muscular twisting (UMT) was observed most frequently in autumn and winter. Spermatozoa were found being stored in the posterior uterus (undergoing UMT) of females, which presented secondary vitellogenic follicles during autumn (Fig. 3). Bundles of sperm have a parallel arrangement with closely packed sperm heads oriented against the pseudostratified epithelium composed of many ciliated and some secretory cells (Fig. 3B–C). The spermatozoa tails align in parallel waves filling the deep furrows of the uterus (Fig. 3B–C). Two females from São Paulo gave birth to newborns on the 18 and 25 February in captivity at the Butantan Institute (S. Cardoso, pers. comm.).
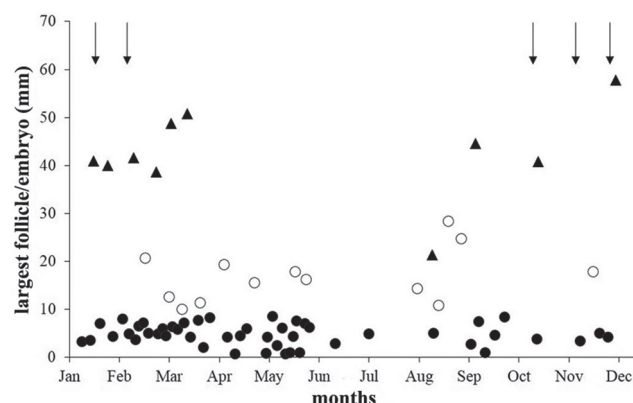
Male reproductive cycle and male–male combat behavior

Testis volume was positively correlated with SVL (F = 40.56, R^2 = 0.4014, P < 0.0001) and this parameter

Figure 2. Female reproductive cycle: seasonal variation in diameter of the largest ovarian follicle or embryo in mature females of *Bothrops moojeni* from São Paulo, southeastern Brazil. Solid circles = primary vitellogenic follicles; open circles = secondary vitellogenic follicles; solid triangles = embryos in the oviducts. Arrows indicate the parturition period according to Leloup (1975), Nogueira et al. (2003), and this study.

differed among seasons (F = 2.8145, *P* = 0.0477). Testis volume was greater during the summer (Fig. 4), when spermatogenesis was observed in the histological analysis (*n* = 6, Fig. 5A), than in autumn and winter. Testes were also larger during the spring than in winter, when the histological analysis confirmed the regression of the seminiferous tubules (*n* = 1, Fig. 5B). The diameter of the ductus deferens was higher during the winter than in autumn (H = 15.2721, *P* = 0.0016; Fig. 6). Two males were observed fighting in summer (28 March 2013) in the wild in the municipality of Paraguaçu Paulista, state of São Paulo, Brazil (M.S. Mayor, pers. comm.; Fig. 7).

Activity patterns

Males were more abundant in autumn than summer (χ^2 = 5.554, *P* = 0.0256), winter (χ^2 = 27, *P* < 0.0001), or spring (χ^2 = 25, *P* < 0.0001) and more abundant in summer than spring (χ^2 = 8.533, *P* = 0.0062) and winter (χ^2 = 9.966,

P = 0.003). Females were more abundant in autumn than summer (χ^2 = 7.367, *P* = 0.0066), winter (χ^2 = 16.095, *P* = 0.0001), and spring (χ^2 = 13.091, *P* = 0.0005; Fig. 8).

DISCUSSION

Seasonal reproductive cycles seem to be characteristic of Neotropical pit vipers of both sexes (Almeida-Santos and Salomão, 2002; Salomão and Almeida-Santos, 2002; Nogueira et al., 2003; Barros et al., 2012, 2014a,b). Although extended vitellogenesis has been observed in *Bothrops moojeni* and other species of *Bothrops* (Hartmann et al., 2004; Barros et al., 2014a,b), it does not seem to be good evidence of an aseasonal cycle because rates of vitellogenesis may be influenced by several factors. Intraspecific variation in the timing of vitellogenesis may be at least partially influenced by female body condition, prey availability and ingestion during the vitellogenic period, and hormonal variations among individuals (Licht,
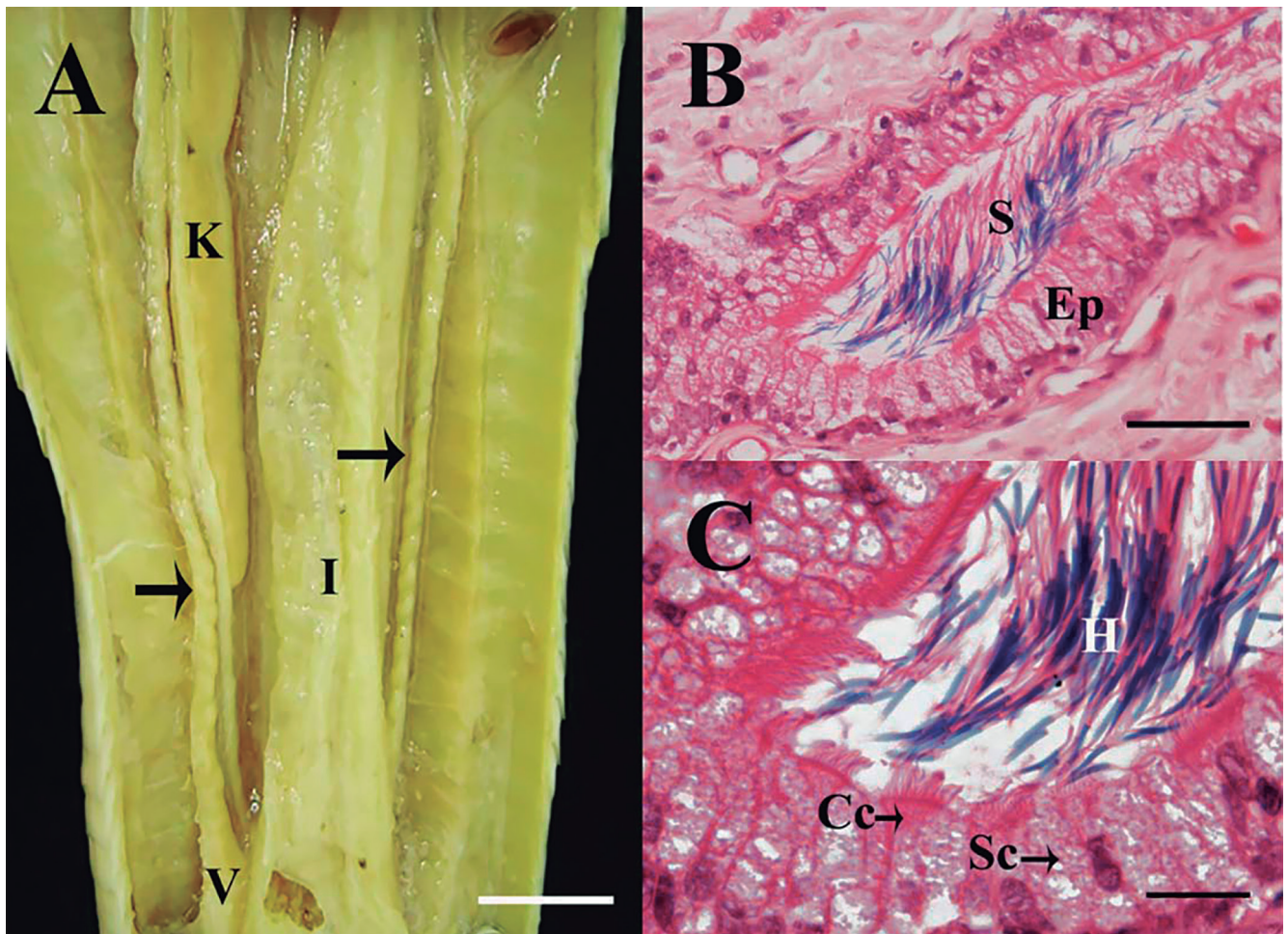

Figure 3. Sperm storage in the twisted uterus (UMT) of *Bothrops moojeni*. **(A)** Macroscopical view showing the vagina (v), intestine (I) and kidneys (k). The arrows indicate the UMT in the posterior uterus. Bar = 3 cm. **(B)** Sperm storage in the deep furrows of the posterior uterus. Bar = 30 μm. C. Higher magnification of B. showing the orientation of sperm heads against the pseudostratified epithelium. Bar = 10 μm. S = sperm; Ep = epithelium; Cc = ciliated cells; Sc = secretory cells; H = spermatozoa heads.

Reproductive Biology of the Brazilian Lancehead, *Bothrops moojeni* (Serpentes, Viperidae), from the State of São Paulo, Southeastern Brazil
Selma Maria Almeida-Santos, Verônica Alberto Barros, Claudio Augusto Rojas, Letícia Ruiz Sueiro, Roberto Harutomi Corrêa Nomura
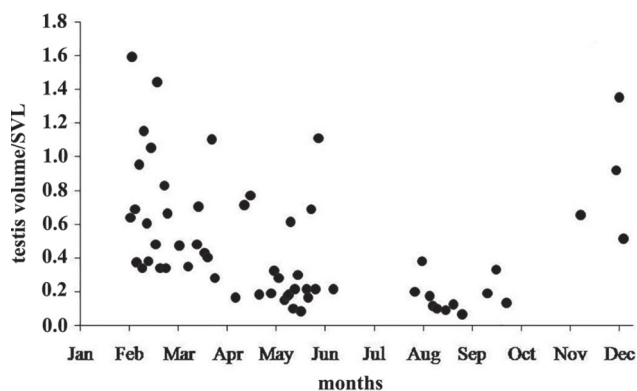
177

Figure 4. Seasonal variation in testes volume in *Bothrops moojeni.*
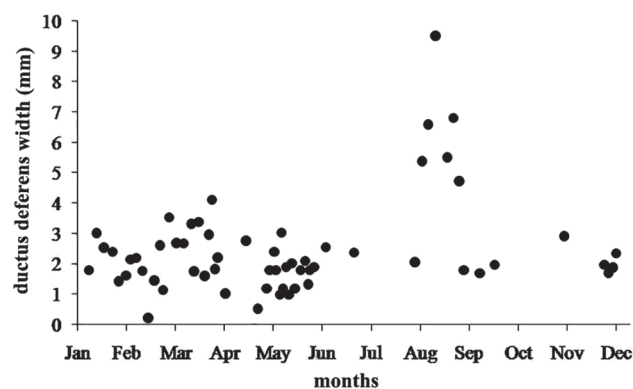

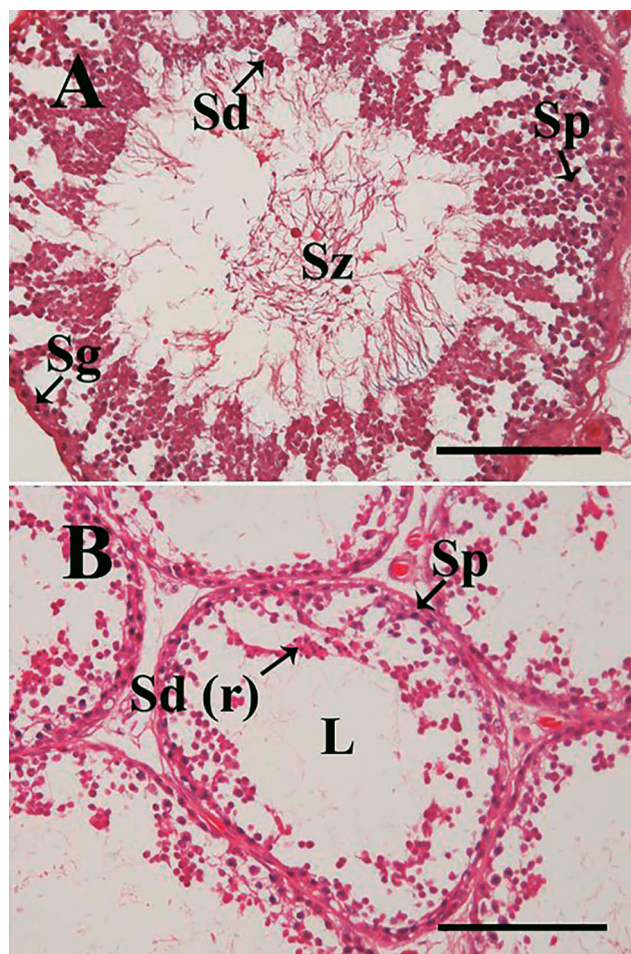**Figure 6.** Seasonal variation in ductus deferens width in *Bothrops moojeni.*

Figure 5. Sections of the testes in *B. moojeni.* **(A)** Spermatogenesis during summer. Bar = 100 μm. **(B)** Regression during winter. Bar = 100 μm. sp: primary spermatocyte; sz: spermatozoa; sd: spermatid; L: lumen; Sg: spermatogonia: Sd (r): remaining spermatid.

Figure 7. Record of ritual combat in the field in *Bothrops moojeni* snakes. Photo: Matheus da Silva Mayor.
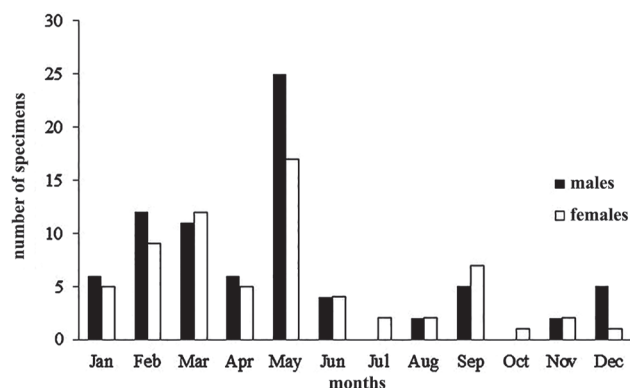
Figure 8. Seasonal abundance of male and female *Bothrops moojeni.*

1984; Cree et al., 1991; Reading, 2004; Mathies, 2011). Further, although there is some variability in the period of ovulation in some individuals, with its occurrence not only during spring but also in winter (July and August; Nogueira et al., 2003; this study), the occurrence of embryos in the oviducts and parturition in restricted periods
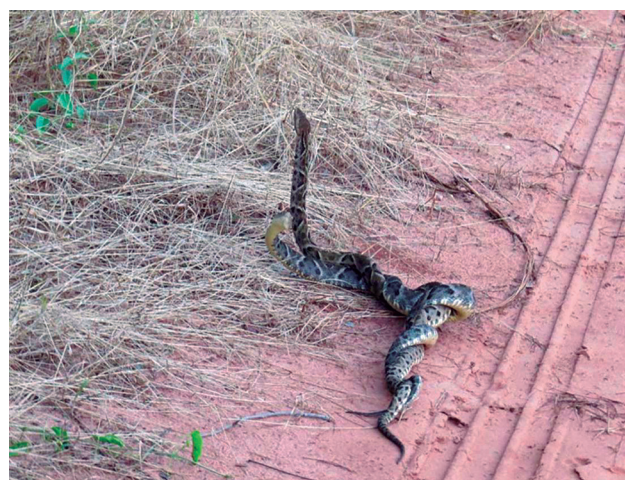
in *B. moojeni* confirm the seasonal characteristic of female reproductive cycle. Leloup (1975) observed births in *B. moojeni* from October–January, with over 60% of births occurring in December in captivity. Nogueira et al. (2003) observed that births were concentrated around December and January and full-term embryos were present only in females collected in December. Here, we described a more extended period for the timing of birth in the population

178

Reproductive Biology of the Brazilian Lancehead, *Bothrops moojeni* (Serpentes, Viperidae), from the State of São Paulo, Southeastern Brazil
Selma Maria Almeida-Santos, Verônica Alberto Barros, Claudio Augusto Rojas, Letícia Ruiz Sueiro, Roberto Harutomi Corrêa Nomura

of *B. moojeni* from southeastern Brazil, with births occurring in late February and embryos present in the oviducts until March. The occurrence of births from summer to early autumn is a striking feature of neotropical pit vipers (Almeida-Santos and Salomão, 2002; Barros et al., 2012).

The presence of sperm stored in the posterior uterus of *Bothrops moojeni* during the mating season (late summer to autumn; Almeida-Santos and Salomão, 2002; Zacariotti et al., 2011) reinforces the importance of female sperm storage as a tactic to synchronize reproductive events in Neotropical pit vipers, as mating and ovulation are dissociated (Almeida-Santos and Salomão, 1997, 2002; Barros et al., 2012, 2014a). Aggregations of sperm in the oviducts can be found in the posterior uterus, where UMT occurs (Almeida-Santos and Salomão, 1997, 2002), and in the posterior infundibulum, where specialized sperm receptacles occur (Siegel et al., 2011).

Although the presence of sperm in the posterior uterus may be interpreted as an artifact of recent mating (Siegel and Sever, 2006), the arrangement of the spermatozoa (parallel conformation with closely packed sperm heads oriented against the epithelium) in the deep furrows of the posterior uterus demonstrate that the posterior uterus is a sperm storage site (Rojas et al., 2015). This conformation is similar to that observed for spermatozoa stored in the infundibular glands of garter snake (*Thamnophis sirtalis* Linnaeus, 1858 and *T. elegans terrestris* Baird and Girard, 1853), which is related to a reduction in sperm activity and energy expenditure, features that are related to sperm storage (Fox, 1956). Seminal fluid physiology changes after sperm and seminal fluid enters the oviduct in *Crotalus durissus terrificus* Linnaeus, 1858 (Marinho et al., 2009). Thus, the posterior oviduct of snakes may also secrete seminal fluid to help maintain the viability of sperm in the posterior oviduct (Marinho et al., 2009).

A seasonal pattern is also observed in the *Bothrops moojeni* male reproductive cycle with spermatogenesis occurring in summer when the testes volume peaked (but see Rojas et al., 2013; Barros et al., 2014a for criticism regarding the use of this macroscopic indicator of spermatogenesis timing), and regression of seminiferous epithelium occurs during winter, when testis volume decreased. All previous studies on the reproductive strategies of male neotropical pit vipers using histological methods to describe spermatogenic cycles showed a strong seasonal pattern in timing of sperm production with the presence of a period of quiescence in the testes (Salomão and Almeida-Santos, 2002; Barros et al., 2012, 2014a,b). Male–male combat observed in the wild during summer occurred simultaneously with sperm production. In addition, there are previous records of combat behavior in captive males during February and March (Leloup, 1975) and copulation in the wild in March (Zacariotti et al., 2011). The occurrence of all these reproductive

events during late summer confirms that the reproductive season of *B. moojeni* starts in summer and may likely continue throughout autumn, when we observed sperm in the female reproductive tract.

A mechanism by which males can maximize access to females is by increasing movement to enhance female encounter rate. Males of several snake species exhibit a peak of activity during the mating season (Aldridge and Brown, 1995). This pattern may be influenced by the mating system adopted by males of *Bothrops moojeni* including a prolonged and active search for females and rituals of combat to compete for reproductive females during the fall, thereby increasing the chances of their sighting and contact during this season (Aldridge and Duvall, 2002).

Selective forces may jointly act on female and male body size because fecundity increases with larger body size (Shine, 1994) and larger males tend to be more successful in male–male combat for access to females (Shine, 1978; Shine, 1980; Madsen and Shine, 1993). Combat behavior is more common in taxa where male-biased sexual size dimorphism occurs (e.g., *Agkistrodon* Palisot de Beauvois, 1799, *Bitis* Gray, 1842, *Crotalus* Linnaeus, 1758, *Calloselasma* Kuhl, 1824, *Sistrurus* Garman, 1884, *Trimeresurus* Lacépède, 1804, and *Vipera* Laurenti, 1768; Shine, 1978, 1994; Almeida-Santos et al., 1999; Almeida-Santos and Salomão, 2002). However, there is a clear female-biased sexual size dimorphism in *B. moojeni* and *Bothrops* in general (Barros, 2016).

Sexual maturation in *Bothrops moojeni* females occurs at a smaller size, at least in the population from São Paulo, than previous reported for this species (Nogueira et al., 2003). Early maturation is a characteristic of both sexes in *B. moojeni* and a closely related species like *B. leucurus* Wagler, 1824 from the Atlantic Forest (Barros et al., 2014a). Both species belong to the *atrox* group and show a recent divergence (Wüster et al., 1999). However, sexual maturation may vary between populations in Neotropical pit vipers (Barros et al., 2012). Thus, a study focusing on the reproduction of *B. moojeni* from central Brazil may possibly reveal some differences on this trait. In addition, there may be differences in other reproductive tactics among different populations of *B. moojeni* as recently reported for *Crotalus durissus* (Barros et al., 2012) and *B. leucurus* (Barros et al., 2014a).

Significant molecular divergence occurs among *Bothrops moojeni* populations from relatively close areas in central Brazil (Dutra et al., 2008). These high levels of population structure in *B. moojeni* may be due to the isolation of several populations. Habitat fragmentation caused by human activities and an increase in crop-producing areas in the Cerrado represents a barrier to gene flow for populations of this species (Dutra et al., 2008). Therefore, populations of *B. moojeni* from São Paulo (southeastern Brazil) and central Brazil are likely disconnected due to the high rates of destruction of the Cerrado between

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Selma Maria Almeida-Santos, Verônica Alberto Barros, Claudio Augusto Rojas, Letícia Ruiz Sueiro, Roberto Harutomi Corrêa Nomura

179

these regions, and a study on *B. moojeni* reproduction in central Brazil would provide interesting insights on intraspecific variation in reproductive traits under the recent conditions of population isolation. These data may provide useful insights for the husbandry of this species in the future if conservation measures are needed.

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181