

Reproductive biology of the neotropical rattlesnake *Crotalus durissus* from northeastern Brazil: a test of phylogenetic conservatism of reproductive patterns

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We studied reproductive patterns in neotropical rattlesnakes (*Crotalus durissus*) from areas with different climatic conditions. The timing of female reproductive events (vitellogenesis, sperm storage and parturition) was identical in both regions, therefore we assume it to be phylogenetically conserved. However, size at maturation in both sexes and male reproductive cycles (timing of spermatogenesis and testicular regression) was markedly different between study areas, suggesting that these reproductive characteristics widely differ across the species' range.

Key words: long-term sperm storage, reproductive cycles, spermatogenesis

INTRODUCTION

Reproductive traits in reptiles can vary substantially among populations (Mathies, 2011). Well-studied examples of variation in sexual maturity and reproductive seasonality in snakes include native and introduced populations of the brown tree snake *Boiga irregularis* in Australia, Papua New Guinea and Guam (Mathies et al., 2010; Savidge et al., 2007; Bull et al., 1997; Whittier & Limpus, 1996; Shine, 1991), the aquatic oviparous snake *Natrix maura* from the Iberian Peninsula (Santos et al., 2005), and the Chinese cobra *Naja atra* (Ji & Wang, 2005). Generally, inter-population variation in reproductive characteristics in squamates (see also for example Vitt, 1992; Tinkle & Ballinger, 1972; Ballinger, 1979) is often attributed to different climatic conditions and/or food availability (Solórzano & Cerdas, 1989; Bronikowski & Arnold, 1999; Pizzatto & Marques, 2006; Mathies et al., 2010).

The absence of variability in reproductive traits among populations of snakes and lizards that live under different climatic conditions may be attributable to phylogenetic inertia (James & Shine, 1985; 1988; Vitt, 1992; Orzack & Sober, 2001). Almeida-Santos & Salomão (1997; 2002) proposed that neotropical pitvipers of the genus *Bothrops* and *Crotalus* retain an ancestral reproductive pattern with regard to timing of mating, male-male fighting, parturition and long-term sperm storage. The neotropical rattlesnake *Crotalus durissus* is well suited to study phylogenetic conservatism of reproductive traits, because the species is widely distributed in open formations with different environmental conditions (disturbed areas, cerrado, caatingas, pampas and relictual savannas in Amazonia,

Campbell & Lamar, 2004). However, data on reproductive cycles have so far only been available for population from disturbed and cerrado areas from the State of São Paulo in southeastern Brazil (Almeida-Santos et al., 2004a; 2004b; Almeida-Santos & Salomão, 1997; 2002; Salomão & Almeida-Santos, 2002).

Males and females of *C. durissus* from southeastern Brazil and other *Crotalus* species from temperate regions (Schuett, 1992; Aldridge & Duvall, 2002) exhibit similar reproductive cycles (Almeida-Santos et al., 2004a; 2004b; Salomão & Almeida-Santos, 2002). Spermatogenesis begins in spring, and reaches its peak in summer (Salomão & Almeida-Santos, 2002); sperm is stored in male deferent ducts until mating occurs in autumn (Almeida-Santos et al., 2004b). Ovulation and fertilization are not synchronous to mating, and females store sperm by means of an uterine muscular twisting (UMT, Almeida-Santos & Salomão, 1997; 2002). Vitellogenesis starts in autumn and continues through winter until ovulation and fertilization occur in spring. Parturition occurs in the end of spring and during summer (Almeida-Santos & Salomão, 2002).

To test the hypothesis that reproductive patterns are phylogenetically conserved in snakes of the genus *Crotalus* from temperate and neotropical regions, we compare the reproductive biology of *C. durissus* from northeastern Brazil with data available from southeastern Brazil. Considering that consistently high temperatures are maintained throughout the year in the northeastern region, whereas low temperatures are common during the winter in the southeastern region (Mendonça & Danni-Oliveira, 2007), we expect variation in reproductive traits among the studied populations.



Fig. 1. Geographical origin of *Crotalus durissus* specimens used in this study. Circles represent samples from the northeastern population (NE) and triangles represent samples from the southeastern population (SE).

MATERIALS AND METHODS

According to Wüster et al. (2005), the three subspecies *C. d. collilineatus*, *C. d. cascavella* and *C. d. terrificus sensu* Hoge (1965) are phylogenetically closely related, and do not constitute independent evolutionary lineages. Thus, we did not adopt the subspecies designation in this study. Northeastern (NE) individuals originated from the states of Bahia, Pernambuco, Paraíba, Rio Grande do Norte, Ceará and Maranhão ($5^{\circ}11'$ to $15^{\circ}31'$ S; $5^{\circ}49'$ to $7^{\circ}32'$ W; Fig. 1), from caatinga, cerrado and disturbed areas. The coldest months were July and August (mean temperature ranging from 13°C to 31.9°C). Rainfall is concentrated between October and March, whereas April – September is dry (Fig. 2); mean annual precipitation varies between 459 and 1214 mm amongst the sampled municipalities. Southeastern (SE) individuals were studied in São Paulo state ($22^{\circ}11'$ to $23^{\circ}58'$ S; $45^{\circ}11'$ to $49^{\circ}03'$ W; Fig. 1), characterized by farmlands and urban areas. The coldest month is July (mean temperature ranging from 7.5°C to 24.1°C). The period between October and March has more rainfall and higher temperatures (Fig. 2). Mean annual precipitation varies between 1211 and 1650 mm amongst the municipalities sampled.

We examined a total of 228 preserved specimens of *C. durissus* (76 adult females, 87 adult males, 48 immatures and 17 newborns) from NE, held in the following collections: Museu de Zoologia da Universidade Estadual de Santa Cruz (MZUESC), Coleção Zoológica Gregório Bondar (CZGB) at CEPEC/CEPLAC (Centro de Estudos e Pesquisa do Cacau/ Comissão Executiva do Plano da Lavoura Cacaueira) and Coleção Herpetológica Alphonse Richard Hoge at Instituto Butantan (IB). We measured snout-vent length (SVL) to the nearest 1 mm, and after dissection the following data were recorded: (1) diameter of the largest ovarian follicle or presence of embryos in females; (2) presence/absence of UMT; (3) oviductal condition (flat or folded); (4) presence of corpora lutea in the ovaries; (5) testicular volume (TV), as determined

using the formula $TV = 4/3 \cdot \Pi \cdot a \cdot b \cdot c$, where a =half of length, b = half of width and c =half of thickness; (6) diameter of the right distal deferent duct close to the cloaca. These measurements were taken to the nearest 0.1 mm.

The presence of spermatozoa in the testes or deferent ducts was used as the main criterion to determine sexual maturity in males. The convoluted aspect of deferent ducts was used as an additional criterion. Females were considered mature if they had follicles in secondary vitellogenesis, and based on the presence of oviductal embryos or corpora lutea in the ovaries or folded oviducts. Individuals that did not present these characteristics were considered immature. Certain individuals that were housed in the collections had already been dissected, some organs were missing and the collection date was not available for some specimens. Therefore, we only included data from these specimens in sexual dimorphism analyses.

Sexual dimorphism (SSD) was measured as $1 - \text{mean adult SVL of the larger sex} / \text{mean adult SVL of the smaller sex}$ (Gibbons & Lovich, 1990; Shine, 1994). Positive and negative values of SSD correspond to females larger than males and vice versa, respectively. The reproductive cycle of mature females was determined by comparing the length of the largest ovarian follicle or oviductal embryo at different times of the year. The distal portion of the uterus in the presence of UMT and secondary vitellogenic follicles served to verify whether sperm was stored in the female reproductive tract. Records of *C. durissus* from NE maintained in captivity at IB from 1973 to 1998 and literature records were examined to obtain data on parturition period and size of newborns.

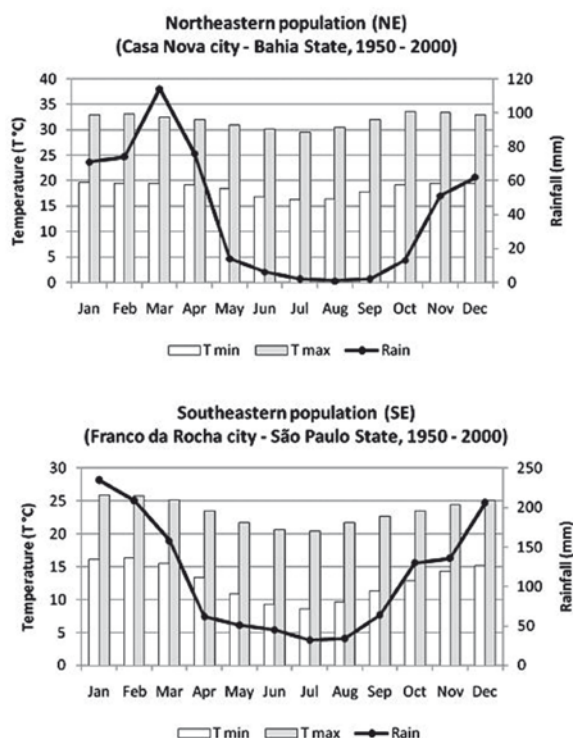


Fig. 2. Climatic patterns in the northeastern (NE) and southeastern (SE) Brazilian areas where the populations considered in this study were sampled.

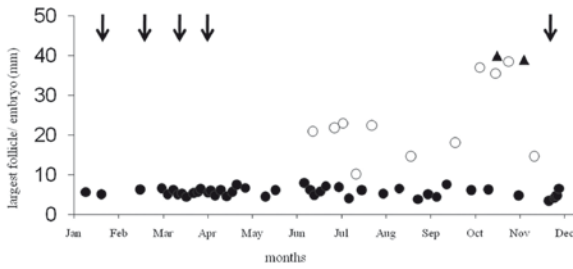


Fig. 3. Seasonal variation in diameter of the largest ovarian follicle or embryo in mature females of *Crotalus durissus* from northeastern Brazil (NE). Solid circles=primary vitellogenic follicles; open circles=secondary vitellogenic follicles; solid triangles=embryos in the oviducts. Arrows indicate the parturition period.

In males, correlations of testes volume and deferent duct diameter with body length (SVL) were tested for by linear regressions. Analysis of covariance (ANCOVA) was employed to compare testes volume in the four seasons of the year, with SVL being the covariate. Differences on deferent duct diameter among seasons were tested by analysis of variance (ANOVA). Testes and the distal portion of deferent ducts of four males per season were collected after dissection in order to analyze the spermatogenic cycle and to determine the presence of sperm, respectively. All samples of testes from the winter period were inappropriate to histological analysis, probably due to inadequate fixation of individuals held in the collections. All organs (uterus, testes and deferent ducts) were preserved in 70% alcohol and processed for light microscopy by the paraffin method, staining 5 μ m sections with Hematoxylin and Eosin Stain (H/E).

Statistical analyses were performed using Statistica (version 7.0) and Bioestat (version 5.00). The analyses were carried out according to Zar (1999), and all variables were tested for normality and homoscedasticity prior to analysis. Map and climatograms were constructed for every municipality sampled using DIVA-GIS (version 7.1.7) and WorldClim Database (current climate: 1950–2000, version 1.3, 10 minutes resolution).

RESULTS

Sexual maturation and dimorphism

All males below 82 cm SVL and all females below 83 cm SVL were sexually immature. Mature males averaged 113.4 cm (SD=12.8, $n=87$, range=82–146), and mature females averaged 111.5 cm SVL (SD=13.8, $n=76$, range=83–147); no significant difference was observed between the sexes ($t=-0.89$, $p=0.37$). The degree of sexual dimorphism (SSD) was -0.02.

Female reproductive cycle

Primary vitellogenic follicles were present throughout the year, but secondary vitellogenic follicles were present from the end of autumn to the end of spring (July to November). Pregnancy was observed in spring (October and November, Figs. 3, 4). UMT was macroscopically observed during autumn and winter in some females

of our sample. The posterior uterus of all females examined during spring and summer was straight and did not show any twisting. Spermatozoa were found being stored in the posterior uterus of females presenting secondary vitellogenic follicles during winter (July, Fig. 5). Parturition was recorded for three females from NE Brazil maintained in captivity at Instituto Butantan. Births occurred in summer (February 1984, 21 newborns; March 1984, 33 newborns) and early autumn (April 1984, 12 newborns, Fig. 4). Neonates of a litter from Pernambuco State averaged 29.4 cm SVL (SD=6.9, $n=17$, range=28–30.5 cm)

Male reproductive cycle

Histological analyses showed that *C. durissus* from NE Brazil present a seasonal spermatogenic cycle. Spermiogenesis occurs at the end of summer and autumn (March to June, $n=5$) and peak spermatogenesis is reached during autumn (June, $n=3$). Complete regression (only spermatogonia and Sertoli cells present in the seminiferous tubules) was detected in late spring (November, $n=4$, Figs. 4, 6).

Testes volume was positively correlated with SVL ($F=14.23$, $r^2=0.18$, $p<0.01$), whereas deferent duct diameter was not ($F=0.74$, $r^2=-0.004$, $p=0.60$). Although an increase in testes volume occurs in autumn (end of March to June, Fig. 7A), no significant differences occurred among the four seasons (ANCOVA, $F=0.43$, $p=0.73$). Sperm was present throughout the year in deferent ducts, although duct diameter did not show significant variation among seasons (ANOVA, $F=1.29$, $p=0.28$, Fig. 7B). A male-male combat episode was observed during autumn (end of May) by a pasture area in the municipality of Bom Jesus da Lapa, Bahia State (A.P. Silva, personal communication).

DISCUSSION

Sexual maturation and dimorphism

Size at sexual maturation is highly variable among *C. durissus* populations. Rattlesnakes from Central America mature at larger sizes (100 cm for females and 101 cm for males, Solórzano & Cerdas, 1988) than populations from South America. Individuals of the *C. durissus* population from SE Brazil may become sexually mature at 56 cm

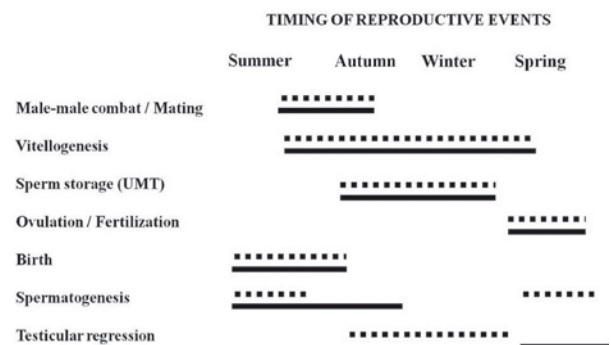


Fig. 4. Timing of reproduction in *Crotalus durissus* populations from northeastern (continuous line) and southeastern Brazil (dotted line).

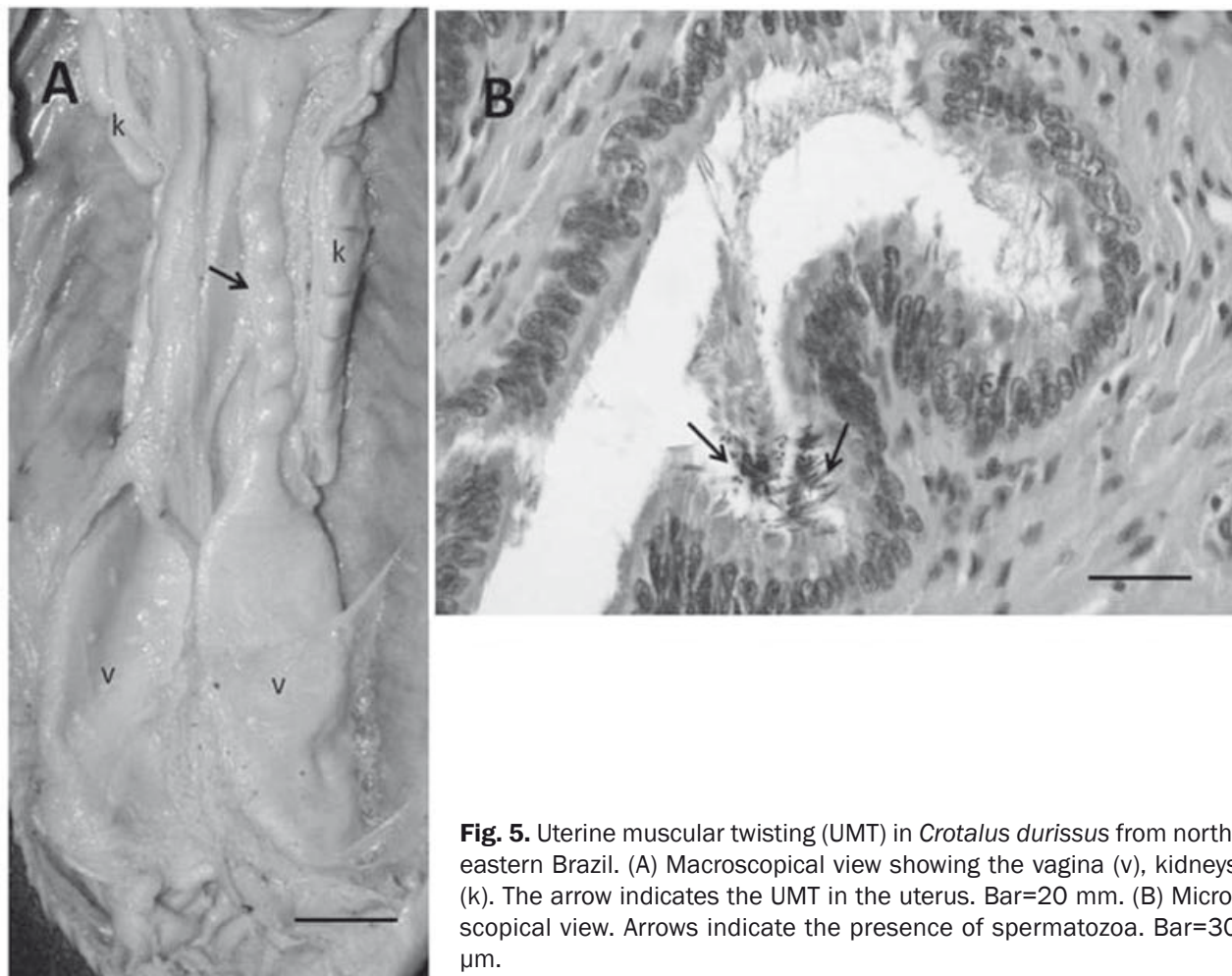


Fig. 5. Uterine muscular twisting (UMT) in *Crotalus durissus* from north-eastern Brazil. (A) Macroscopical view showing the vagina (v), kidneys (k). The arrow indicates the UMT in the uterus. Bar=20 mm. (B) Microscopical view. Arrows indicate the presence of spermatozoa. Bar=30 µm.

(males) and 76 cm (females, Salomão & Almeida-Santos, 2002; Almeida-Santos, 2005). Such intraspecific differences in sexual maturation size may reflect local adaptations of *C. durissus* populations. Individuals from NE and Central America seem to allocate more energy to growth before starting reproductive activities (Shine, 1980; Aubret et al., 2002). On the other hand, becoming sexually mature at a smaller size means that allocation of resources to reproduction begins earlier in SE populations, and less energy may be available for growth (Bonnet et al., 1998). Thus, *C. durissus* females from SE are smaller and produce smaller clutches (♀ SVL=98.5±8.5 cm; clutch size=11.7±5.1, Almeida-Santos, 2005) than females from NE (SVL=111.5±13.84 cm; clutch size=12 to 33 newborns; Cordeiro et al., 1981; Lira-da-Silva et al., 1994) and Central America (♀ SVL=127 cm, range 100–160 mm; clutch size=22.9±5.9, Solórzano & Cerdas, 1988). Data on the frequency of reproductive events are needed for measures of lifetime reproductive success, and to assess differences in reproductive costs among *C. durissus* populations.

Males are larger than females in most species of genus *Crotalus* (Shine, 1994; Klauber, 1972). However, males and females of *C. durissus* from NE Brazil do not exhibit sexual dimorphism in relation to body size (SVL). According to Solórzano & Cerdas (1988), males of *C. durissus* from Central America have a higher total length than females. Male snakes usually have longer tails than females (Shine et al., 1999), and the absence of sexual

SVL dimorphism in *C. durissus* needs further confirmation with larger samples. Selective forces may jointly act on female and male body size, because fecundity increases with larger body sizes (Shine, 1994), and larger males are more successful in male-male combats and access to females (Shine, 1978; Shine, 1980; Madsen & Shine, 1993).

Female reproductive cycle

The timing of female reproductive events is identical in NE and SE populations, despite different climatic conditions. Secondary vitellogenesis occurs from the end of autumn throughout the winter until ovulation in spring. Females store sperm over winter by means of a uterine muscular twisting (UMT) in both populations. As ovulation and mating are not synchronous, long-term sperm storage is important to guarantee fertilization in spring (Schuett, 1992). Sperm storage may also be a strategy to promote sperm competition: by delaying fertilization, females may gain fitness benefits by allowing sperm from different males to compete (Fox, 1963; Conner & Crews, 1980; Sever & Brizzi, 1998). Sperm storage is rather unexpected for snake species in tropical areas (Saint-Girons, 1982). Under favourable climatic conditions such as in the Brazilian northeastern region, it is rather surprising that ovulation in *C. durissus* is not synchronized with copulation.

Siegel & Sever (2006) questioned the existence of UMT in *C. durissus*, based on a single specimen. Here,

we provide macroscopical evidence of the existence of UMT in the posterior uterus. Histological analysis also shows that sperm was present in the UMT of *C. durissus* females during winter. As mating has never been observed during winter, we conclude that presence of sperm is not due to recent mating activity, but evidence for sperm storage. Sperm may be maintained viably while being stored in the posterior uterus by secretions of the female oviducts (Marinho et al., 2009) or by secretions of male origin (Siegel & Sever, 2008). Simple tubular glands

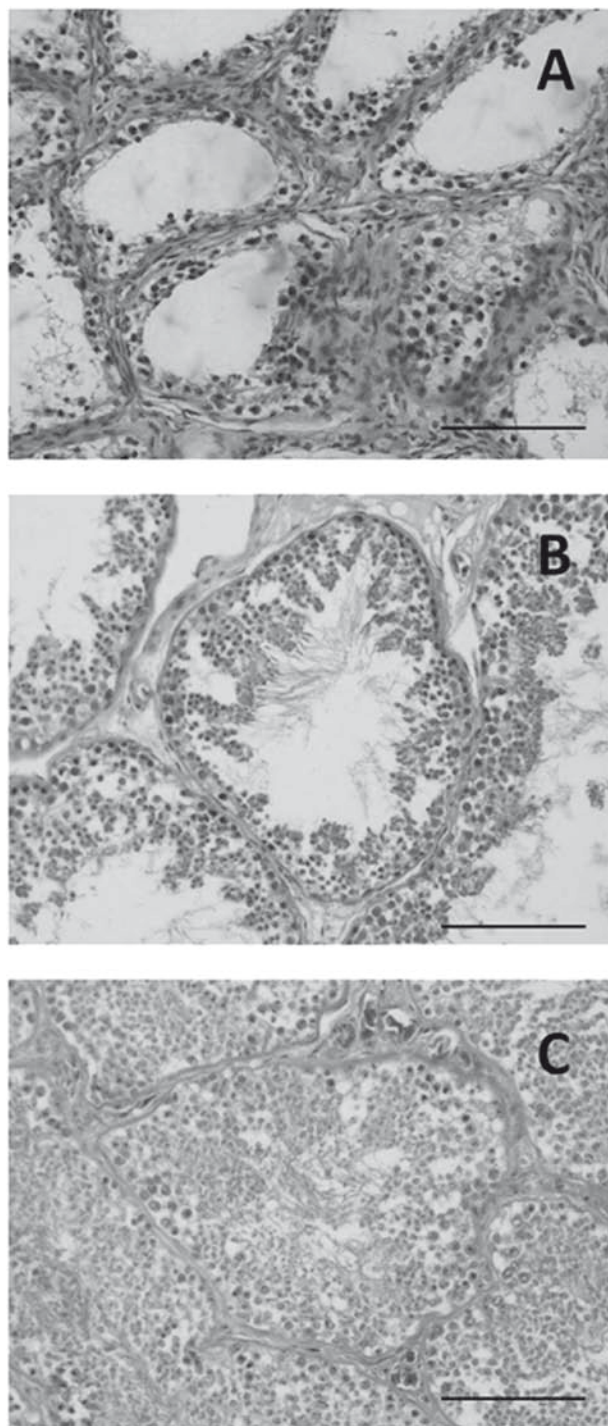


Fig. 6. Sections of the testes in *Crotalus durissus* from Northeastern Brazil. (A) Inactive period (November - spring). (B) Spermatids and the first spermatozoa (March - summer). (C) Epithelium maximal height, peak spermatogenesis (June - autumn). Bars=100 µm.

that may be sperm storage tubules were found in the posterior infundibulum (Siegel & Sever, 2006). Although no sperm was found in these glands, their existence suggests that *C. durissus* may also store sperm in the posterior infundibulum during spring until ovulation and fertilization. More research is needed to clarify the role of the infundibular glands in *C. durissus*.

Parturition also occurs during summer and early autumn in both populations (Cordeiro et al., 1981; Lira-da-Silva, 1994; Almeida-Santos & Salomão, 1997; 2002). The size of newborns from SE (SVL=29.7±4.1 cm, Almeida-Santos, 2005) is very similar to the size of newborns of a NE clutch described in this study. However, according to Cordeiro et al. (1981) and Lira-da-Silva et al. (1994), newborns of *C. durissus* from Bahia and Maranhão States in NE Brazil may be larger than 34 cm. Additional data are needed to allow for a more accurate comparison.

Similarly to conserved female reproductive cycles described for *Micrurus* (Marques, 1996), the reproductive patterns observed for *C. durissus* resemble those described for Viperidae from temperate regions (Schuett, 1992; Aldridge & Duvall, 2002), despite the occurrence of a continuous vitellogenic cycle throughout the winter and the absence of hibernation (Almeida-Santos & Salomão, 1997). Other squamates which occur in both temperate and tropical areas also display the reproductive pattern expected in temperate areas (James & Shine, 1985; 1988; Vitt, 1992; Marques, 1996). Phylogenetic inertia may be a plausible explanation for this observation (cf. Orzack & Sober, 2001). On the other hand, some species of snakes from the neotropical region exhibit intraspecific variation in female reproductive cycles. Timing of mating and parturition are different in two populations of the pitviper *Bothrops asper* isolated by mountains in Costa Rica (Solórzano & Cerdas, 1989). Two populations of *Waglerophis merremii* from geographically adjacent regions with distinct climates (southeastern Brazil vs. southern Brazil) exhibit different timing of vitellogenesis and egg-laying (Pizzatto et al., 2008). Vitellogenesis may be more or less synchronized among females of *Liophis miliaris* (Pizzatto & Marques, 2006). Reproductive cycles of other snake species from neotropical regions (e.g. *Leptodeira anulata*, Pizzatto et al., 2008; Fitch, 1970 and *Tantilla melanocephala*, Marques & Puerto, 1998; Santos-Costa et al., 2006) seem to be seasonal or aseasonal depending on latitude; however, more data is needed to confirm these patterns (Mathies, 2011). Thus, the timing of female reproductive events in snakes may be conservative in a lineage, despite being influenced by factors such as climate conditions and food availability (Pizzatto et al., 2008; Brown & Shine, 2006; Ji & Wang, 2005; Santos et al., 2005).

Male reproductive cycle

Crotalus durissus males from NE Brazil did not exhibit a conserved pattern of reproductive activity. Inter-population variation in the reproductive cycle of males is less studied than in females, focusing on testes volume or testes size/ mass as an indicator of timing of spermatogenesis (Mathies, 2011). Testicular cycles (seasonal variation in testis mass) differ in two populations

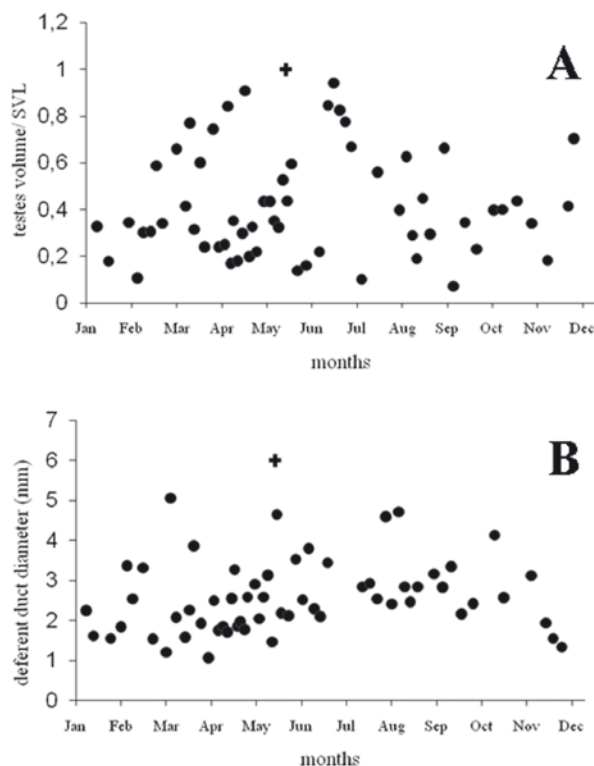


Fig. 7. (A) Seasonal variation in the relative testes volume in *Crotalus durissus* northeastern population (NE). (B) Seasonal variation in the deferent duct diameter (mm) in *Crotalus durissus* northeastern population (NE). The cross indicates male-male combat occurrence.

of the pitviper *B. asper* isolated by mountains in Costa Rica (Solórzano & Cerdas, 1989). However, without histological evidence this assessment may be misleading (Mathies, 2011). In *Boiga irregularis* from Guam and Papua New Guinea, spermatogenesis occurs throughout the year, while males from southeastern Queensland in Australia only exhibit seasonal testicular regression (Bull et al., 1997; Mathies et al., 2010).

Different timing of spermatogenesis and testicular regression among *C. durissus* populations from NE and SE Brazil may be interpreted as a plastic response to environmental conditions (Stearns, 1976). Male snakes of the genus *Crotalus* from temperate areas (Saint-Girons, 1982; Aldridge & Duvall, 2002) and *C. durissus* from SE Brazil (Salomão & Almeida-Santos, 2002) present post-nuptial, dissociated or type I reproductive cycles according to the classification proposed by Saint-Girons (1982): spermatogenesis occurs in the warmer season and spermatozoa are stored throughout the winter in the deferent ducts or in the female reproductive tract. Histological analyses showed that testicular regression occurred in autumn and winter (colder seasons), while spermatogenesis began in spring and reached its peak in summer (warmer season) in males of the SE population (Salomão & Almeida-Santos, 2002). Sperm is stored by males in the deferent ducts until mating occurs in autumn (when testes are inactive) and after copulation, and by females throughout the winter in the uterus, until ovulation occurs in spring (Almeida-Santos et al., 2004b; Almeida-Santos & Salomão, 1997).

The process of spermatogenesis is apparently under the

control of body temperature (Aldridge & Duvall, 2002). Thus, the consistently high temperatures in the NE region would allow males to conduct spermatogenesis at any time of the year. Our results show that testicular regression occurs in spring, while spermatogenesis begins in summer and reaches its peak during autumn, simultaneously to male-male combat and probably to mating. The mating season may coincide with spermatogenesis in some species of viperid snakes from temperate regions, although these events occur in the warmer seasons (Saint-Girons, 1982; Aldridge & Duvall, 2002).

Although sperm storage in the female reproductive tract throughout the winter is obligatory in NE populations, the male reproductive cycle may be classified as prenuptial, associated or type III according to Saint-Girons (1982) and Schuett (1992) because spermatogenesis is completed towards the end of mating period. Despite seasonal spermatogenesis, sperm is present throughout the year in the deferent ducts. Studies considering the sexual segment of the kidney (cf. Sever et al., 2008) and abdominal fat (cf. Santos & Lorente, 2004) should be conducted to verify if there are differential reproductive costs between SE and NE *C. durissus* populations representing different male reproductive strategies. Temperature variation across different regions, especially the maintenance of high temperatures during autumn and winter in Northeastern Brazil, may be a key factor to explain intraspecific variation and evolutionary plasticity in *C. durissus* male reproductive cycles. More research, including a common garden experiment which standardizes environmental factors (Ji & Wang, 2005), will be essential to determine if timing of spermatogenesis is based on phenotypic variation or a local adaptation of *C. durissus* males.

ACKNOWLEDGEMENTS

We thank V.J. Germano and Cristian A. Gomes for their technical assistance and Lilian R. B. Mariutti for the English review. Francisco L. Franco (IB), Jacques Delabie (CEPLAC) and Antonio J. Argôlo (MZUESC) allowed access to collections. This study is based on an MSc thesis undertaken by Verônica A. Barros, funded by FAPESP (2008/56169-5).

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Accepted: 21 September 2011