

Long-Term Sperm Storage in the Female Neotropical Rattlesnake *Crotalus durissus terrificus* (Viperidae: Crotalinae)

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Abstract: This paper reports the muscular twisting and convolution of the uterus in *Crotalus durissus terrificus* as a strategy to store sperm over winter (long-term sperm storage). Aspects of the female reproductive cycle are reported such as mating season (austral autumn), size of follicles (0.5 to 3.0 cm), time of ovulation (austral spring), and gestation period (4 to 5 months). Morphological and physiological changes were observed in the uterus of adult females soon after mating. This condition lasted for only one season (austral winter) and after ovulation the uterus relaxed and spermatozoa ascended the oviducts. The anatomy of the genital tract is described and the terminology is discussed in comparison with other temperate relatives (*C. viridis viridis*). The importance of such physiological process is hypothesized as a way to avoid waste of vitellogenic follicles, which are not ready to be fertilized at the time of mating. Due to the asynchrony of the reproductive events in this species, long-term sperm storage is an obligatory component.

Key words: *Crotalus*; Reproduction; Delayed fertilization; Long-term sperm storage; Copulatory plug

Long-term sperm storage (LTSS) and maintenance of sperm in the female genital tract are a common reproductive strategy in squamates, particularly in pit vipers from temperate regions, e.g. the genera *Agkistrodon* (Fukada, 1986; Isogawa and Kato, 1995), *Sistrurus*, *Crotalus* (Schuett, 1992) and also the African night-adder, *Causus* (Woodward, 1933).

Winter sperm storage, whether in seminal receptacles or in the caudal oviduct is related to the asynchrony between the mating period (summer and/or autumn) and delayed ovulation (the following spring), and thus its existence would ensure fertilization (Schuett, 1992). Delayed fertilization (spring) would ensure proper temperature for the female during gestation and high availability of food for offspring at birth (summer), playing the same role as spring mating, as proposed by Shine (1977).

The presence of such a strategy in snakes has led to an interesting discussion about the possible increase of the egg fertilization rates per clutch (Madsen et al., 1992). It is known that mated female *Vipera berus* show contraction and convolution of the uterus, which is supposed to block the posterior part of the uterus from later sperm entry, or even prevent sperm leakage, possibly precluding bi- or multipaternal

litters (Andrén and Nilson, 1987; Andrén et al., 1997; Schuett, 1992; Stille and Niklasson, 1987; Stille et al., 1986).

Despite the debate about this subject concerning temperate vipers and pit vipers (Andrén and Nilson, 1987; Ludwig and Rahn, 1943; Nilson and Andrén, 1982; Schuett, 1992), next to nothing is known regarding neotropical pit vipers. The rattlesnake *Crotalus durissus* is widely distributed from North to South America, that is, in temperate and tropical regions (Campbell and Lamar, 1989). The reproductive cycle of Brazilian representative *C. durissus* is quite similar to that of temperate pit vipers with combat rituals and mating only during austral autumn (Santos et al., 1990) and ovulation in austral spring (pers. observation), that is a postnuptial gametogenic cycle (Almeida-Santos et al., 1997; Langlada et al., 1973). However, Brazilian *C. durissus* have some distinct features, such as the lack of a period of hibernation and continuous vitellogenesis through the winter (Salomão et al., 1995).

Under these circumstances, there are topics about the reproductive biology of neotropical rattlesnakes which remain unclear and raise some questions concerning the existence of sperm storage, the type of strategy for storing sperm, the length of time spermatozoa can be

stored, and the period of gestation.

The aim of this work was to investigate the genital tract of the female neotropical rattlesnake *Crotalus durissus* in an attempt to check the presence of long-term sperm storage; describe the strategy of maintenance of sperm during the winter; determine more precisely the time and period of gestation in this species; and discuss the hypothesis of a copulatory plug in viperid snakes. We will also make some comments about the terminology of the genital tract in pit vipers.

MATERIAL AND METHODS

A first sample of 120 adult female [snout-vent length = 89.97 ± 2.15 ($\bar{X} \pm SD$)mm] of *C. durissus* from the State of São Paulo, Southeastern Brazil, identified as *C. d. terrificus*, had their genital tracts macroscopically observed monthly in 1992 (10 per month). Many of these snakes arrived at the Instituto Butantan severely injured (when caught by people either bitten by rattlesnakes in fields, or by people who were working on farms and in agricultural areas). The presence of vitellogenic follicles in the ovary and oviduct was recorded, a smear was taken to detect the presence and motility of sperm in the vagina, uterus and oviduct, and the uterus was examined. From the total number of snakes

examined, a subsample of eleven females in secondary vitellogenesis (with sperm in the uterus) had the right uteri fixed in situ (formalin 10% and Bouin fluid) and removed for histological and histochemical analysis from just after the mating period (April-May, austral autumn) up till ovulation (September, austral spring). Sections (2–3 μ m) were stained with hematoxylin-eosin, Mallory's trichrome, toluidin-fuchsin blue, periodic acid-Schiff (PAS), Alcian blue (AB) and bromophenol blue (BB).

A second sample of four gravid females was kept in captivity from soon after mating time until parturition (January, austral summer), and then their genital tract observed and prepared the same way as described above for histology and histochemical analysis. A third sample of six gravid females was also kept in captivity soon after mating time and isolated from males after the offspring were born in 1990. They have been fed ad libitum and observed until this time of writing (1997) to check if any litter would be produced. All the specimens from the three sub-samples were chosen randomly and examined soon after their arrival at Instituto Butantan.

Complementary observations of the female genital tract of museum specimens of *Crotalus viridis viridis* (N=5) from Texas and New Mexico, USA, captured in April/May (spring)

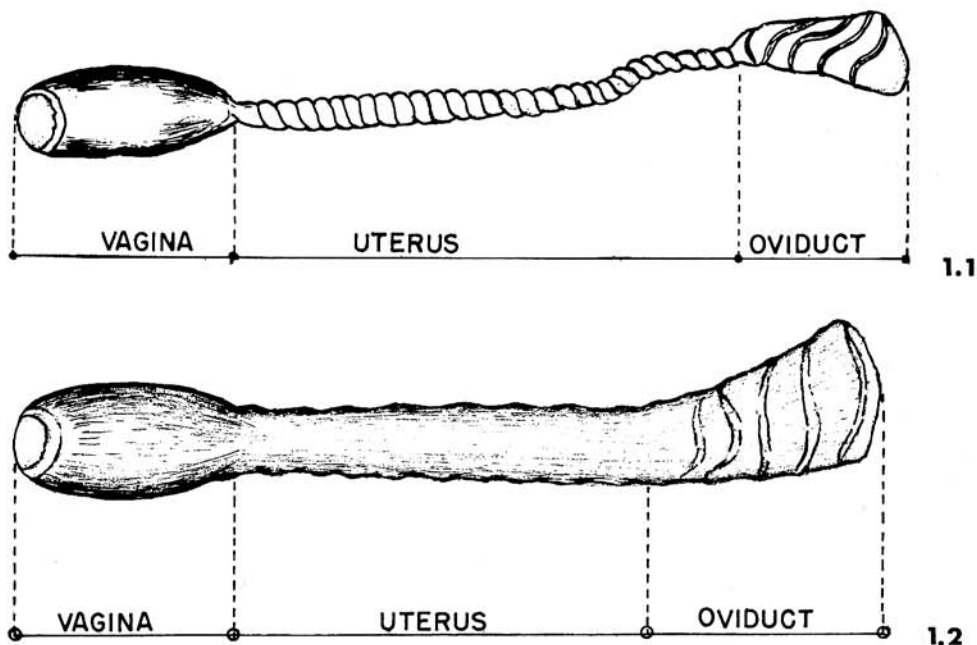


FIG. 1. Genital tract of *Crotalus d. terrificus*. 1.1: The oviduct, twisted uterus, and the vagina pouch of a mated female in May; 1.2: The oviduct, uterus, and vagina of a post-partum female in January.

(IB Nos. 2310; 14973; 20436; 20447 and 28444) were performed to compare the condition and length of the vagina and the uterus, relative to the SVL. Comparing this information to the data from *C. d. terrificus* allowed us to discuss similar data available in the literature.

RESULTS

Morphology of genital tract.—Macroscopical survey confirmed by histological analysis of the genital tract showed three distinct divisions: the oviduct, the uterus and an extremely large vaginal pouch (Figs. 1.1 and 1.2). Their length (N=5) was 20.10 ± 2.62 cm for the oviduct,

4.25 ± 0.34 cm for the uterus, and 3.10 ± 0.59 cm for the vagina. These measurements corresponded to 0.231, 0.049, and 0.036 of the SVL, respectively. The limits between these three structures were easily seen both macroscopically and through microscopical examination. First, the differences in the diameter between the oviduct and uterus and the uterus and the vagina are quite distinct even in post-partum snakes. Second, the texture of the musculature made the uterus more opaque than the oviduct.

The oviduct was soft and less rigid than either the uterus or the vagina. In both vitellogenic

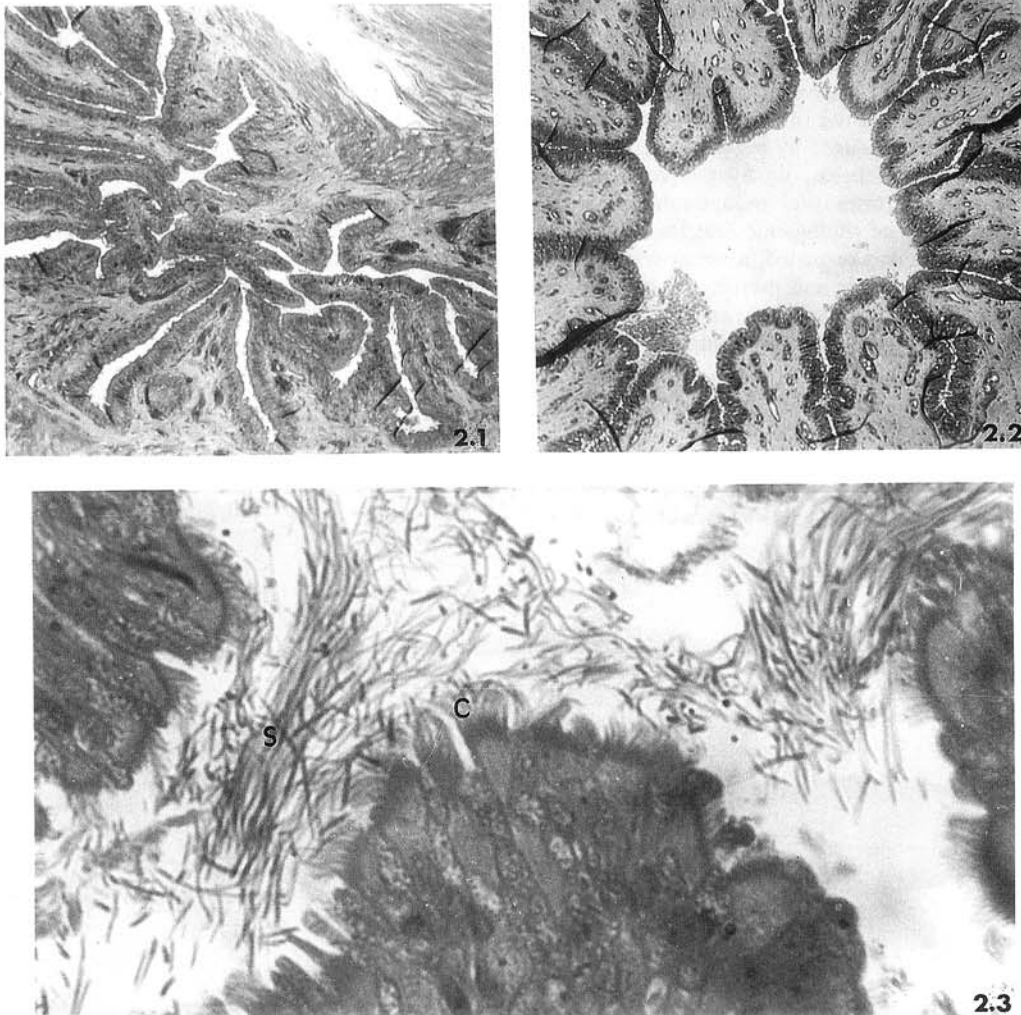


FIG. 2. Transversal section of the uterus of *C. d. terrificus*. 2.1: A mated female in June, showing the epithelium; 2.2: A post-partum female in January; 2.3: A mated female in July, showing the cilia of the columnar epithelium (c) and the spermatozoa (s).

TABLE 1. Monthly number of *Crotalus d. terrificus* (N=10) in each breeding condition.

Condition of snakes	Months											
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Sperm and twisted uterus	0	0	0	8	10	10	10	9	2	0	0	0
Vitellogenic follicles or embryos (oviduct)	5	0	0	0	0	0	0	0	8	10	10	10
Non-vitellogenic (follicles <5 mm)	5	10	10	2	0	0	0	1	0	0	0	0

(first sample) and post-partum (second sample) cases, the musculature of the oviduct was ring-shaped. In vitellogenic females an increase in the size ($1.5\times$) of the oviduct chambers (about 2.0 cm width) was noticed, whereas in the post-partum specimens they appeared to be folded.

The uterus was characterized by possessing longitudinal smooth ciliated musculature which was twisted, rigid, and well defined in the vitellogenic females (Fig. 2.1), to a much greater extent than in post-partum individuals, which presented a uterus diameter approximately five times greater than the twisted form (Fig. 2.2). This situation was observed in vitellogenic females from soon after mating in April and May (austral autumn), until ovulation in September (austral spring) when the uterus had more relaxed musculature (about two times more than the twisted condition—see Figs. 1.1 and 1.2). There was a layer of mucous cells (PAS and AB positive and BB negative) just under the ciliated uterine epithelium, which appeared to be more pronounced and organized in the acini of the vitellogenic than in post-partum snakes. The specimens in vitellogenesis possessed a folded central lumen of the uterus which appeared to be reduced and compressed, and motile spermatozoa were seen attached to the cilia (Fig. 2.3). No carrier matrix (material which stains positively with PAS and becomes associated with sperm) was observed in the lumen region while the uterus was twisted. There seemed to have been no physical obstruction along the convoluted uterus, since a needle (1.0 mm diameter) could be inserted in the uterus duct. Despite this, it was difficult to remove the contents of the uterus without squeezing it. In the post-partum females the central lumen of the uterus was wider and more easily visible. This indicated that this twisting of the uterus may hinder the passage of spermatozoa both to the oviduct and also back to the vagina.

The vagina possessed a wider chamber with longitudinal musculature wall, similar to that in the uterus, but much thicker. In post-partum females sperm was never found inside the genital tract. Neither vitellogenic or post-partum

females had seminal receptacles.

Macroscopical examination of the female genital tract in *C. v. viridis* showed characteristics of length (vagina = 2.57 ± 0.77 cm; uterus = 3.62 ± 0.84 cm) and proportions to SVL (vagina = 0.033; uterus = 0.047) similar to those found in *C. d. terrificus*. In *C. v. viridis* the uterus was more opaque and easily differentiated from both vagina and oviduct due to differences in their diameter as in *C. d. terrificus*. Contorsion and convolution of the uterus were observed in four of the five *C. v. viridis* examined. A Student t test failed to show significant differences ($p < 0.05$) between *C. v. viridis* and *C. d. terrificus* in the length and proportions of vagina and uterus to SVL.

Sperm storage results.—Table 1 shows the number of females of the first sample with sperm in the uterus, the presence of a twisted and convoluted uterus, and the month when ovulation was recorded. Size of vitellogenic follicles varied from 0.5 cm (primary vitellogenesis) up to 3.0 cm (secondary vitellogenesis). At the latter size they were observed to migrate to the oviduct. Motile sperm were detected in the uterus of 96% (47/50) of the females from soon after the mating period until ovulation. In April and August some females examined (two and one respectively) had neither sperm in the genital tract nor the twisting and convolution, but the follicles which were still in the ovary, and were of the same size (about 30.0 mm) as those observed in mated females. In September eight females had their vitellogenic follicles in the oviduct and the musculature of the uterus was already relaxed. However, two females had the uterus still convoluted and twisted and the vitellogenic follicles were still in the ovary. Whenever the smear test showed the presence of sperm in the uterus, it (the uterus) was firmly twisted. All the females which showed spermatozoa in the uterus were considered to have mated. Mating has been recorded in Instituto Butantan from April to May with only one exception recorded during the first week of June (late austral autumn) (pers. observation).

None of the six females of *C. d. terrificus*

isolated from males in 1990 (third sample) produced any litter.

DISCUSSION

Concerning the anatomy of the genital tract of *C. d. terrificus*, some authors do not take into consideration the existence of a differentiated muscular portion contiguous to the vaginal pouch and oviduct, the so-called uterus. However, such authors present some differentiations in the shape between the anterior and the posterior part of what they just call the oviduct. This is the case described in *V. aspis* (Lécuru-Renous and Platel, 1970) and *Bothrops jararaca* (Gomes and Puerto, 1993). Ludwig and Rahn (1943) pointed out in *C. viridis viridis* a region of the oviduct possessing tubular glands, which they called the uterus. Although their diagram is quite exact regarding the shape and size of the structures, they considered the vagina a bit longer than what it really is and did not mention that the uterus has a thicker musculature than the oviduct, besides the presence of tubular glands. Such discrepancy related to the length of vagina and uterus was mentioned by Aldridge (1992), but without any further explanations. The comparisons between *C. v. viridis* and *C. d. terrificus* carried out here showed the exact limits between both structures. The vagina itself in both species consists only of a chamber which diminishes drastically in diameter at the point where the uterus begins. This is the first time this issue has been discussed in detail and precise drawings of the genital tract of tropical pit vipers indicating the limits of the vagina, uterus, and oviduct can also be seen in Abdalla (1994) and Langlada et al. (1994) (*B. jararaca* and *C. durissus terrificus*, respectively).

The twisting and convolution observed in the uterus of *C. d. terrificus* result in an obstruction which inhibits the passage of the spermatozoa to the oviduct until ovulation and the subsequent fertilization occur (spring). These morphological changes act as a kind of physiological copulatory plug. This is supported by the results of the smear test which revealed no sperm at all in the oviduct during the period when the plug was observed (four months in winter). Ovulation seems to stimulate the relaxation of the uterine musculature and spermatozoa ascend the oviduct, probably aided by muscular propulsion supplemented by ciliary movement as has also been observed in *Thamnophis sirtalis parietalis* (Halpert et al., 1982) and in turtles (Parker, 1931). The fact that the copulatory plug lasts only over winter explains the fact that

captive female rattlesnakes were not able to produce any litter following the previous litter after they had been isolated from males. This fact supports the statement of Langlada et al. (1973) who reported the absence of seminal receptacles at the level of the infundibulum, differing from some colubrid snakes such as *Tantilla coronata* (Colubrinae) (Aldridge, 1992) and *T. s. parietalis* (Natricinae) (Halpert et al., 1982) and even from some crotalines such as *Agkistrodon blomhoffi* (Isogawa and Kato, 1995).

Winter sperm storage is necessary because mating is restricted to autumn (Almeida-Santos et al., 1997). Thus, females with sperm in the uterus certainly mated the previous season and those without sperm in the uterus or vitellogenic follicles were always non-vitellogenic (follicles smaller than 0.5 cm), probably because they had given birth in the previous summer (post-partum).

The strongly PAS+ and AB+ uterine columnar epithelium is disorganized in post-partum females, perhaps as a consequence of the expulsion of the offspring, causing the uterine layer to slough. The mucous substances produced by these cells probably serve as a nutritional source for the spermatozoa during storage. This nutritional substance has also been observed in *T. s. parietalis* in which it helps to transport the sperm (carrier matrix) (Halpert et al., 1982) and in turtles (Motz and Callard, 1991). Its presence explains the spermatozoa survival throughout the winter when they were always motile. The absence of carrier matrix when the copulatory plug is present may be interpreted as a lack of sperm transport to the oviduct.

The idea of a physiological copulatory plug does not preclude the possibility of intraseason multiple copulation, sperm competition, and, therefore, multiple paternity, as suggested by Stille et al. (1986), Stille and Niklasson (1987) and Madsen et al. (1992) in *V. berus*. In *C. d. terrificus*, however, it seems more likely to be a female strategy to guarantee delayed fertilization rather than a male strategy for reproductive success.

Taking into consideration the spring ovulation, the winter sperm storage, the absence of seminal receptacles at the level of the infundibulum, and the birth in summer (December to February), the gestation period in *C. d. terrificus* is four to five months. Moreover, the long-term sperm storage (LTSS) is obligatory due to the asynchrony between the reproductive events. Otherwise, sperm would reach the oviduct when follicles were not ready (autumn), causing the

waste of both clutch and sperm.

Although the questions proposed have been answered, there are still some doubts about the stimulus which promotes the uterine contraction soon after mating, the length of time the uterus remains contracted and the stimulus which synchronizes uterine relaxation with ovulation. There are some reports of the effects of the renal sexual segment secretion on the uterine musculature contraction of *V. berus* (Nilson and Andrén, 1982) and the effects of oxytocin on the contraction of the uterus of *B. jararaca* (Abdalla, 1994; Abdalla et al., 1996). However, the existence of hormonal control of the uterus sensitivity remains unclear and more investigation is required.

The fact that the copulatory plug, and therefore LTSS, is present in *Crotalus* of both temperate (Klauber, 1972; Schuett, 1992) and tropical areas may mean the presence of a genetic component determining the pattern of the reproductive cycle in this genus. So phylogeny is another factor influencing reproduction and perhaps carries as much weight as the ecological one. However, further research has to be carried out in an attempt to test such a hypothesis.

ACKNOWLEDGEMENTS.—We thank Joaquim Cavalheiro and Valdir José Germano for helping to select the snakes and looking after the offspring in the bioterium. We also thank Carlos Jared for help in making the photographs and plates. We also thank Marcelo Ribeiro Duarte, Rosana dos Santos Jordão, and Hebert Ferrarezzi for reading and the fruitful discussion which resulted in this paper. We also thank Francisco do Carmo Costa for revising the final version of the manuscript.

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要旨 新熱帯産ガラガラヘビ *Crotalus durissus terrificus* 雌の長期精子貯蔵

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ガラガラヘビ *Crotalus durissus terrificus* の子宮にみられる回旋と筋肉収縮を、冬を越えて精子を貯蔵する（長期精子貯蔵）ための戦略として報告した。交尾期（秋季）、濾胞の大きさ（5-30 mm）、排卵の時期（春季）、妊娠期間（4-5カ月）など、雌の繁殖周期について述べた。雌の子宮には、交尾後すぐに形態学的、生理学的変化が観察された。この状態は1季節（冬季）続くだけで、排卵後に子宮に弛緩し、精子が卵管をさかのぼる。生殖管の解剖形態を記述し、

温帯産の近縁種プレーリーガラガラヘビとの関連で、語句の用法について論議した。交尾期にはまだ受精の準備ができていない卵黄形成後の濾胞を、無駄にしないような生理学的過程の重要性を仮定すると、この種では繁殖に関する出来事が時間的に同調しないため、長期精子貯蔵をせざるを得ないと考えられる。

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