

Sperm storage in males of the snake *Crotalus durissus terrificus* (Crotalinae: Viperidae) in southeastern Brazil

Selma M. Almeida-Santos^a, Iara L. Laporta-Ferreira^a, Marta M. Antoniazzi^b, Carlos Jared^{b,*}

^aLaboratory of Herpetology, Instituto Butantan, Av. Dr Vital Brazil 1500, CEP 05503-900, São Paulo, Brazil

^bLaboratory of Cellular Biology, Instituto Butantan, Av. Dr Vital Brazil 1500, CEP 05503-900, São Paulo, Brazil

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Abstract

Seasonal variations in spermatozoa numbers and in sperm motility along the vas deferens in *Crotalus durissus terrificus* from southeastern Brazil were analyzed. Our data demonstrate storage and motility of the spermatozoa along the vas deferens throughout the year. This is characteristic of a postnuptial reproductive cycle, usually found in snakes living in temperate climates. We describe similarities in reproductive cycle patterns found in the tropical nonhibernator *C. durissus terrificus* and in hibernator snakes from temperate zones. Our results show that in *C. durissus terrificus*, a significant difference in spermatozoa counts occurs between winter and summer. Higher numbers of spermatozoa in summer and autumn, due to intense spermiogenesis, coincides with the mating season in autumn. These data indicate that after spermiogenesis in summer, the males combine the peak of sperm storage to the period females are attractive. Mating, however, is not linked to ovulation, and the sperm is stored in the females during winter until fertilization occurs in spring. In the males, after mating, spermatozoon counts low. In spring, they gradually increase, turning again the highest in summer and autumn. During spermiogenesis in the convoluted vas deferens, spermatozoa gain motility, enhancing their performance along their way towards the distal portion.

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1. Introduction

The effects of environment on the reproductive physiology of tropical snakes are little known, especially in males (Seigel and Ford, 1987; Schuett, 1992; Schuett et al., 2002). Study of testicular cycles of certain species has suggested that the spermatozoa are active through most of the year (Seigel and Ford, 1987).

Volsøe (1944) and Saint-Girons (1982) were the first authors who identified different categories in the spermatogenic cycles of snakes, which were summarized by Schuett (1992) as type I (aestival or postnuptial), type II (mixed), type III (vernal or prenuptial), and type IV (continuous or seasonal) (for subtypes, see Schuett, 1992). In type I cycle, spermatogenesis initiates in spring and ends with spermiogenesis in late summer or early autumn. The spermatozoa

are then stored in the vas deferens throughout winter, during hibernation, or occasionally in the oviducts of the females. These previous definitions together with data on ecology and natural history contributed to the better understanding of snake mating systems in temperate climates, and favored the characterization of spermatogenesis and reproductive cycles, as described by Aldridge (1979, 1993, 2002), Jacob et al. (1987), Johnson et al. (1982), and Goldberg and Rosen (2000).

In tropical and equatorial regions, data characterizing snake male reproductive cycles are scarce, although it is known that climatic conditions allow spermatogenesis to proceed at any time during the year, and it is always prenuptial (Saint-Girons, 1982). Salomão and Almeida-Santos (2002) observed that spermatogenic activity in *Crotalus durissus terrificus* of southeastern Brazil begins in spring and reaches a peak in production in summer. These findings demonstrate that although this species lives in a tropical region, its reproductive cycle is characterized as type

* Corresponding author. Fax: +55 11 3257 8905.

E-mail address: carlosjared@uol.com.br (C. Jared).

I (aestival or postnuptial) (Saint-Girons, 1982; Schuett, 1992). In this type of cycle, sperm storage is indispensable (Seigel and Ford, 1987) because there is a temporal dissociation between spermatogenesis and mating. However, data on numerical variation in spermatozoa during the year, or on the storage process, are still insufficient or imprecise.

The neotropical rattlesnake *C. durissus* has a wide distribution in Americas, ranging from Mexico to northern Argentina (Campbell and Lamar, 1989). In southeastern Brazil, *C. durissus terrificus* is reproductively active from late summer (March) to early winter (July) (Salomão et al., 1995). In autumn (April to June), the period of sexual activity, males engage in ritualized fights (combat) and winners presumably gain priority of access to females (Almeida-Santos et al., 1990; Almeida-Santos et al., 1999).

The aim of this work is to analyze the relationship between seasonal variation of testicular activity and sperm storage in males of *C. durissus terrificus*. Similarities in reproductive cycle patterns in this rattlesnake and others from temperate climates are discussed.

2. Material and methods

Adult males ($n=72$) of *C. durissus terrificus* Laurenti, 1768, used in this study were (mean \pm S.D.) 915 \pm 89 mm (range=800–1070 mm) TL and 585 \pm 198 g (range=285–990 g) body mass; all had been brought to the Instituto Butantan, from the vicinity of São Paulo City, southeastern Brazil.

From 1999 to 2000, three recently collected snakes were sacrificed every 10 days, using 30 mg/kg of Nembutal administrated subcutaneously (Chudzinski et al., 1989). The average room temperature while the snakes were maintained in captivity (maximum of 1 week) was 22 °C. These specimens were also used for other studies concerning the reproductive cycle (Salomão and Almeida-Santos, 2002) and the ultrastructure of the reproductive system.

For a more precise reference, three regions were recognized in the vas deferens: proximal, median, and distal (Fig. 1). The structures were removed by laparotomy (Langlada et al., 1973), and then stretched out and elongated on a glass slide for semen extraction. For this purpose, a compression over the whole extension of the two vasa deferentia was applied. Of the total amount of semen obtained, part was used for spermatozoa concentration count and the other part for analysis of motility and survival-time tests. Sperm motility was used as a parameter for determining the survival-time test. One drop of nondiluted semen was placed on a glass slide maintained at room temperature (± 22 °C) and observed on a light microscope (magnification, 400 \times). The grade of motility was then determined for each one of the regions (proximal, median, and distal) by visual criteria. After that, observations were made every 2 h for 10 h. Thus, the survival-time was defined as the interval from semen extraction to the time sperm was no longer motile. Counts were carried out in a Neubauer chamber after dilution in Ringer's solution (Hoar and Hickman, 1967) in a proportion of 1:5. For better spermatozoa stabilization, the chamber was first flushed with formalin vapor. The same survival-time tests were applied to the sperm extracted from snakes ($n=5$) kept in the freezer (-10 °C) just after death for 24 h.

For statistical analysis, ANOVA and Tukey tests were used. Microphotographs were made from fresh material. Mating seasons were determined by observation of copulations in the Instituto Butantan's semi-extensive captivity ($n=6$) and also in the field ($n=1$).

3. Results

The vas deferens (Figs. 1 and 2) of *C. durissus terrificus* is convoluted (Fig. 2), and in this form occupy approximately one-third of the body length. When freed from the

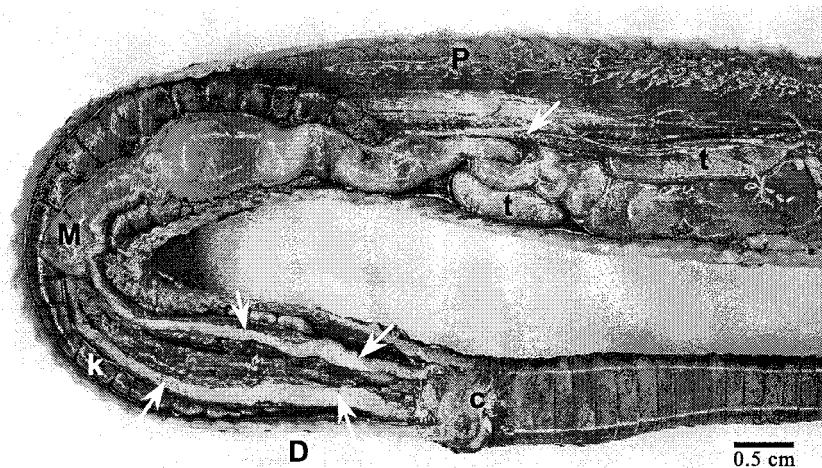


Fig. 1. *C. durissus terrificus*. Dissection of the genital tract of a male. Vasa deferentia, arrows; t—testicles; k—kidney; c—cloaca. Regions: D—distal; M—median; P—proximal.

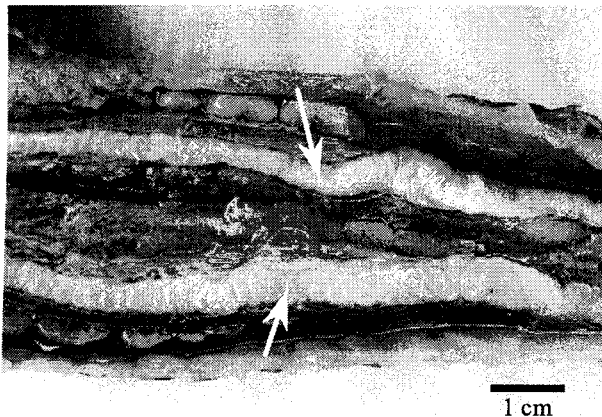


Fig. 2. Higher magnification of Fig. 1, showing the convoluted arrangement of vasa deferentia (arrows).

peritoneal membrane and manually distended, its length is about six times the total body length, although the right vas deferens is always slightly longer than the left.

The total volume of semen obtained from each male varied from 0.8 to 1.0 ml, proportionally to the size of the snake. No seasonal variation in semen volume was observed. Sperm color varied from white to ivory.

Sperm motility increases from the proximal to the distal region of the vas deferens, especially in the distal third, which presents a thicker diameter when compared to the other regions (Fig. 1), and larger sperm concentration counts. The survival-time of nondiluted spermatozoa exposed to the laboratory environment was approximately 8 h, at an average temperature of 20 °C in winter and 24 °C in summer. Spermatozoa extracted from snakes kept in the freezer after death for 24 h, when defrosted, still showed motility.

The light microscope showed that the spermatozoa have spicule-shaped tips in the acrosomes (Fig. 3).

Spermatozoa were observed to be present in the vas deferens of *C. durissus terrificus* at all seasons during the 2 years of study (Table 1 and Fig. 4). Over this period, no

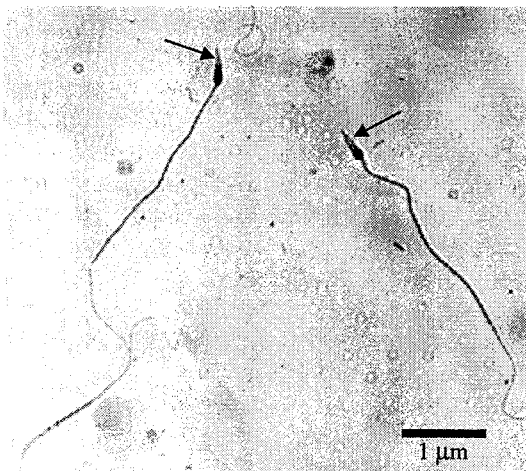


Fig. 3. Spermatozoa from the distal vas deferens from *C. durissus terrificus*. The arrows point to the spicule-shaped acrosomes.

Table 1

Means and standard error of spermatozoa concentration collected over two successive years

Season	N	Spermatozoa count ($\times 10^5/\text{mm}^3$)
Winter (Jun, Jul, Aug)	18	38.21 \pm 0.34
Spring (Sept, Oct, Nov)	18	40.68 \pm 0.14
Summer (Dec, Jan, Feb)	18	41.68 \pm 0.11
Autumn (Mar, Apr, May)	18	41.73 \pm 0.08

Statistical differences: $P_{\text{winter spring}} < 0.001$, $P_{\text{spring summer}} < 0.01$, $P_{\text{summer autumn}} > 0.05$.

significant difference was seen in spermatozoa numbers at the same season in different years ($F=71.38$, $df=143$, $P>0.05$).

A comparison of spermatozoa counts between winter and spring shows that the mean count in winter (38.21 \pm 0.34) was significantly lower than that obtained in spring (40.68 \pm 0.14) ($P<0.001$), characterizing an increase in spermatozoa numbers along the months. Comparison between spring and summer (41.68 \pm 0.11) shows a significant increase of sperm numbers ($P<0.01$). The highest counts were found in summer and autumn (41.73 \pm 0.08), with no significant differences among these two seasons ($P>0.05$). When compared to summer and autumn, winter and spring were characterized by a lower spermatozoa count (Table 1 and Fig. 4). Copulation was observed only during autumn (Fig. 4), both in the semi-extensive captivity and in the field.

Fig. 4 shows an outline of seasonal male and female reproductive events demonstrating that there is a synchronism among the highest counts of spermatozoa in males, vitellogenesis, and mating (in autumn), and an asynchronism between spermiogenesis and mating, since fertilization occurs only in spring, after a period of sperm storage in the female uteri during winter. The figure also shows that the lower counts of spermatozoa in males occur after mating, during winter.

4. Discussion

The process of sperm storage in snakes is better known in females (Fox, 1956, 1977; Schuett, 1992). However, since the studies of Volsoe (1944), Shine (1977), and Saint-Girons (1982), this phenomenon is known to occur also in males.

In reptiles, the vas deferens is the site of sperm storage in males, and differently from mammals, the epididymis does not participate in sperm maturation or storage (Jones, 1998; Sever et al., 2002). We observed that in *C. durissus terrificus*, the spermatozoa would become increasingly more motile along their journey from the proximal to the distal region of the vas deferens. In the distal region, they are in the maximum of their motility, suggesting that the internal environment along the male genital tract enhances somehow spermatozoa maturation and performance. In this species, the convoluted configuration of vas deferens seems

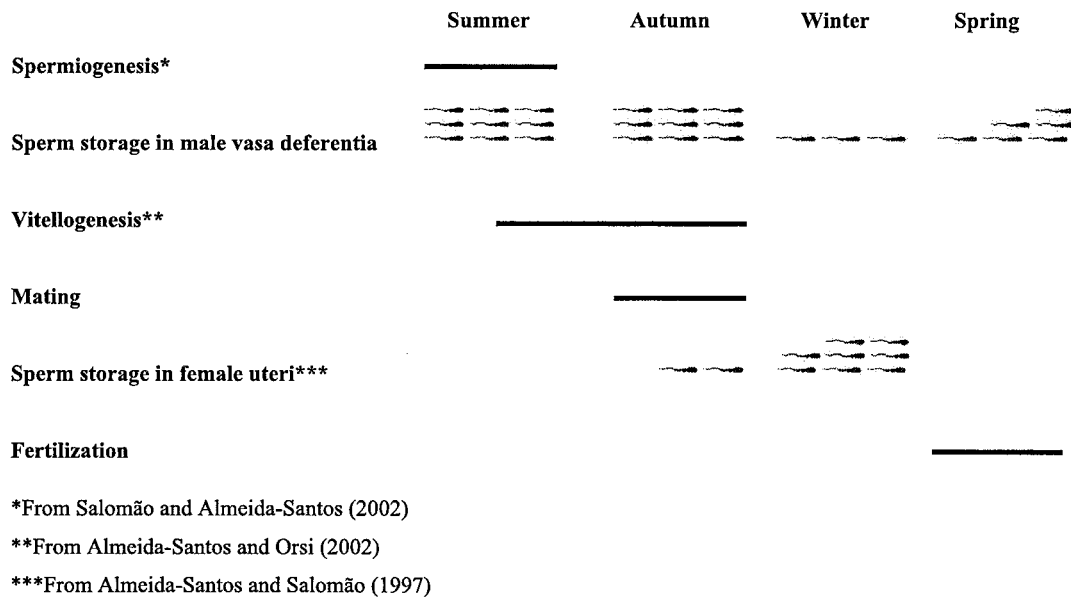


Fig. 4. *C. durissus terrificus*—seasonal reproductive events and spermatozoa occurrence in genital tract of males and females. (—) lowest spermatozoa counts; (⋈) increase in spermatozoa counts; (⋈) highest spermatozoa counts.

to be directly associated to the elongation of the way of spermatozoa during their maturation (Fox, 1977; Tsui and Licht, 1974). This configuration must also favor storage and concentration of sperm.

Diller and Wallace (1984) working with *Crotalus viridis* from Idaho, USA, observed that the spermatozoa remain in the vas deferens throughout the year, including the hibernation period. Aldridge (1979, 1993, 2002) described the same process in various subspecies of this rattlesnake, affirming that spermatogenesis occurs during summer and the spermatozoa are stored in the vas deferens until mating. In populations with short-term sperm storage (STSS), the sperm is stored in male vas deferens over the winter, whereas in populations with long-term sperm storage (LTSS), the sperm is stored in the female oviduct over the winter (Aldridge and Duvall, 2002). In certain populations, LTSS is an obligatory component of the reproductive cycle of the rattlesnake *C. viridis viridis* (Schuett, 1992). Yokoyama and Yoshida (1993) found sperm stored in the posterior region of vas deferens in the Japanese viperid *Trimeresurus flavoviridis*. Both in *C. viridis* and *T. flavoviridis*, the reproductive cycle, according to Saint-Girons (1982) and Schuett (1992), can be classified as postnuptial or type I (aestival), characteristic of snakes from temperate climates.

Salomão and Almeida-Santos (2002) reported that spermatogenesis in the nonhibernator *C. durissus terrificus* begins in spring. It was observed in this work that during autumn and winter, spermatozoa are present in vas deferens. However, in these seasons, according to Belluomini et al. (1966), there is no evidence of spermatogenesis, and in addition, a clear testicular regression was found (Salomão and Almeida-Santos, 2002). All these data are a strong indication that male tropical *C. durissus terrificus* have the ability of storing sperm in autumn and winter, as other

rattlesnakes from temperate regions do, independent of whether they live in the North or South hemispheres, where seasons are temporally inverse.

Although spermatozoa of *C. durissus terrificus* are present throughout the year, their counts are the lowest during winter—a fact also observed by Mello and Belluomini (1965)—and are the highest in summer and autumn, due to the high temperatures favoring testicular recrudescence in early spring, when spermatogenesis begins (Salomão and Almeida-Santos, 2002). Furthermore, spermiogenesis peaks in summer (Salomão and Almeida-Santos, 2002). This cycle is apparently under the control of body temperature. Aldridge (1975) found that spermatogenesis in *C. viridis viridis* was initiated by warm environmental temperatures and not by photoperiod.

Tsui and Licht (1974), working with spermatozoa of the American colubrid *Thamnophis sirtalis*, did not find difference in motility at temperatures ranging from 8 to 30 °C. In the present work, it was observed that the spermatozoa of tropical *C. durissus terrificus* remain alive and motile even after exposure to temperatures of −10 °C, apparently not being affected by freezing. Thus, in the specimens examined, the reduction in numbers of spermatozoa in winter (as shown in Table 1 and Fig. 4) must be exclusively due to their use in copulation during autumn. Similarly, Johnson et al. (1982), working with *Agkistrodon piscivorus* from Alabama, have demonstrated that spermatozoa are stored in the vas deferens through the winter and reduce in number soon after copulation in spring.

Copulation in *C. durissus terrificus* was observed only during autumn. However, it could potentially occur in spring because, during this season, spermatozoa are also present in vas deferens. Sever et al. (2002) reported that in the American colubrid *Seminatrix pygaea*, although the

sperm is stored in males throughout the year, in females, it is found in storage tubules of the oviduct only in late spring (Sever and Ryan, 1999). From these observations, we conclude that the mating period would be determined by the females, because sperm was never found in the oviducts during summer or autumn.

In *C. durissus terrificus*, vitellogenesis and mating season are synchronous (Almeida-Santos and Orsi, 2002) and coincide with the highest counts of spermatozoa in males especially in autumn. These data indicate that after spermiogenesis in summer, the males combine the peak of sperm storage to the period vitellogenesis is completed, and females enter estrus, signaling they are receptive for copulation (Aldridge and Duvall, 2002). All these observations corroborate the hypothesis that females are the determinant of the mating period. After copulation, females store the sperm in uteri during autumn and winter (Almeida-Santos and Salomão, 1997) and fertilization occurs in the spring.

The age of the sperm in male vas deferens used during copulation has never been determined (Aldridge and Duvall, 2002). However, through the analysis of the reproductive events, both in males and females, it is possible to estimate this age at a minimum of 1 year. *C. durissus terrificus* has an annual cycle (Salomão and Almeida-Santos, 2002), and in the beginning of a new spermatogenic season, sperm is usually found in vas deferens, probably remnant from previous cycle. These observations lead to the conclusion that sperm produced in spring, together with the remnant from previous season (or seasons), is stored throughout the year (with higher counts in late summer and autumn). After being transferred to the female uterus by copulation in autumn, storage continues during winter in the female, and finally in spring, sperm is used for fertilization, as it has already been demonstrated by Almeida-Santos and Salomão (1997) for this species. Since spermatogenesis occurs in summer in all species of temperate North American Crotalinae snakes examined (Saint-Girons, 1982), the actual age of the sperm, whether snakes mate in summer, spring, or both, is identical (Aldridge and Duvall, 2002).

Saint-Girons (1982) suggests that the advantage of male sperm storage would be the possibility for the male to fertilize numerous females. However, in order to support this idea, it would be necessary first to determine the medium volume of sperm used in one copulation, and then to associate it with the total volume stored in vas deferens.

Storage of spermatozoa is usually considered as a factor in the adaptation of snakes to very cold climates (Shine, 1977). Our data demonstrate that the storage pattern in the vas deferens of tropical nonhibernator *C. durissus terrificus* is similar to that of the hibernator north American species *C. viridis*, *C. scutellatus*, *C. atrox*, and *A. piscivorus* (Schuett, 1992; Aldridge, 2002). Since *C. durissus terrificus* lives in a region where storage does not seem necessary, besides similarities due to pitviper phylogeny (Parkinson et al., 2000), the maintenance of this reproductive strategy may be the result of adaptations to other physiological factors.

On the other hand, sperm storage seems to be a characteristic common to all viperids (Schuett, 1992; Aldridge and Duvall, 2002) and is a result of selection of mating seasons in species living in different environments. According to Aldridge and Duvall (2002), the timing for mating within species tends to be similar, but among species is variable, suggesting that the mating season is an ecologically plastic trait.

Thus, besides its importance in the study of snake biology, sperm storage could be an important additional factor in the analysis of phylogenetic questions in this group.

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