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Reproductive cycle of the Neotropical *Crotalus durissus terrificus*: I. Seasonal levels and interplay between steroid hormones and vasotocinase

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Abstract

Crotaline snakes present delayed fertilization and sperm storage because secondary vitellogenesis is not completed by the time of mating. The release of vitellogenesis and synchrony between ovulation and fertilization suggest a steroidal modulation. We investigated changes of sexual steroid levels during reproduction in the Neotropical rattlesnake *Crotalus durissus terrificus*, analyzing macroscopical variations of reproductive condition (vitellogenesis, pregnancy, and post-partum) and plasma levels of estradiol, progesterone, and vasotocinase cystine aminopeptidase (CAP) activity over 2 years. Data showed 44.4% non-reproductive snakes (40.1% primary vitellogenesis and 4.3% post-partum) and 55.6% reproductive (36.8% secondary vitellogenesis and 18.8% pregnant). Estradiol was low in spring and summer, increasing in autumn till it peaked in winter. Estradiol in secondary vitellogenesis was significantly higher than in primary vitellogenesis, or in pregnant and post-partum females, Progesterone dropped significantly in autumn compared to summer, winter, and spring. Pregnant females showed the highest levels of progesterone compared to primary or secondary vitellogenesis, or post-partum females. CAP activity showed lowest values in reproductive females in autumn and greatest levels in post-partum females. A significant negative linear relationship was obtained between CAP activity and estradiol. The combination of morphological observations, levels of steroids and CAP activity allowed us to suggest a similar morphological reproductive pattern between temperate and tropical rattlesnakes, and to infer the role of estradiol, progesterone and CAP activity on vitellogenesis, gestation and sperm storage, respectively.

Keywords: *Crotalus*; Reproduction; Estradiol; Progesterone; Vasotocinase

1. Introduction

Most crotaline snakes show a seasonal biennial reproductive austral 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, 1997, 2002; Schuett, 1992). In the viviparous Neotropical rattlesnake *Crotalus durissus terrificus*, LTSS occurs because secondary vitellogenesis is not completed by the time mating occurs, otherwise, sperm would reach the oviduct when follicles were not ready, causing the waste of clutch and sperm. Although it has been reported that ovaries produce substances with estrogenic and progestational activity, the relationship between all these physiological events and possible plasma steroid hormone changes in this snake is completely unknown.

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Reproductive events in reptiles such as the development of the genital tract have been shown to be dependent on ovarian hormones (Jones and Guillette, 1982; Mead et al., 1981). A decrease in weight and a diminished secretory activity of oviductal glands were noted in the genital tract following ovariectomy in lizards and snakes (Mead et al., 1981). Administration of estradiol to ovariectomized garter snake (*Thamnophis elegans*) partially restored epithelial cell height, glandular activity and width of the longitudinal muscle layer of the oviduct (Girling, 2002). Although little is known about the function of estrogen, it seems to be the primary stimulus to vitellogenesis in most non-mammalian vertebrates (Callard et al., 1990). In snakes, this situation remains controversial. Several studies suggest that changes in estradiol levels are independent of vitellogenesis (Bonnet et al., 1994; Crews, 1992; Saint Girons et al., 1993), mainly for those species in which vitellogenesis is not dependent on body reserves as it is the case of *Thamnophis sirtalis parietalis* (Whittier and Crews, 1990). However, the administration of exogenous 17β-estradiol stimulates RNA synthesis of vitellogenin, precursor of vitellus, in the squamate liver (see Callard et al., 1990 for review). Vitellus is then transported by blood to the oocytes to promote follicle growth (Crews and Garstka, 1982).

In mammals, progesterone has been shown to participate in the maintenance of the uterus in a quiescent state, in the first stage of the pregnancy, when the ratio of progesterone to estrogen is high (see Wray, 1993 for review). The role of progesterone in reproduction remains unclear when reptiles are considered, although high plasma levels of progesterone have been reported during pregnancy in *Thamnophis elegans* (Highfill and Mead, 1975) and, more recently, in a viper (*Vipera aspis*) (Bonnet et al., 2001; Naulleau and Fleury, 1990).

On the other hand, arginine vasotocin (AVT), a non-mammalian neurohypophyseal hormone homologous to arginine vasopressin (AVP) and oxytocin (OT) (for review see Acher, 1990), is known to play a role in oviposition and parturition in vivo in lizards and the chicken (Jones and Guillette, 1982; Koike et al., 1988; Saito et al., 1987; Shimada et al., 1986). Therefore, it seems likely that AVT could also modulate reproduction and LTSS in the rattlesnake *C. d. terrificus*. The presence of high levels of circulating enzyme, the vasotocinase cystine aminopeptidase (CAP) with neurohypophyseal peptide hydrolytic activity, is a peculiar feature of the hormonal AVT and AVP-OT systems which had been detected only in primate gestation condition (Matsumoto and Mori, 1998; Mizutani et al., 1995). However, in female *Bothrops jararaca*, CAP activity is detected in different stages of the reproductive cycle and corresponds to the potential physiological bioavailability of AVT (Silveira et al., 1992, 1998).

Thus, this work aims at clarifying the association between the macroscopical morphological aspects which characterize the reproductive cycle and plasma level changes of estradiol, progesterone, and CAP activity in the Neotropical rattlesnake *C. d. terrificus*.

2. Materials and methods

2.1. Animals

A sample of 117 adult females of *C. d. terrificus* captured from the wild in the State of São Paulo, Southeastern Brazil, (identified by the Laboratory of Herpetology, Instituto Butantan) was examined. Depending on the availability of snakes arrived at Instituto Butantan up to five animals per month were used for macroscopical observations and blood collection over two years, due to the biennial reproductive cycle presented by rattlesnakes (Almeida-Santos and Orsi, 2002; Almeida-Santos and Salomão, 1997). In all experimental procedures, snakes were anesthetized with 30 mg/kg subcutaneous of sodium pentobarbital (Cristália—Brazil). Snout–vent length—SVL (cm), tail length—TL (cm), and body mass (g) were recorded for each specimen. Animal care and procedures used were in accordance with the guidelines of the Animal Ethics Committee of Instituto Butantan.

2.2. Macroscopical morphological observations

After ventral dissection, snakes had the genital tract macroscopically examined for the presence and size of vitellogenic follicles in the ovary and oviduct and for the condition of the uterine musculature to determine the reproductive stages according to Almeida-Santos and Orsi (2002). Females were divided into two categories of maturation 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) (see Aldridge, 1979 for details). Due to the limited availability of snakes, pregnant snakes and those in a state of secondary vitellogenesis were not subdivided into different categories of follicle size or developmental stages. Ovulation was determined to have taken place when follicles had migrated from the ovary to the oviduct, signalling the onset of embryogenesis or pregnancy, whether the development of embryo was visible or not. Post-partum condition was inferred from the appearance of the uterus, usually relaxed and flaccid, as well as by the presence of corpora lutea, indicating birth in the previous summer.

2.3. Blood collection

Individual blood samples (8.0 ml) were collected from the hepatic vein in a polyethylene tube containing

1 U/ml of heparin (Liquemine, Roche, Brazil) and were centrifuged at 10,000*g*, 4 °C for 50 min. The plasma obtained was stored at −20 °C up to 3 months until analysis.

2.4. Sexual hormone determination

Plasma was thawed at room temperature and the estradiol and the progesterone levels were measured by radioimmunoassay using ^{125}I-estradiol-coat-acount estradiol kit and ^{125}I-progesterone-coat-acount progesterone kit (Diagnostic Products, Los Angeles, CA), respectively, according to instructions of manufacturer. Results were expressed as picogram of estradiol/ml of plasma or nanogram of progesterone/ml of plasma. The intra-assay ($n=24$) and inter-assay ($n=6$) coefficients of variation to estradiol and progesterone were 5.4 and 7.1% and 7.8 and 9.5%, respectively.

2.5. Protein determination

Plasma protein concentration was determined by Bradford (1976) method (Bio-Rad protein assay, Hercules, CA) using bovine serum albumin (BSA) (Sigma, St. Louis, MO) as standard.

2.6. Vasotocinase CAP activity

CAP activity was quantified by fluorimetric assay according to Silveira et al. (2001). CAP activity was expressed as picomoles of substrate hydrolyzed per minute (UAP) per milligram of protein. Assays were linear with respect to time of hydrolysis and protein content.

2.7. Statistical analysis

The χ^2 test was used to test for differences among the frequency of females in different reproductive conditions during the seasons of the year ($P<0.05$). One-way analysis of variance (ANOVA) was performed to detect significant differences in estradiol and progesterone levels among different seasons and different reproductive conditions, followed by the Newman–Keuls test when differences were detected. The results are reported as means ± SEM, n representing the number of animals, and statistical significance assumed was $P<0.05$. CAP activity data were analyzed using the Graph-Pad Prism (GraphPad Software, San Diego, CA) and Instat software. Regression analysis was performed to obtain standard curves for protein and enzyme activity. To estimate the corresponding correlation coefficients, regression analysis were performed between paired combinations of values of CAP activity and estradiol and CAP activity and progesterone.

3. Results

3.1. Reproductive cycle

Snakes used in this study were (means ± SEM, $n=117$) 88.88 ± 0.77 cm SVL, 6.54 ± 0.07 cm TL, and 639.41 ± 22.14 g body mass. From the total, 47 (40.1%) females were in primary vitellogenesis and 5 (4.3%) were post-partum (non-reproductive). The remaining were reproductive namely 43 (36.8%) in secondary vitellogenesis and 22 (18.8%) pregnant (Table 1). Primary vitellogenesis was observed throughout the year, but mainly in

Table 1
Number of female *C. d. terrificus* in different reproductive conditions in different months and seasons of the year

Season of the year	Months	Reproductive conditions				Total
		Primary vitellogenesis	Secondary vitellogenesis	Pregnant	Post-partum	
Summer	January	6	1	3	0	10
	February	5	2	0	1	8
	March	5	3	1	0	9
Autumn	April	3	8	0	0	11
	May	4	6	0	1	11
	June	2	9	0	0	11
Winter	July	4	4	0	2	10
	August	2	7	3	0	12
	September	3	3	8	0	14
Spring	October	1	0	3	0	4
	November	7	0	4	0	11
	December	5	0	0	1	6
Total		47	43	22	5	117

Statistical analysis showed no significant differences between the frequency of primary vitellogenesis and secondary vitellogenesis ($\chi^2=0.0222$; df = 1; $P=0.8815$).

spring and summer, whereas secondary vitellogenesis occurred from January until September (mid-summer to late winter) (Table 1; Figs. 1 and 2). Similar proportion of non-reproductive ($n=52$; 44.4%) and reproductive ($n=65$; 55.6%) snakes in the sample confirms the seasonal biennial cycle ($\chi^2=0.623$; $P=0.430$; df = 1). Ovulation occurs in September and October and pregnancy from September to April. Post-partum snakes were detected from December to August (Table 1; Fig. 1).

3.2. Sexual hormone levels throughout the year

In non-reproductive *C. d. terrificus* plasma estradiol (pg/ml) remained low during all seasons (summer 18.55 ± 2.88, $n=16$; autumn 25.21 ± 4.18, $n=8$; winter 21.60 ± 4.13, $n=12$; and spring 14.44 ± 1.88, $n=14$). However, in reproductive snakes estradiol gradually increased in autumn (71.63 ± 16.84, $n=22$) until it peaked in winter (167.40 ± 45.47, $n=14$; $P<0.05$), compared to summer and spring (26.10 ± 4.29, $n=9$; 10.97 ± 1.40, $n=11$, respectively) (Fig. 3A).

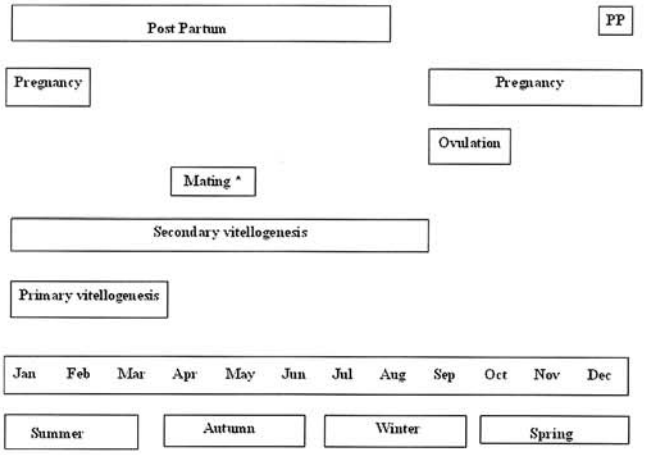

Fig. 1. Timing of the events in the reproductive cycle of the neotropical rattlesnake *C. d. terrificus* (PP, post-partum females); * data obtained from Almeida-Santos and Salomão (1997).

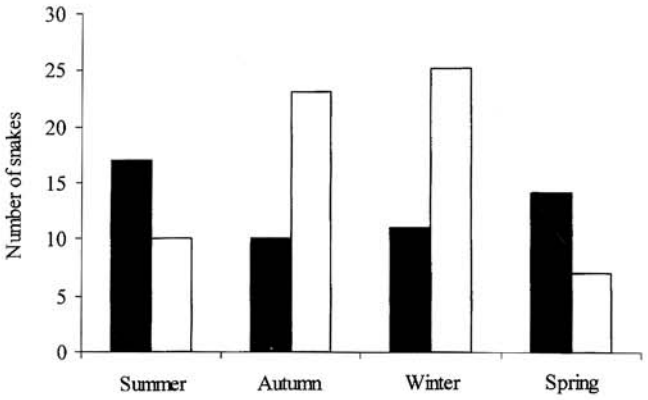

Fig. 2. Number of female *C. d. terrificus* in reproductive (open bars) and non-reproductive condition in the seasons of the year ($\chi^2=13.44$; $P=0.0038$; df = 3).

In non-reproductive rattlesnakes plasma progesterone levels (ng/ml) remained low in all seasons (summer 5.23 ± 3.43, $n=15$; autumn 1.52 ± 0.50, $n=8$; winter 1.50 ± 0.42, $n=12$; and spring 2.74 ± 1.43, $n=12$). In reproductive snakes progesterone levels were significantly higher in summer, winter, and spring (16.53 ± 5.47, $n=9$; 12.02 ± 4.94, $n=13$; and 23.80 ± 2.49, $n=11$, respectively), than in autumn (1.60 ± 0.33, $n=21$; $P<0.05$) (Fig. 3B).

Estradiol levels found during secondary vitellogenesis (103.40 ± 20.76, $n=40$) were significantly higher than during primary vitellogenesis, pregnancy or post-partum (17.95 ± 1.60, $n=44$; 19.30 ± 3.27, $n=18$; and 26.79 ± 7.55, $n=6$, respectively; $P<0.05$) (Fig. 4A).

Pregnant females showed the highest levels of progesterone (25.34 ± 2.77, $n=18$) (Fig. 4B). However, low levels of progesterone were observed during primary or secondary vitellogenesis and in post-partum snakes (2.55 ± 1.17, $n=43$; 4.02 ± 1.45, $n=40$; and 6.16 ± 2.56, $n=6$, respectively; $P<0.05$) (Fig. 4B).

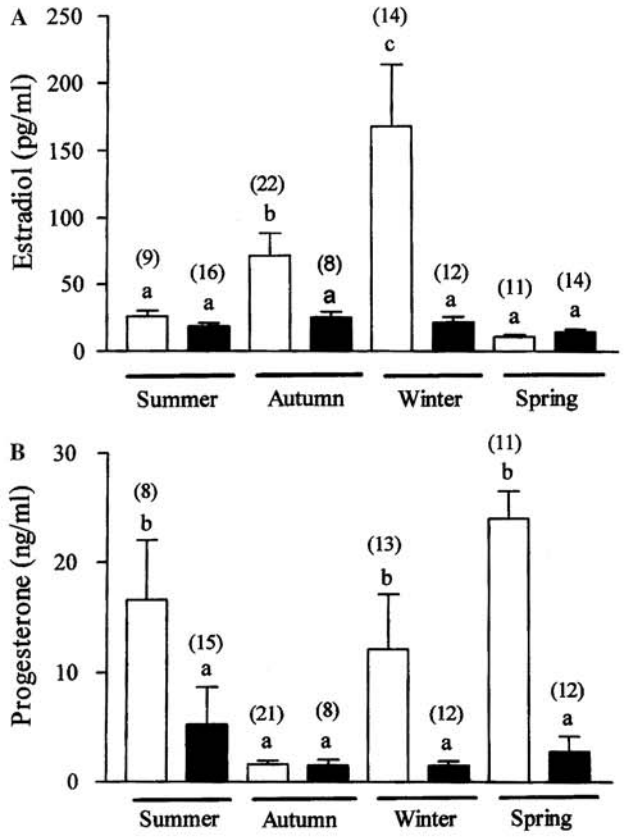

Fig. 3. Seasonal distribution of plasma estradiol (A) and progesterone (B) in *C. d. terrificus* in reproductive (open bars) and non-reproductive females. Each bar and vertical line represent means ± SEM. Number of experiments are in parentheses. Different letters indicate statistical significant difference ($P<0.05$, ANOVA–Newman–Keuls test).

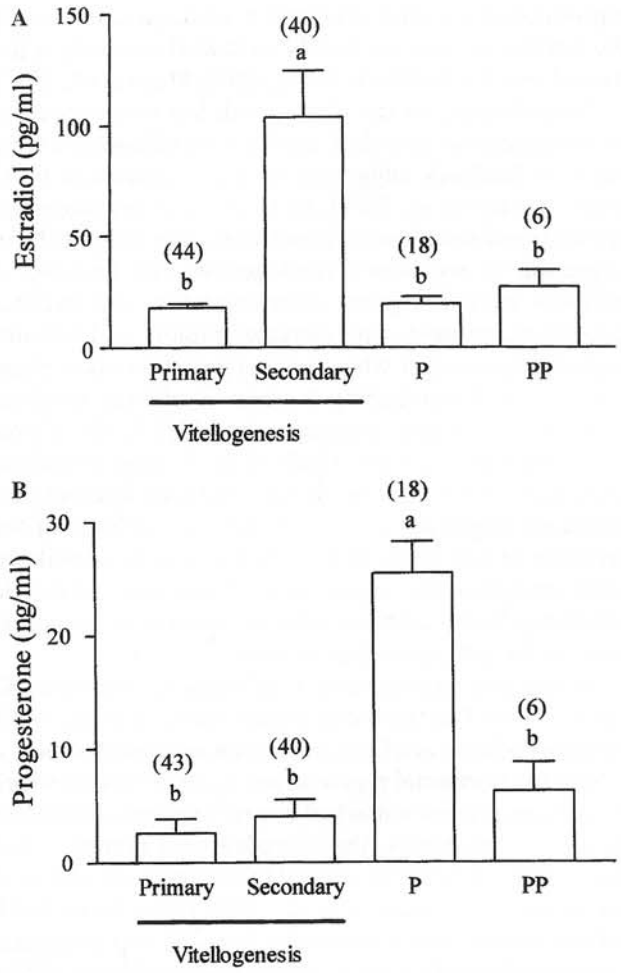

Fig. 4. Seasonal distribution of plasma estradiol (A) and progesterone (B) in *C. d. terrificus* in primary and secondary vitellogenesis, pregnant (P) and post-partum (PP). Each bar and vertical line represent means ± SEM. Number of experiments are in parentheses. Different letters indicate statistical significant difference ($P < 0.05$, ANOVA–Newman–Keuls test).

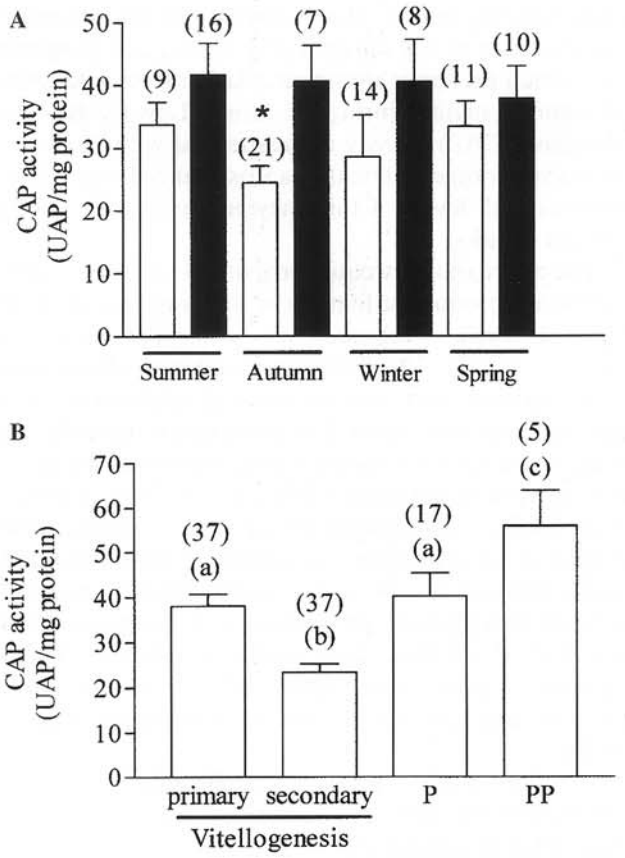

Fig. 5. Seasonal values of plasma vasotocinase CAP activity (UAP = picomoles of substrate hydrolyzed per minute per milligram protein) in *C. d. terrificus* in reproductive (open bars) and non-reproductive conditions. ANOVA reveals no significant statistical difference among all values ($P = 0.07$), and values of reproductive ($P = 0.38$), or non-reproductive snakes ($P = 0.96$). Asterisk indicates significant statistical difference between the reproductive conditions in the same season (Student's two-tailed t test, $P < 0.009$ (A); Plasma CAP levels in *C. d. terrificus* during primary vitellogenesis, secondary vitellogenesis, pregnancy (P) and post-partum (PP) conditions. Different letters indicate statistical significant difference ($P < 0.05$, ANOVA–Newman–Keuls test) (B). Each bar and vertical line represent means ± SEM. Number of animals are in parentheses.

3.3. Vasotocinase CAP activity during the reproductive cycle and its relationship with sexual hormones

No significant differences in CAP activity were observed between reproductive (spring 33.4 ± 4, $n = 11$; summer 33.8 ± 3.6, $n = 9$; autumn 24.5 ± 2.7, $n = 21$; and winter 28.6 ± 6.7, $n = 14$) or non-reproductive snakes (spring 37.8 ± 5.1, $n = 10$; summer 41.6 ± 5, $n = 16$; autumn 40.6 ± 5.7, $n = 7$; and winter 40.5 ± 6.7, $n = 8$) among the seasons. However, a significant difference was found between reproductive and non-reproductive snakes in autumn ($P < 0.05$) (Fig. 5A). Post-partum snakes showed the highest levels of CAP activity (56.0 ± 7.9, $n = 5$), contrasting with the lowest levels during secondary vitellogenesis (23.4 ± 1.8, $n = 37$). CAP activity during primary vitellogenesis and pregnancy showed intermediate values (38.1 ± 2.7, $n = 37$; 40.3 ± 5.1, $n = 17$, respectively) (Fig. 5B). A significant negative linear relationship was obtained between CAP activity and estradiol ($r = 0.24$, $F = 5.98$, $P = 0.016$, slope $= -1.27 \pm 0.52$, $n = 96$). The value of the intercept demonstrates that the decrease of estradiol influences CAP activity in a linear fashion below a threshold of 98.4 ± 19.8 pg/ml. No significant linear or non-linear correlation coefficients between progesterone and CAP activity were obtained ($r = 0.17$, $F = 2.52$, $P = 0.11$, slope $= 0.11 \pm 0.07$, $n = 96$).

4. Discussion

The rattlesnake *C. d. terrificus* presents a seasonal biennial reproductive cycle also referred as post-nuptial dissociated cycle by Crews and Garstka (1982), in which the main events are under the control of estradiol, which

predominates in the blood stream during secondary vitellogenesis in late autumn and winter, and progesterone, which predominates in circulation from late winter till summer, mainly during pregnancy. Low levels of vasotocinase (CAP) activity are associated with the period of sperm storage (Almeida-Santos and Salomão, 2002) whereas high levels of this enzyme are typical of post-partum females.

The approximately equal proportions of reproductive and non-reproductive females in the sample confirm the biennial cycle proposed by Almeida-Santos and Salomão (1997). These authors combined observations of the genital tract and presence of abdominal fat to infer reproductive status. It is important to note the relevance of direct macroscopical observations of the genital tract instead of palpation (Bonnet et al., 2001; Lourdais et al., 2002) or radiography (Naulleau and Fleury, 1990), frequently used in vipers to determine the reproductive status. Dissections allowed a more precise correlation between morphology, physiology, and hormonal condition in *C. d. terrificus*, since results of palpation or radiography may be easily distorted due to the robust physical structure and abdominal body lipids of rattlesnakes.

In this study, primary vitellogenesis occurred throughout the year, but secondary vitellogenesis predominated in autumn and winter, when Almeida-Santos and Salomão (2002) recorded mating. However, ovulation is restricted to September and October (early spring). After that, gestation, which lasts about 4 months, starts (Almeida-Santos and Salomão, 1997) and continues until late summer. Thus, the period between the birth (late summer) and the onset of secondary vitellogenesis (autumn) in *C. d. terrificus* is relatively short, preventing females from regaining the body lipids (Janeiro-Cinquini et al., 1995) necessary to initiate follicular yolking in the same year. This explains why rattlesnakes have biennial (in tropical species—this work) or even longer (some temperate species—rattlesnakes) (Brown, 1991; Diller and Wallace, 1984; Goldberg, 2000) reproductive cycles.

Physiological regulation of reproduction in reptiles and snakes has been also discussed with respect to dependence on and control of the endocrine system (Whittier and Tokarz, 1992). In *C. d. terrificus*, estradiol shifts significantly during reproduction, increasing through the autumn, mating season, and showing a peak in winter, when vitellogenesis reaches its maximum, and decreasing significantly in spring when fertilization occurs. 17β-estradiol has been described as a promoter of mobilization of maternal reserves and vitellogenesis in *Vipera aspis* (Bonnet et al., 1994, 2001), indicating that this steroid may be playing the same role in the rattlesnake, taking into account similar aspects of their reproductive ecology such as viviparity and LTSS (Andrén et al., 1997). Exogenous estradiol influences reptile reproduction, causing oviductal hypertrophy, increasing the number of mucous glands, oviductal secretory activity and vascularization (Girling, 2002; Mead et al., 1981)

Progesterone, on the other hand, has been viewed as an antagonist to estradiol, inhibiting vitellogenesis via a negative feedback suggested by their correlation in *V. aspis* (Bonnet et al., 2001). In *C. d. terrificus*, progesterone dropped significantly in autumn, coinciding with the beginning of secondary vitellogenesis and increase of estradiol levels. In spring, when ovulation and fertilization occur, progesterone increases rapidly and remains high during summer when most of gestation takes place. The seasonal relationship between ovulation, development of luteal tissue, pregnancy and high levels of progesterone has also been observed in *V. aspis* (Naulleau and Fleury, 1990). The possibility that high levels of progesterone might act as a vitellogenesis inhibitor in the presence of low levels of estradiol should be considered when analyzing the importance of hormone balance in rebuilding body reserve condition, a necessary precondition for the next reproductive cycle.

Acting as a vasotocinase, CAP regulates the bioavailability of AVT in the blood stream (Silveira et al., 1998). Aminopeptidase activities have been suggested to play a role in the hormonal reproductive cycle of rats, in which proestrous present a marked rise of Tyr-aminopeptidase in the hypothalamus, the amygdale and pituitary, and Arg-aminopeptidase in several brain areas, as well as in the serum (De Gandarias et al., 1996, 1990). Serum CAP activity in mice is modulated by estrogen and progesterone in males and females (Matsumoto and Mori, 1998). It is interesting to note that CAP activity was variable in reproductive rattlesnakes, dropping particularly in autumn, which coincides with mating, LTSS through a uterine muscular twisting (UMT) and secondary vitellogenesis. On the other hand, the highest levels of CAP activity were observed in post-partum females. In the rattlesnake our data suggested that CAP activity is also an estrogen-influenced aminopeptidase, as in the neotropical pitviper *B. jararaca*. AVT is the OT homologue hormone which promotes in vitro uterus contraction in *C. d. terrificus* (Yamanouye et al., 2004). Since levels of CAP activity are associated with the dynamical process of AVT bioavailability, low levels of CAP activity would permit AVT to favour UMT maintenance soon after mating, whereas high levels of CAP activity (with low estradiol) in post-partum would reflect the need to reduce AVT released after parturition. Low CAP activity coincides with the highest levels of estradiol in secondary vitellogenesis, suggesting a short-term feedback between AVT and CAP activity under up regulation by estradiol, as similarly reported in rats between AVP and CAP activity (Matsumoto and Mori, 1998; Prieto et al., 2002).

In conclusion, our data suggest a similar morphological pattern in the reproductive cycles of temperate and

tropical rattlesnakes, despite the fact that they occupy widely varying ecological conditions (Aldridge, 1979; Salomão et al., 1995; Schuett et al., 2002). Such similarity might be attributed to phylogenetic conservatism, which appears to have maintained the ancestral condition throughout time in *C. durissus*, the only species of the genus which reached South America in the Pliocene (Vanzolini, 1986), directing maintenance of sexually related behaviors and physiological mechanisms. The combination of morphological observations and hormonal data obtained in this study allowed us to infer the role of estradiol, progesterone and CAP activity on vitellogenesis, gestation and LTSS, respectively.

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