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Reproductive cycle of the Neotropical *Crotalus durissus terrificus*: II. Establishment and maintenance of the uterine muscular twisting, a strategy for long-term sperm storage

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Abstract

Crotaline snakes store sperm by means of a uterine musculature twisting (UMT). We investigated the influence of plasma levels of estradiol and progesterone and vasotocinase cystine aminopeptidase (CAP) activity on UMT formation and maintenance, and the in vitro uterine reactivity for AVT in *Crotalus durissus terrificus* in primary or secondary vitellogenesis with or without UMT. Frequency of females in secondary vitellogenesis with UMT is significantly higher than in primary one. Estradiol levels did not vary in all conditions studied, however, significantly low levels of progesterone were found in snakes in secondary vitellogenesis with UMT compared to those without it. UMT is always observed when high levels of estradiol and low levels of progesterone are detected. CAP activity did not change in the presence of UMT. AVT produced concentration–response contractions of the isolated uterus of snakes in all stages analysed and the pD_2 value and maximum contractile response were significantly higher in primary vitellogenesis without UMT than in other reproductive conditions, indicating that uterus of those snakes presents a higher contractile capacity which may favour UMT establishment. In conclusion, we show a relationship of UMT and estradiol/progesterone balance and a possible participation of AVT in UMT formation and maintenance in the Neotropical rattlesnake.

Keywords: *Crotalus*; Reproduction; Uterine muscular twisting; Sexual hormones; Arginine vasotocin

1. Introduction

Crotaline snakes have been observed to show a biennial reproductive pattern that includes autumnal courtship and mating and spring ovulation, resulting in delayed fertilization and obligatory winter long term sperm storage (LTSS) (Almeida-Santos and Salomão, 2002; Schuett, 1992). In the Neotropical rattlesnake *Crotalus durissus terrificus* LTSS and maintenance of sperm in the female genital tract occur in the posterior region of the uterus which becomes convoluted and contracted. This mechanism described initially as a copulatory plug (Almeida-Santos and Salomão, 1997) is in fact an uterine muscular twisting (UMT), which keeps viable sperm and guarantees the success of reproduction despite uncoupled mating and fertilization. Due to the fact that UMT has been described in other phylogenetic relates pitvipers (Almeida-Santos and Salomão, 2002) and in vipers (Andrén and Nilson, 1987), Almeida-Santos and Salomão (2002) suggested UMT as an ancestral reproductive strategy in viperid snakes.

In *C. d. terrificus* spring ovulation seems to stimulate the relaxation of the uterine musculature and spermato-

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zoa ascend the oviduct. Thus, UMT lasts only over winter, explaining why captive female rattlesnakes have not been observed to produce any litter following the previous litter after they had been isolated from males (Almeida-Santos and Salomão, 1997). The stimulus, which promotes UMT soon after mating, the period of time UMT persists, and the stimulus, which promotes the synchronic uterine relaxation with ovulation, is completely unknown.

Steroid sexual hormones, namely estradiol and progesterone orchestrate most of events that occur during the reproductive cycle. We show that estradiol and progesterone play a role in the reproduction of the Neotropical rattlesnakes *C. d. terrificus* especially vitellogenesis and gestation, respectively (Almeida-Santos et al., 2004), and consequently may participate in the contraction of the uterus resulting in the UMT formation.

In Almeida-Santos et al. (2004) we show that the cystine aminopeptidase (CAP) activity, which reflects the bioavailability of arginine vasotocin (AVT) in the blood stream, changes during reproductive cycle. The lowest activity of this enzyme was found in *C. d. terrificus* in secondary vitellogenesis females in which uterus contraction is necessary both to maintain UMT, as well as to expel follicles by the time of ovulation, and the highest levels were found in post-partum stage in which uterus remain relaxed and flaccid. AVT is a non-mammalian neurohypophyseal hormone homologous to arginine vasopressin (AVP) and oxytocin (OT) (for review see Acher, 1990). Seasonal uterus (Fergusson and Bradshaw, 1992; Jones and Guillette, 1982) and oviduct (Jones et al., 1987) contractile effect has been induced in vitro under influence of AVT, which is also known to play a role in oviposition and parturition in vivo in lizard and the chicken (Jones and Guillette, 1982; Koike et al., 1988; Saito et al., 1987; Shimada et al., 1986).

Thus, this work aims at clarifying the influence of sexual hormones and AVT/CAP activity in the UMT establishment and maintenance in the Neotropical rattlesnake *C. d. terrificus* analysing monthly over a period of 2 years the in vitro uterine reactivity for AVT, and plasma levels of CAP, estradiol, and progesterone during primary or secondary vitellogenesis with or without UMT condition.

2. Materials and methods

2.1. Animals and determination of the stage of reproductive cycle

All animals used in this study ($n = 117$) were the same sample from Almeida-Santos et al. (2004), as well as the macroscopical morphological observations for determining the stage of reproductive cycle. Briefly, for this study, females were divided into two reproductive categories, namely primary vitellogenesis (follicles up to 1.0 cm) and secondary vitellogenesis when follicles receive vitellus deposition (from 1.0 cm up to size of ovulation—about 3.0 cm) (see Aldridge, 1979, 1982 for details). The condition of the uterus was verified by dissection to determine the presence of UMT. Uterine smear was performed to detect the presence of sperm (live or dead) and hence, recent mating whenever the UMT was observed. Animal care and procedures used here were in accordance with guidelines of the Animal Ethics Committee of Instituto Butantan.

2.2. Blood collection, protein determination, sexual hormone determination, and CAP activity

Blood samples were the same used in Almeida-Santos et al. (2004). Blood collection, plasma protein, estradiol or progesterone and CAP activity determination were done as described in the above-mentioned study.

2.3. Isolated uterus preparation

A segment of about 5 cm long from the posterior uterus between the anterior vagina and the medial part of the oviduct (where UMT occurs) was removed and mounted as described by Abdalla et al. (1996) with some modifications. Briefly, uterine segments (1.0 cm) were suspended in an organ chamber and connected to a transducer (Ampère or Ugo Basile) for measurement of isometric tension. The organ chamber was filled with a nutrient solution (composition in mM: NaCl 150.0; K_2HPO_4 1.6; KH_2PO_4 0.4; $MgSO_4$ 1.2; $CaCl_2$ 1.8; and glucose 5.0) and bubbled with air. The experiments were performed at 30 °C. The uterine segments were placed under 1.0*g* of tension and allowed to equilibrate for 60 min. Uteri from snakes in primary or secondary vitellogenesis with or without UMT were used to construct non-cumulative concentration–response curves for AVT.

2.4. Statistical analysis

The χ^2 test was used to test for differences among the frequencies of females in primary vitellogenesis with and without UMT or females in secondary vitellogenesis with or without UMT. CAP activity data were analyzed using the Graph-Pad Prism (GraphPad Software, San Diego, CA) and Instat software. The concentration–response curves for AVT were fitted through a non-linear regression and the pD_2 values ($-\log EC_{50}$) were calculated using the curve-fitting program Graph-Pad Prism. Regression analysis was performed to obtain standard curves for protein and CAP activity measurements. One-way analysis of variance (ANOVA) was performed to detect differences in uterine reactivity for AVT among different reproductive conditions, followed by the

Newman–Keuls test when differences were detected. The results are reported as means ± SEM, with n representing the number of animals analyzed and statistical significance assumed was $P < 0.05$. Unpaired two-tailed Student's t test was performed to detect differences of CAP activity, estradiol and progesterone levels in the presence or absence of UMT in the same reproductive condition.

3. Results

3.1. Presence of UMT during reproductive cycle

From the total ($n = 90$), 47 (52.3%) females examined were in primary vitellogenesis [12 (13.4%) with and 35 (38.9%) without UMT], 43 (47.7%) in secondary vitellogenesis [30 (33.3%) with and 13 (14.4%) without UMT] (Table 1). Other 27 snakes were in pregnant or post-partum conditions (Almeida-Santos et al., 2004). Interestingly, the presence of UMT (Fig. 1) in females in secondary vitellogenesis was observed from February until September, whereas in primary vitellogenesis condition was observed throughout the year, except in the months of April, June, October, and December. The presence of sperm was detected in all females in secondary vitellogenesis with UMT, however, females in primary vitellogenesis with UMT the uterine smear revealed absence of sperm. χ^2 analysis showed a significant dependent relationship between the reproductive condition (vitellogenesis) and the presence or absence of UMT. Thus, the frequency of females in primary vitellogenesis with UMT is lower than that of secondary vitellogenesis with UMT. On the other hand, the frequency of females in primary vitellogenesis condition without UMT is higher than that of secondary vitellogenesis without UMT ($\chi^2 = 15.41$; $df = 1$; $P < 0.001$).

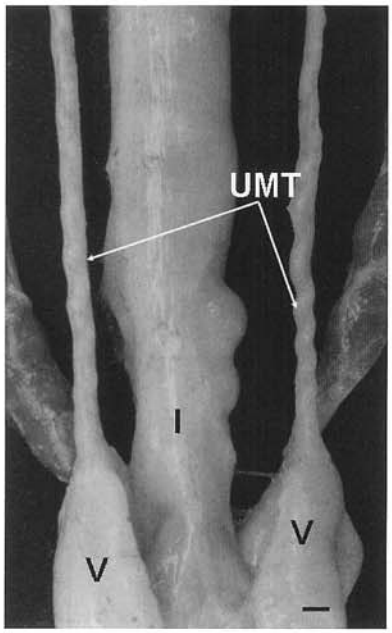

Fig. 1. Uterine muscular twisting (UMT) in *C. d. terrificus*. V, vagina; I, intestine. Bar, 39 mm.

3.2. UMT and sexual hormone levels

Estradiol plasma levels found in primary with or without UMT females (22.46 ± 3.59 pg/ml, $n = 11$ and 16.71 ± 1.50 pg/ml, $n = 32$, respectively), or in secondary vitellogenesis with or without UMT females (102.84 ± 25.6, $n = 26$ and 109.49 ± 34.20, $n = 14$, respectively), were

Table 1
Number of female *C. d. terrificus* in primary and secondary vitellogenesis throughout the year ($n = 90$) (UMT, uterine muscular twisting)

Season of the year	Months	Reproductive conditions			
		Primary vitellogenesis without UMT	Primary vitellogenesis with UMT	Secondary vitellogenesis without UMT	Secondary vitellogenesis with UMT
Summer	January	4	2	1	0
	February	4	1	0	2
	March	3	2	1	2
Autumn	April	3	0	2	6
	May	1	3	2	4
	June	2	0	2	7
Winter	July	3	1	0	4
	August	1	1	4	3
	September	2	1	1	2
Spring	October	1	0	0	0
	November	6	1	0	0
	December	5	0	0	0
Total		35	12	13	30

Statistical analysis showed significant differences between the frequency of primary vitellogenesis without UMT and secondary vitellogenesis without UMT ($\chi^2 = 15.41$; $df = 1$; $P < 0.001$).

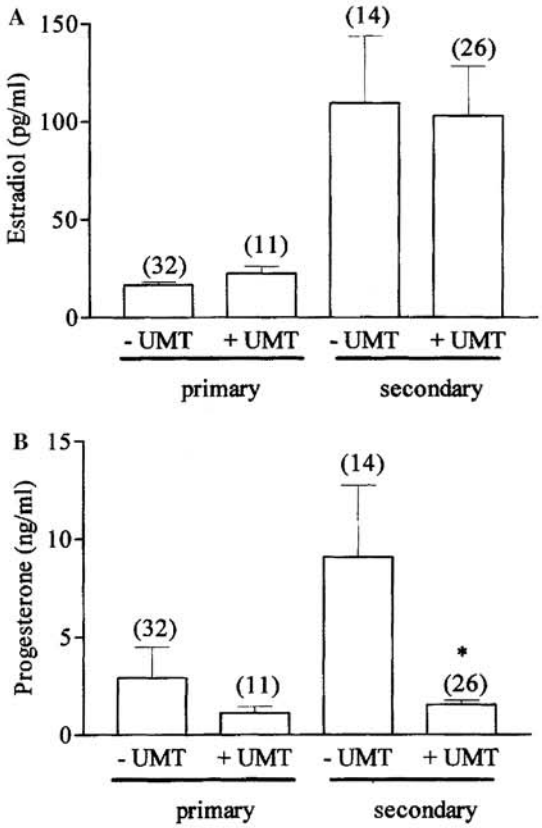

Fig. 2. Plasma estradiol (A) and progesterone (B) levels in female *C. d. terrificus* in both primary vitellogenesis and secondary vitellogenesis (with or without UMT). Each bar and vertical line represent mean ± SEM. Number of experiments are in parentheses. *Significant difference between with and without UMT (Student's *t* test, $P < 0.05$).

not significantly different (Fig. 2A), despite significant difference between primary and secondary condition found in Almeida-Santos et al. (2004). Progesterone plasma levels in primary vitellogenesis with or without UMT (2.90 ± 1.60 ng/ml, $n = 32$; 1.10 ± 0.34 ng/ml, $n = 10$) were not significantly different, however, a significant difference ($P < 0.05$) between secondary vitellogenesis females without UMT (9.08 ± 3.64 ng/ml, $n = 14$) and secondary vitellogenesis with UMT (1.53 ± 0.22 ng/ml, $n = 26$) was found (Fig. 2B). It is noteworthy, the fact that UMT was observed only when high levels of estradiol and low levels of progesterone were detected simultaneously.

3.3. The relationship between plasma vasotocinase CAP activity and the presence of UMT

Levels of CAP activity expressed as UAP (picomole of substrate hydrolyzed per minute) per milligram of protein appear significantly low in secondary vitellogenesis females as shown in our previous study (Almeida-Santos et al., 2004). However, Fig. 3 shows that plasma CAP activity levels are independent of the presence of UMT under the same reproductive condition. CAP activity in females in primary vitellogenesis with or without UMT was 32.7 ± 5.3 ($n = 9$) and 39.8 ± 3.1 ($n = 28$), respectively, and in secondary vitellogenesis with or without UMT was 24.6 ± 2.2 ($n = 23$) and 21.3 ± 2.97 ($n = 14$), respectively.

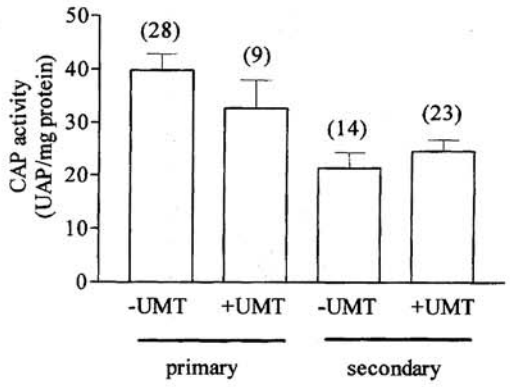

Fig. 3. Plasma vasotocinase CAP activity levels, expressed as UAP (picomoles of substrate hydrolyzed per minute) per milligram of protein, measured throughout two years in female *C. d. terrificus* in the absence (−) or presence (+) of UMT during primary vitellogenesis and secondary vitellogenesis conditions. No differences between the presence or absence of UMT were detected under the same reproductive condition (unpaired two-tailed Student's *t* test, $P < 0.05$). Each bar and vertical line represent means ± SEM. Number of animals is in parentheses.

3.4. Uterus sensitivity to AVT during the reproductive cycle

AVT produced concentration–response-dependent contractions of isolated uterus from snakes in all reproductive conditions studied. The pD_2 value for AVT in the uteri from snakes in primary vitellogenesis without UMT (8.04 ± 0.03, $n = 6$) was significantly lower than those found in uteri from snakes in primary vitellogenesis with UMT (8.43 ± 0.07, $n = 5$) or secondary vitellogenesis without or with UMT (8.42 ± 0.05, $n = 3$; 8.22 ± 0.01, $n = 10$, respectively) ($P < 0.05$, Table 2). Interestingly the maximum response in uteri from snakes in primary vitellogenesis without UMT (82.31 ± 18.75 mg of tension/mg of wet tissue, $n = 4$) was greater than the maximum response in uteri from snakes in primary vitellogenesis with UMT (36.78 ± 12.71 mg of tension/mg of wet tissue, $n = 4$) or secondary vitellogenesis without or with UMT (34.31, $n = 2$ and 42.06 ± 7.22 mg of tension/mg of wet tissue, $n = 7$, respectively) ($P < 0.05$, Table 2).

The presence of UMT in secondary viltellogenesis did not change either the sensitivity or the efficacy of AVT as occurred in primary vitellogenesis. AVT also produced concentration–response-dependent contractions of isolated uteri from pregnant or post-partum snakes and the pD_2 values were 8.54 ± 0.22, $n = 6$ and 8.19 ± 0.17, $n = 5$, respectively (Table 2). The maximum response in both reproductive conditions were 21.84 ± 2.77, ($n = 6$) and 25.64 ± 9.60 ($n = 4$) mg of tension per milligram of wet tissue, respectively. Both pD_2 value and the maximum

Table 2
Contractile effect of AVT on the isolated uterus of *C. d. terrificus* in different stages of the reproductive cycle (UMT, uterine muscular twisting)

Stage of reproductive cycle	pD_2 ($-\log EC_{50}$)	Maximum response (mg of tension/mg of tissue)
Primary vitellogenesis without UMT	$8.04 \pm 0.03^{*}$ (6)	$82.31 \pm 18.75^{*}$ (4)
Primary vitellogenesis with UMT	8.43 ± 0.07 (5)	36.78 ± 12.71 (4)
Secondary vitellogenesis without UMT	8.42 ± 0.06 (3)	34.31 (2)
Secondary vitellogenesis with UMT	8.22 ± 0.01 (10)	42.06 ± 7.22 (7)
Pregnant	8.54 ± 0.22 (6)	21.84 ± 2.77 (6)
Post-partum	8.19 ± 0.17 (5)	25.64 ± 9.60 (4)

Values represent means ± SEM. The number of experiments are given in parentheses.
* Value significantly different from other reproductive stages.

response in snakes in primary vitellogenesis without UMT were significantly different from snakes in pregnant or post-partum ($P < 0.05$, Table 2).

4. Discussion

Crotalus durissus terrificus carries out winter long-term sperm storage (LTSS) by means of a uterine muscular twisting (UMT). In this paper, we showed that the establishment and maintenance of this phenomenon is modulated by a balance between estradiol and progesterone plasma levels. On the other hand, uteri of snakes in primary vitellogenesis without UMT present a higher contractile capacity in presence of AVT, indicating a favourable condition for UMT establishment.

The first evidence for a long survival of sperm in uterus with maintenance of fertilization capacity in snakes was verified in the colubrid snake genus *Thamnophis* by Rahn (1940). In rattlesnakes the presence of sperm in the female genital tract was early observed in the posterior region of oviduct (Ludwing and Rahn, 1943), but evidences for winter LTSS were reported much later by Almeida-Santos and Salomão (1997, 2002). However, different from *Thamnophis* where the sperm can be found anteriously to the uterus in the so-called infundibulum much time before fertilization (Halpert et al., 1982) , in the Neotropical rattlesnake sperm remain in the part of uterus with UMT till the time ovulation takes place.

UMT is a peculiar strategy for LTSS, which guarantees the viability of sperm during at least one season and consequently success of reproduction despite uncoupled mating and fertilization (Almeida-Santos and Salomão, 2002). This phenomenon was first described in *Vipera berus* as a contraction of a sphincter muscle of the uterus to prevent additional successful mating (Nilson and Andrén, 1982). Eventually, Almeida-Santos and Salomão (1997) showed that in *C. d. terrificus* this uterus contraction is in fact a muscular twisting and convolution which is established soon after autumnal mating and persists till spring ovulation, keeping live sperm for about 4 months.

In this study, we verified the presence of UMT in females in primary and secondary vitellogenesis, however, the frequency of primary vitellogenesis females with UMT was very low compared to secondary vitellogenesis snakes with UMT. In primary vitellogenesis animals no sperm was found in uterine smear and we noticed that UMT could be mechanically induced during experimental handling. The low frequency of UMT in snakes in primary vitellogenesis associated with the absence of sperm in their uteri, besides the possibility of obtaining their contraction through touching could explain the relatively low but widespread occurrence of UMT in such reproductive stage throughout the year, indicating that this condition seems to be unnatural.

The mechanisms involved in the UMT formation and maintenance were unknown. Data in the literature show that secretion from renal sexual segment from males is able to contract uterus (Nilson and Andrén, 1982). Renal sexual segment is a hypertrophied segment of the distal tubule of male snakes, which can be stimulated into secretory function by androgens (Prasad and Reddy, 1972). Burtner et al. (1965) first described the renal sexual segment in rattlesnakes and this segment is seasonally enlarged during mating and spermatogenesis (Aldridge, 2002; Regaud and Policard, 1903; Schuett et al., 2002). Therefore, the establishment of UMT in snakes could be influenced by the secretion of renal sexual segment added to sperm and conveyed to the female genital tract during mating.

In this study, we showed that sexual hormones may also modulate UMT establishment but mainly its maintenance. Simultaneous high levels of estradiol and low levels of progesterone seem to be appropriated physiological conditions to stimulate formation and maintenance of the UMT in females in secondary vitellogenesis, allowing the optimization of the LTSS reproductive strategy. High levels of estradiol are related to the deposition of vitellus in the follicles (see Almeida-Santos et al., 2004), whereas, low levels of progesterone may help the maintenance of the contraction of the uterus (UMT), since this hormone is known to decrease the contractile activity of oviduct and uterus musculature (Veith, 1974) and is an obligatory component of gestation success in viviparous snakes (Callard et al., 1978; Kleis-San Francisco and Callard, 1986; Panigel, 1956).

On the other hand, AVT seems to play a more important role in the maintenance than in formation of UMT, since no differences were found here on CAP activity between secondary vitellogenesis with and without UMT, despite our previous data (Almeida-Santos et al., 2004) have shown low plasma CAP activity, and therefore high bioavailability of circulating AVT in *C. d. terrificus* in secondary vitellogenesis comparatively to other reproductive stages. AVT is a non-mammalian neurohypophyseal hormone which has the ability to contract uterus and oviduct (Fergusson and Bradshaw, 1992; Jones and Guillette, 1982) and participates in ovoposition and parturition (Jones and Guillette, 1982; Koike et al., 1988; Saito et al., 1987; Shimada et al., 1986), both situations, which need contraction. In addition, seasonal variations of such contractions have been recorded (Jones et al., 1987). However, our findings of high efficiency of AVT to contract uteri from snakes in primary vitellogenesis may indicate that AVT could also be implied in UMT establishment, and at the same time explain the occurrence of mechanical UMT formation in such reproductive condition coincidently with absence of sperm throughout the year, indicating that no previous mating had taken place and reinforcing its artificial nature.

In conclusion, this study showed the importance of sexual hormones, especially the balance of estradiol and progesterone, and AVT in establishing and maintaining the UMT after mating, to keep viable sperm inside uterus during at least one season (LTSS) and consequently guarantees fertilization in the Neotropical rattlesnakes *C. d. terrificus*.

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