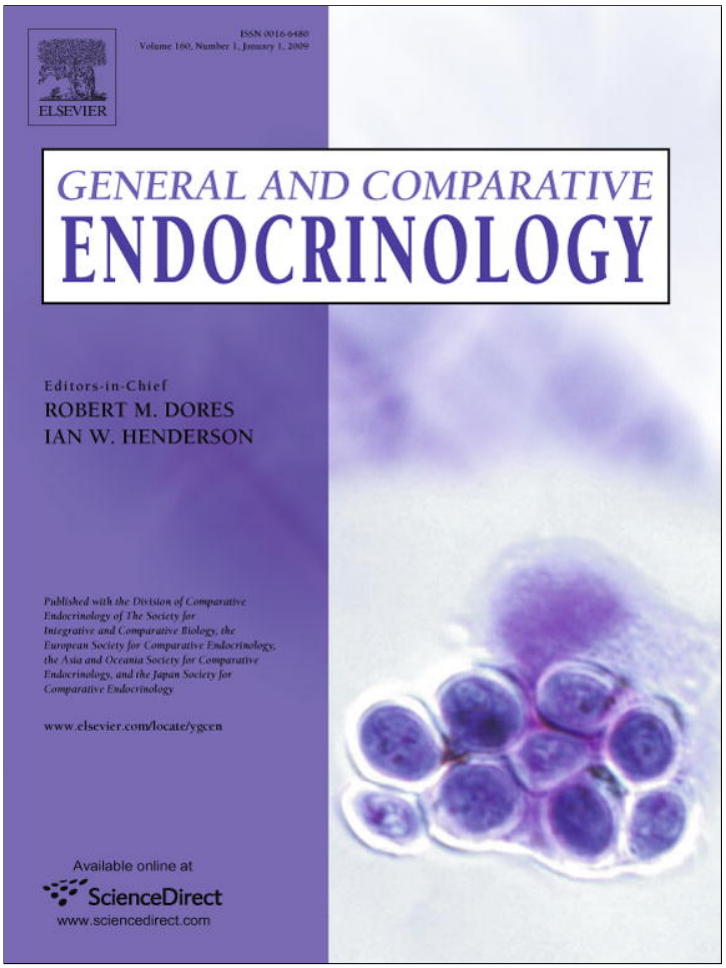

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Seasonal variation of peptidase activities in the reproductive tract of *Crotalus durissus terrificus*

Camila Eduardo Marinho [a,c], Almeida-Santos SM [b], Simone Cristina Yamasaki [a], Paulo Flavio Silveira [a,*]

[a] Laboratory of Pharmacology, Instituto Butantan, Av. Vital Brasil, 1500, São Paulo, SP 05503-900, Brazil
[b] Laboratory of Ecology and Evolution, Instituto Butantan, Av. Vital Brasil, 1500, São Paulo, SP 05503-900, Brazil
[c] Department of Physiology, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP 05508-900, Brazil

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ABSTRACT

Seasonal quantitative patterns of acid (APA), basic (APB), puromycin-sensitive (APN-PS) and puromycin-insensitive neutral (APN-PI), cystyl (CAP), dipeptidyl IV (DPPIV), type-1 pyroglutamyl (PAP-I) and prolyl-imino (PIP) aminopeptidases and prolyl oligopeptidase (POP) activities in soluble (SF) and solubilized membrane-bound (MF) fractions from ductus deferens, vagina and uterus were studied to evaluate their relationships with the reproductive cycle and the extensive long-term spermatozoa storage (LTSS) of the Neotropical rattlesnake *Crotalus durissus terrificus*. APB, PIP and POP were detected only in SF, while other peptidases were detected in SF and MF. APB, APN-PI and APN-PS were predominant in most tissues in all seasons. Peptidase activities had a common pattern of increment during the dry season (winter/autumn), which coincides with the mating period (autumn) and LTSS in the female (winter), as well as the reduction of spermatozoa motility and maintenance of fertilization capacity of spermatozoa. The high CAP activity in the soluble fraction of the vagina during winter, compared to summer (time of parturition) and spring, coincides with the relaxation of this tissue. In the soluble fraction, the low PAP-I activity of the ductus deferens coincided with its high activity in the vagina during the winter; and the inverse occurred in summer, which is consistent with the physiological process of preserving spermatozoon viability. In conclusion, the studied peptidase activities had seasonal and tissue-specific characteristics, which suggest a relevant role in the reproductive physiology of *C. d. terrificus*.

1. Introduction

Many vipers (Serpentes: Viperidae), as an obligatory component of their reproductive cycle, show remarkable long-term sperm storage (LTSS) with maintenance of fertilization capacity (Schuett, 1992; Isogawa and Kato, 1995; Almeida-Santos and Salomão, 1997; Almeida-Santos et al., 2004b). In the South American rattlesnake (*Crotalus durissus terrificus*), spermatogenesis occurs in spring (austral), peaks in summer and spermatozoa are stored in the ductus deferens until mating, which occurs during mid autumn. Post-mating storage of sperm in females occurs in the posterior region of the oviducts (posterior uterus) throughout winter until ovulation and fertilization in spring (Schuett, 1992; Almeida-Santos and Salomão, 1997). LTSS in the female has been considered as an adaptation of snakes from temperate regions (Shine, 1977). It is thus intriguing that a rattlesnake species from the Neotropics exhibits a pattern of sperm storage (males and females) similar to that of snakes from temperate regions (Schuett, 1992; Almeida-Santos et al., 2004b).

The male reproductive tract of *C. d. terrificus* is a paired system (i.e., two testes, two ductus deferens, two epididymis, two hemipenes). An additional system includes the paired kidneys, and contains the unique sexual segment of the kidneys (SSK) that is located in the posterior region of nephron tubules (Sever et al., 2002). Differently from mammals, the epididymis of reptiles does not participate in sperm maturation and storage (Sever et al., 2002). However, the present study aims to focus on the ductus deferens owing to their role as sperm storage organs in male reptiles. The ductus deferens connect the testicle with the genital papilla in the cloaca, near the basis of the hemipenis, since reptiles do not have penile urethra (Aldridge et al., 1990; Vasse, 1994). Three regions of the ductus deferens can be distinguished: proximal (testicular), medial and distal (cloacal). Along the year, spermatozoa of *C. d. terrificus* can be found in the ductus deferens (Salomão and Almeida-Santos, 2002). The number of spermatozoa is increased in summer and autumn (which is related to the mating in the middle of autumn), compared to winter (post-mating) (Almeida-Santos et al., 2004b). During the mating (autumn), the percentage of total

* Corresponding author. Fax: +55 11 3726 7222/2133.
E-mail address: pefesil@butantan.gov.br (P.F. Silveira).

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spermatozoa defects of *C. d. terrificus* is lower compared to the winter, as well as the serum levels of testosterone is lower in winter than in summer (Zacariotti, 2004). As in other squamate reptiles, the female reproductive tract of *C. d. terrificus* is also a paired system (i.e., two ovaries, two oviducts), which are bilaterally and asymmetrically positioned (e.g., Ghelman, 1998). Regions of the oviduct are the infundibulum, uterus (anterior and posterior) and vagina (Almeida-Santos and Orsi, 2002). In many rattlesnakes, the ovarian cycle consists in two vitellogenic phases: primary (quiescent period) and secondary (the active phase of the follicle development) (Aldridge, 1979; Almeida-Santos and Orsi, 2002). In *C. d. terrificus*, primary vitellogenesis occurs early in the year, while secondary vitellogenesis occurs mainly in autumn and winter (Almeida-Santos and Salomão, 2002). In this snake it has been assumed that the LTSS in the female could be related to the uterine contraction, which occurs in the post-mating, promoting a chronic muscular constriction that narrows the uterus lumen, thus retaining the semen within it until the ovulation and subsequent fertilization (Almeida-Santos and Salomão, 1997) and/or blocking the entrance of other spermatozoa in the posterior portion of the uterus (Andrén et al., 1997). However, the role of chronic muscular constriction as a mechanism for LTSS has been under criticism based on the presence of traditional anatomical structures currently associated with sperm storage, such as sperm storage tubules, in Cottonmouth snake, *Agkistrodon piscivorous* (Siegel and Sever, 2008). Anyway, the stimulus to the long-term uterine contraction of *C. d. terrificus* has been reported to be dependent of the direct action of vasotocin (AVT), which is modulated by estradiol and progesterone during vitellogenesis (Almeida-Santos et al., 2004a). In this turn, the maintenance of this long-term uterine contraction depends on the high levels of estradiol and low levels of vasotocinase, which is an enzyme activity that is downregulated by estradiol (Yamanouye et al., 2004). In mammals, this activity corresponds to the cystyl aminopeptidase enzyme, CAP (EC 3.4.11.3), which hydrolyses oxytocin (OXT) and vasopressin (AVP). The functional role of CAP in regulating these peptides is well established during pregnancy in primates (Davison et al., 1993). Circulating CAP activity varies seasonally in females of *C. d. terrificus* (Almeida-Santos et al., 2004a). In mammals, fluctuation of other aminopeptidase activities, related to peptides that are important to the reproductive physiology, according to the reproductive cycle, is well described (de Gandarias et al., 1988; Mitchell and Denker, 1991). This class of enzymes also includes acid aminopeptidase (APA, EC 3.4.11.7), which hydrolyses angiotensin (Ang) I[1] and Ang II (Kugler, 1982); basic aminopeptidase (APB, EC 3.4.11.6), which hydrolyses bradykinin (BK), kallidin, methionine-enkephalin and somatostatin (Barret et al., 1998); neutral aminopeptidases puromycin-sentitive (APN-PS, EC 3.4.11.14) and -insensitive (APN-PI, EC 3.4.11.2), which hydrolyze enkephalin (Fernández et al., 2002) and promote the formation of Ang IV from Ang III (Kugler, 1982) and BK from kallidin (Mizutani et al., 1993); dipeptidyl-peptidase IV (DPPIV, EC 3.4.14.5), which hydrolyses substance P and endorphin-2 (Barret et al., 1998); type-1 pyroglutamyl aminopeptidase (PAP or PAP-I, EC 3.4.19.3), which hydrolyses the luteinizing-hormone releasing hormone (LHRH), thyrotropin-releasing hormone (TRH) and the fertilization promoting peptide (FPP) (O'Cuinn et al., 1990). Another important peptidase which hydrolyses Ang II, substance P, BK, OXT, AVP, LHRH (Barret et al., 1998), TRH and FPP (Siviter and Cockle, 1995) is the prolyl oligopeptidase (POP, EC 3.4.21.26).

The main goal in our study was to evaluate, in males and females of *C. d. terrificus,* seasonal activity levels of APA, APB, APN-PS, APN-PI, CAP, DPPIV, PAP-I, POP and prolyl-iminopeptidase (PIP) in soluble and solubilized membrane-bound fractions of tissues in which semen passes through or is stored (e.g., ductus deferens, posterior uterus and vagina).

2. Material and methods

2.1. Animals

The use of snakes for this research was approved by the Ethics Committee of the Instituto Butantan (CEUAIB), protocol 193/04, in agreement with the Ethical Principles for Experiments on Animals of the Brazilian Council Directive (COBEA). In the present study, adult snakes (*C. d. terrificus*, Serpentes, Viperidae, Crotalinae) were captured from natural environment in the states of São Paulo and Minas Gerais, and identified by the Laboratory of Herpetology of the Instituto Butantan. Snakes were housed individually in wood cages (inside length × width × height of 35 × 26 × 22 cm) and acclimated to controlled conditions of temperature (25 °C), humidity (65.3 ± 0.9%) and photoperiod (12 h light: 12 h dark–lights on at 6:00 am) in a restricted-access room for a period of 10 days.

In all procedures snakes were anesthetized with CO_2 exposure for 3 h. The snout vent-length—SVL (cm), tail length—TL (cm) and body wt (g) of each specimen were recorded. After the ventral dissection, the reproductive tract was examined macroscopically. Pregnant snakes were discarded. The male ductus deferens and the female uterus and vagina were collected.

2.2. Collection of tissues and the fractionation procedure

The ductus deferens, uterus and vagina were removed by laparotomy (Langlada et al., 1994) from anesthetized snakes. Considering that different areas of the ductus deferens and vagina could have distinct physiological roles, these entire organs were taken systematically to assure similar components for each analysis. Only the posterior portion of the uterus was used, considering its importance, since after mating the spermatozoa are maintained in this region until ovulation and fertilization (Almeida-Santos and Salomão, 1997). These organs were opened, stretched out and elongated on polystyrene plates. To remove mucus, spermatozoa and other fluids, a compression with a cell scraper (TPP—Techno Plastic Products AG, Switzerland) over the whole extension of the tissues was applied. These organs were then washed in 10 mM Tris–HCl buffer, pH 7.4, at a slow infusion rate using a syringe, in order to assure the complete withdrawal of secretions that could remain in the lumen.

In order to obtain soluble (SF) and solubilized membrane-bound (MF) fractions, individual samples of ductus deferens and uterus were homogenized in 10 volumes and vagina in 5 volumes (wt [g]/volume [mL]) of 10 mM Tris–HCl buffer (pH 7.4), with a Teflon pestle in a glass potter (2 min at 800 rpm) and ultracentrifuged (100,000 *g* for 35 min) (Hitachi model HIMAC CP56GII). The resulting supernatants corresponded to the SF. To avoid contamination with the SF, the resulting pellet was washed three times with the same buffer and was then homogenized (2 min at 800 rpm) in 10 mM Tris–HCl buffer plus 0.1% Triton X-100 (Calbiochem, USA) (pH 7.4), and then ultracentrifuged (100,000 *g* for 35 min). The resulting supernatants corresponded to the MF. All steps were carried out at 4 °C. After the fractionation procedure, SF and MF were

[1] *Abbreviations used:* Ang, angiotensin; APA, acid aminopeptidase; APB, basic aminopeptidase; APN-PI, puromycin-insensitive neutral aminopeptidase; APN-PS, puromycin-sensitive neutral aminopeptidase; AVP, arginine vasopressin; AVT, arginine vasotocin; BK, bradykinin; BSA, bovine serum albumin; CAP, cystyl aminopeptidase; DMSO, dimethyl sulfoxide; DPPIV, dipeptidyl-peptidase IV; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MF, solubilized membrane-bound fraction; FPP, fertilization promoting peptide; SF, soluble fraction; GHRH, growth hormone releasing hormone; LDH, lactate dehydrogenase; LHRH, luteinizing-hormone releasing hormone; LTSS, long-term sperm storage; NADH, β-nicotinamide adenine dinucleotide reduced form; OXT, oxytocin; PAP or PAP-I, type-1 pyroglutamyl aminopeptidase; PIP, prolyl iminopeptidase; POP, prolyl oligopeptidase; SSK, sexual segment of the kidneys (SSK); SVL, snout vent-length; TL, tail length; TRH, thyrotropin-releasing hormone.

transferred to polystyrene tubes and stored at −80 °C until the lactate dehydrogenase, protein and peptidase activities assays.

2.3. Lactate dehydrogenase (LDH)

As a marker for the fractionation procedure, LDH activity was determined (Bergmeyer and Brent, 1972), in samples of 3 μL of SF and MF, in triplicates, with 297 μL of NADH (β-nicotinamide adenine dinucleotide, reduced form) (Sigma, USA), dissolved in 100 mM phosphate buffer, pH 7.4, containing 1.6 mM pyruvate (Sigma, USA) and 200 mM NaCl. LDH assay was performed in 96-well microplates (Corning, Co., USA), at 340 nm, at zero and 10 min, in the Bio-Tek Power Wave X® spectrophotometer absorbance reader. Values of LDH activity were obtained by subtracting the absorbance read at 10 min from time zero of incubation at 37 °C, and extrapolated by comparison with a standard curve of NADH dissolved in 100 mM phosphate buffer, pH 7.4, containing 200 mM NaCl. LDH activity was expressed as mmol NADH oxidized/min/mg protein.

2.4. Protein

Protein content was measured at 630 nm, in triplicates, using a Bio-Rad protein assay kit (Bio-Rad Laboratories, USA) (Bradford, 1976), in the Bio-Tek Power Wave X® spectrophotometer absorbance reader. Protein contents were extrapolated by comparison with standard curves of bovine serum albumin (BSA) (Sigma, USA) in the same diluent as the samples.

2.5. Peptidase activities

Peptidase activities were quantified on the basis of the amount of 4-methoxy-β-naphthylamine (Sigma, USA) (for DPPIV and CAP) or β-naphthylamine (Sigma, USA) (for all other peptidases) released (Gasparello-Clemente et al., 2003; Varona et al., 2003). This release is the result of the incubation in 96-well flat bottom microplates (30 min, 37 °C) of 20–50 μL SF and MF, with prewarmed substrate solution at concentrations of 0.125 mM (APA, APN-PS, APN-PI, CAP, PAP-I, PIP and POP), 0.2 mM (DPPIV) and 0.5 mM (APB) in respective 0.05 M buffers containing BSA 0.1 mg/mL in a total volume of 300 μL. The content of naphthylamine was estimated fluorometrically (microplate fluorescence reader Bio-Tek FL600FA) at 460/40 nm emission wavelength and 360/40 nm excitation wavelength. The fluorescence value obtained at zero time (blank) was subtracted and the relative fluorescence was then converted to picomoles of β-naphthylamine or 4-methoxy-β-naphthylamine by comparison with a correspondent standard curve of β-naphthylamine (Sigma, USA) or 4-methoxy-β-naphthylamine (Sigma, USA), which was dissolved in the same diluent as the incubation. Peptidase activities were expressed as picomoles of hydrolyzed substrate per min (UP) per milligram of protein. The existence of a linear relationship between time of hydrolysis and protein content in the fluorometric assay was a previous condition. Considering enzyme activity measures as a comparative tool, the possible unspecific degradation during homogenization was not considered.

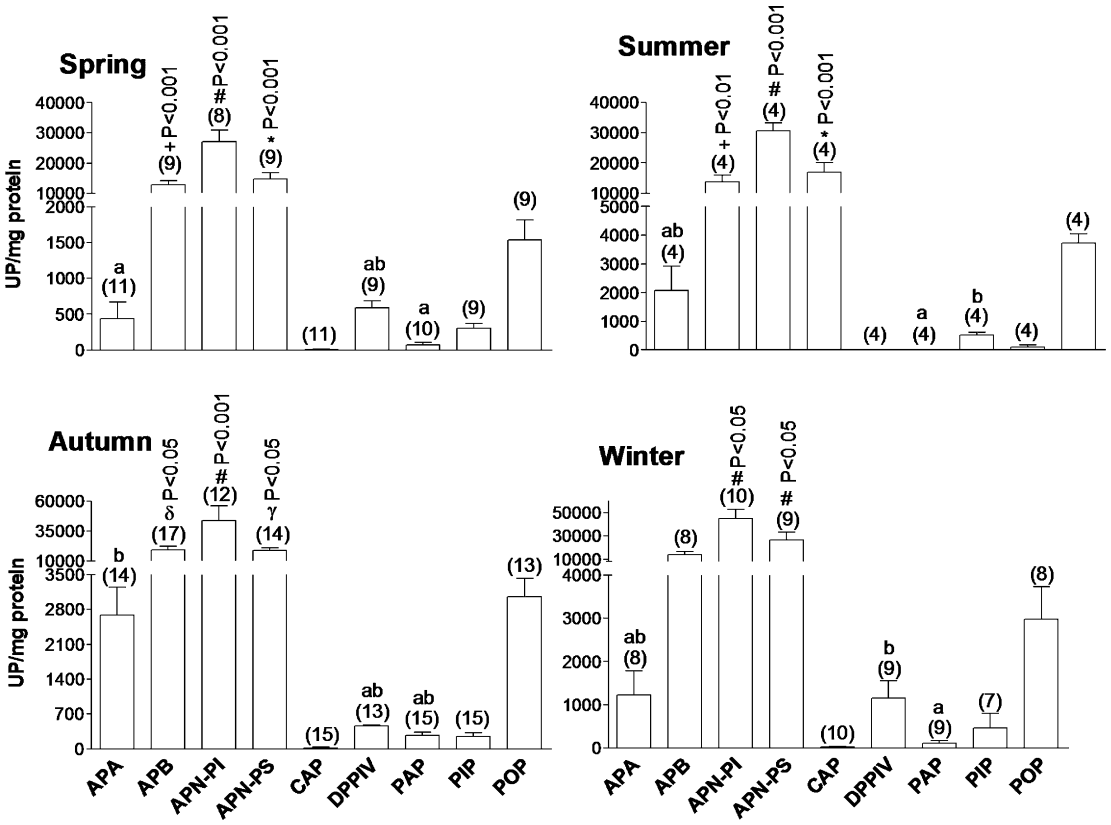

Fig. 1. Variations of activity levels (means ± SEM) of acid (APA), basic (APB), puromycin-insensitive (APN-PI) and -sensitive neutral (APN-PS), cystyl (CAP), dipeptidyl-peptidase IV (DPPIV), type-I pyroglutamyl (PAP), prolyl-imino (PIP) and prolyl oligopeptidase (POP) in soluble fraction of the ductus deferens of *C. d. terrificus* in the wet (spring/summer) and dry (autumn/winter) seasons. Number of animals is given in parentheses. UP = picomoles of hydrolyzed substrate/min. Different letters indicate seasonal variation of a peptidase activity (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni $P < 0.05$). Differences, in the same season, related to: #, all activities; ∗, all activities, except APB; +, all activities, except APN-PS; δ, all activities, except APN-PS, POP, APA; γ, all activities, except APB and POP (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni: P is indicated above each bar).

The following substrates and conditions were used:

- APA, L-aspartic acid α-(β-naphthylamide) (Sigma, USA) (solubilized in 0.012 N NaOH) in Tris–HCl buffer, pH 7.4, with 1 mM $MnCl_2$;
- APB, L-arginine β-naphthylamide (Sigma, USA) (solubilized in H_2O) in phosphate buffer, pH 6.5, with 150 mM NaCl and 0.02 mM puromycin (Sigma, USA);
- APN, L-alanine-β-naphthylamide (Sigma, USA) (solubilized in 0.012 N HCl) in phosphate buffer, pH 7.4, with 1 mM D,L-dithiothreitol (DTT) (Sigma, USA), with or without 0.02 mM puromycin-*APN-PI* activity results of the incubation with puromycin, while *APN-PS* is the result of values of incubates without puromycin minus those with puromycin;
- CAP, H-Cys-4-methoxy-β-naphthylamide (Bachem Bioscience Inc., USA) (solubilized in 0.012 N HCl) Tris–maleate, pH 5.9;
- DPPIV, glycyl-L-proline-4-methoxy-β-naphthylamide (Bachem Bioscience Inc., USA) (solubilized in dimethyl sulfoxide, DMSO, Sigma, USA) in Tris–HCl buffer, pH 8.3;
- PAP-I, L-pyroglutamic acid-β-naphthylamide (Sigma, USA) (solubilized in DMSO) in phosphate buffer, pH 7.4, with 2 mM DTT [DTT inhibits PAP-II and activates PAP-I (O'Cuinn et al., 1990)] and 2 mM ethylenediaminetetraacetic acid (EDTA) (Merck, Brazil);
- PIP, L-proline-β-naphthylamide (Sigma, USA) (solubilized in DMSO) in phosphate buffer, pH 7.4;
- POP, Z-Gly-Pro-β-naphthylamide (Bachem Bioscience Inc., USA) (solubilized in DMSO) in phosphate buffer, pH 7.4, with 2 mM DTT.

2.6. Statistical analysis

Data were analyzed statistically using GraphPad Prism™ and Instat™ software packages. Regression analysis was performed to obtain standard curves of protein, β-naphthylamine and methoxy-β-naphthylamine. Analysis of variance (ANOVA), followed by Bonferroni test, compared values of peptidase activity individually in the examined tissues, and among all peptidase activities in the same season. Student's *t-test* was performed to compare paired values of biometric parameters and LDH of SF and MF. Differences were considered statistically significant at an alpha level of $P < 0.05$.

3. Results

3.1. Biometric evaluation, sampling of specimen, vitellogenic phase and the incidence of semen stored in the uterus

Snakes used in this study had (means ± SEM) for males: SVL = 90.18 ± 1.72 cm, TL = 10.28 ± 0.35 cm and 589.91 ± 42.10 g body wt ($n = 44$); females: SVL = 87.66 ± 2.02 cm, TL = 6.50 ± 0.12 cm and 633.42 ± 42.50 g body wt ($n = 43$). These values correspond to the adult morphometric pattern. TL was significantly higher in males than in females (unpaired, two-side Student's t-test, $P < 0.0001$).

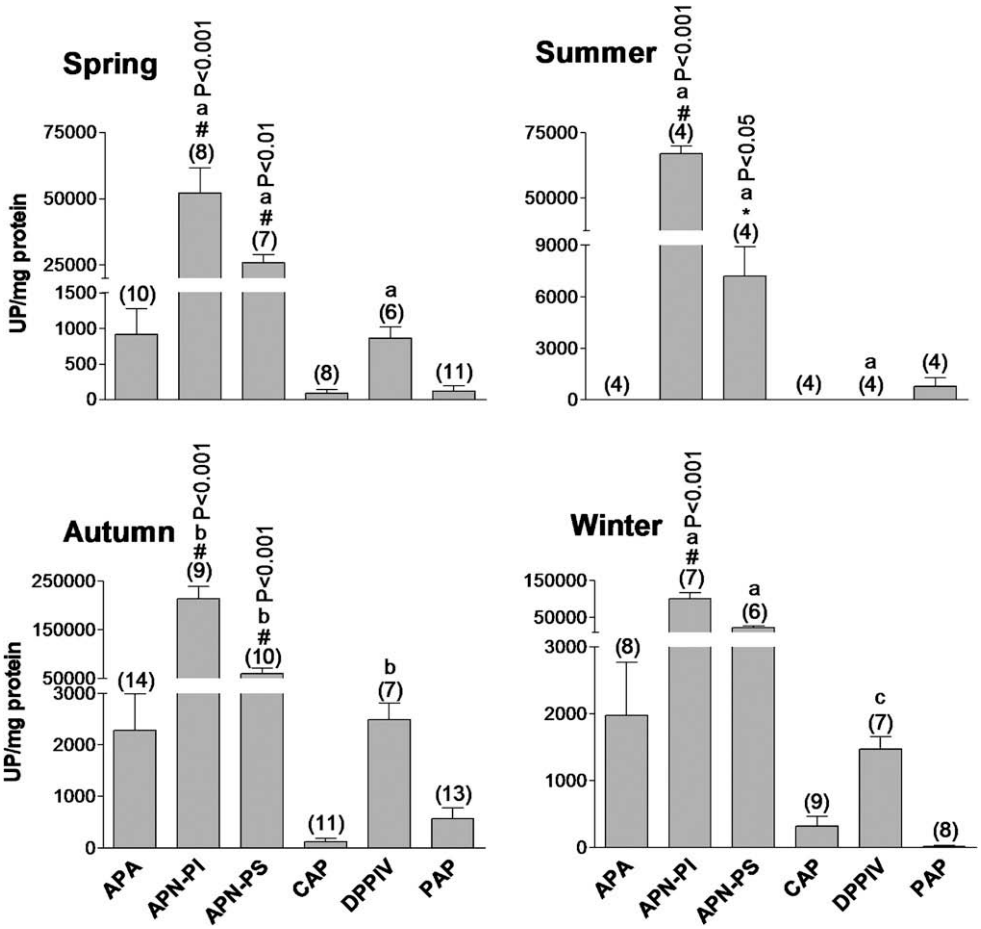

Fig. 2. Variations of activity levels (means ± SEM) of acid (APA), puromycin-insensitive (APN-PI) and -sensitive neutral (APN-PS), cystyl (CAP), dipeptidyl-peptidase IV (DPPIV) and type-I pyroglutamyl (PAP) in solubilized membrane-bound fraction of the ductus deferens of *C. d. terrificus* in the wet (spring/summer) and dry (autumn/winter) seasons. Number of animals is given in parentheses. UP = picomoles of hydrolyzed substrate/min. Different letters indicate seasonal variation of a peptidase activity (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni $P < 0.05$). Differences, in the same season, related to: #, all activities; *, all activities, except PAP (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni: P is indicated above each bar).

A total of 43 non-pregnant females used in this study were collected during 18 months, 23.26% in spring, 13.95% in summer, 37.21% in autumn and 25.58% in winter. The sampling was similar for males. Reproductive females (i.e., secondary vitellogenesis or pregnant) and non-reproductive (i.e., primary vitellogenesis or post-partum) were collected in a ratio of 39.02% and 60.98%, respectively. Semen can be found in the ductus deferens throughout the year. Among 43 samples, eight had semen within the uterus (six in winter and only two in autumn). In reproductive females, 29.63% had semen stored in the uterus, both in autumn and winter. Considering only the females collected in winter, 54.54% had semen stored in the uterus.

3.2. Lactate dehydrogenase

LDH activity (mmol oxidized NADH/min/mg protein) ranged from 0.56 ± 0.07 to 1.67 ± 0.19 in SF and 0.20 ± 0.02 to 0.51 ± 0.07 in MF, thus it was always higher (about threefold-higher) in SF than MF.

3.3. Peptidase activities

Figs. 1–6 show that, in general, independently of seasonal fluctuations, the detectable peptidase activities were APA, APB, APN-PI, APN-PS, CAP, DPPIV, PAP-I, PIP and POP in SF, while APA, APN-PI, APN-PS, CAP, DPPIV and PAP-I activities were in SF and MF. Moreover, in general, independently of seasonal fluctuations, APN-PI and APN-PS (SF and MF) and APB activities were marked in all examined tissues, and APN-PI was the most pronounced activity in SF and MF.

The activities APA, APB, APN-PI, APN-PS, CAP, DPPIV, PAP-I and POP in SF and, APA, APN-PI, APN-PS and DPPIV in MF, varied seasonally (Figs. 1–6). In the SF of the ductus deferens, APA activity was higher in autumn than in spring, DPPIV was higher in winter than in summer, while PAP-I was higher in summer than in spring and winter (Fig. 1). Moreover, in the ductus deferens MF, APN-PI, APN-PS and DPPIV activities were higher in autumn than in other seasons, while the other activities were not altered during all seasons (Fig. 2). In the uterus SF, APA activity was higher in winter than in autumn (Fig. 3). In the uterus MF, APA activity was higher in winter than in other seasons (Fig. 4). APN-PS activity in the uterus SF was higher in winter than in autumn (Fig. 3). In the uterus MF, APN-PI activity was higher in winter than in autumn (Fig. 4). Fig. 5 shows that in the vagina SF, APA, APB and PAP-I activities were higher in winter than in other seasons, while APN-PS activity was higher in winter than in autumn. CAP activity in SF of the vagina was higher in winter than in spring and summer (Fig. 5). Fig. 6 shows that in vagina MF, APA activity was higher in winter than in other seasons, while APN-PS was higher in winter than in summer.

4. Discussion

4.1. General considerations

Some variables could not be controlled in our experimental protocols. For example, due to the relative low number of available samples of females, we were unable to robustly compare peptidase activity in reproductive and non-reproductive groups (Almeida-Santos and Orsi, 2002). Geographic variations did not seem to influence our results, as snakes were provided from locations where weather was similar, and in this sense the tropical weather is markedly divided only in the wet season (spring/summer) and

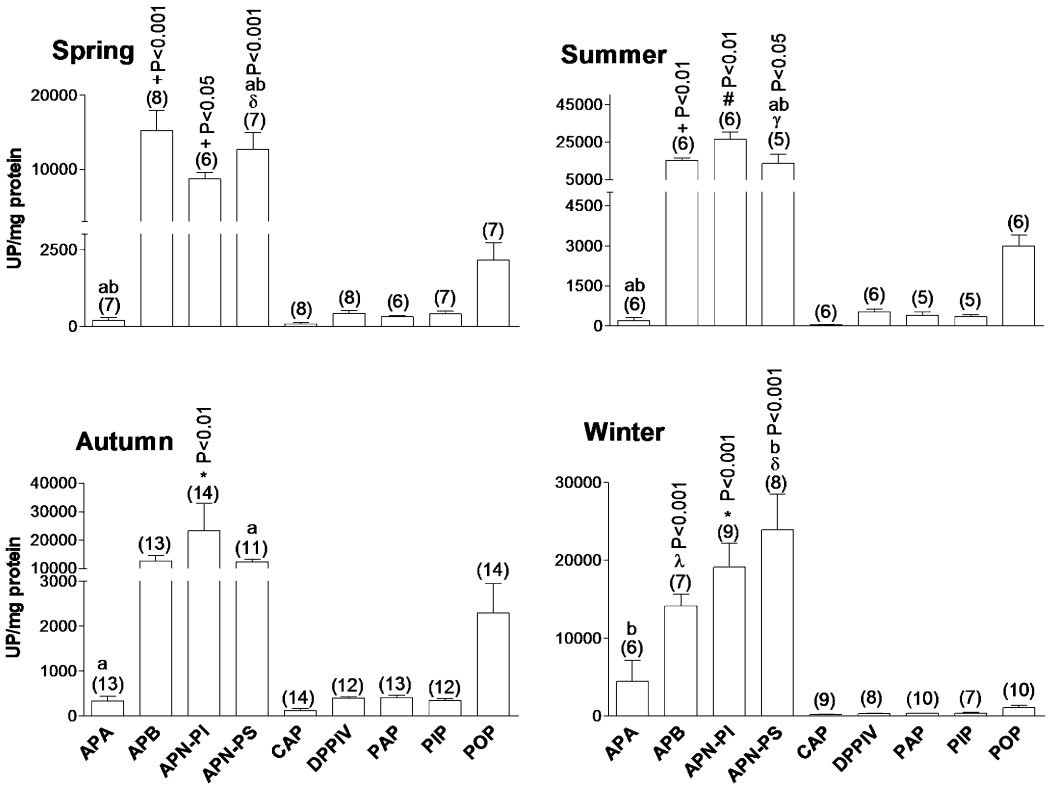

Fig. 3. Variations of activity levels (means ± SEM) of acid (APA), basic (APB), puromycin-insensitive (APN-PI) and -sensitive neutral (APN-PS), cystyl (CAP), dipeptidyl-peptidase IV (DPPIV), type-I pyroglutamyl (PAP), prolyl-imino (PIP) and prolyl oligopeptidase (POP) in soluble fraction of the uterus of *C. d. terrificus* in the wet (spring/summer) and dry (autumn/winter) seasons. Number of animals is given in the parentheses. UP = picomoles of hydrolyzed substrate/min. Different letters indicate seasonal variation of a peptidase activity (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni $P < 0.05$). Differences, in the same season, related to: #, all activities; ⁎, all activities, except APB, APN-PS; +, all activities, except APN-PS; δ, all activities, except APB, APN-PI; γ, all activities, except APB; λ, all activities, except APA, APN-PI, APN-PS (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni: P is indicated above each bar).

the dry season (autumn/winter). We excluded circadian variations as a source of possible error because materials were obtained during the same time of day, with a maximum difference of 90 min. However, it is possible that due to these limitations some differences could not be revealed in the present study.

4.2. Reproductive cycle and macroscopic aspects of the female reproductive tract

According to Almeida-Santos and Orsi (2002), female tropical rattlesnakes (*C. d. terrificus*) can be classified into two groups, i.e., reproductive (secondary vitellogenesis and pregnant) and non-reproductive (primary vitellogenesis and post-partum). We expected a similar proportion of specimens in primary and secondary vitellogenesis in our samples, which means 50% of the snakes should be in each group. Thus, we also expected the presence of semen in the majority of females in the secondary vitellogenesis during autumn, period of mating (Schuett, 1992; Almeida-Santos and Salomão, 1997). Considering the number of samples in all seasons, the incidences of non-reproductive (about 61%) and reproductive females (about 39%) are not in equal ratio as the expected in a same year. These results suggest an inefficiency to complete the reproductive process in 12 months. Furthermore, the low incidence of semen stored in the uterus (in about 50% of the examined females that were in reproductive conditions) suggests a low frequency of copulation in our samples. Copulation and reproduction in many species of snakes is affected by energy and nutrient accumulation (Aubret et al., 2002; Bonnet et al., 1994), which, in turn, is influenced by the environment. In addition, the low incidence of semen stored in the uterus in autumn (2/16) can reflect that in natural environment snakes could be seeking a pair and their capture occurred before the mating. Moreover, considering that the mating of *C. d. terrificus* occurs only during mid autumn (Schuett, 1992; Almeida-Santos and Salomão, 1997), the comparison between the incidences of semen stored in the uterus in both seasons (in a total of eight specimens, six occurred in winter and only two in autumn) corroborates with the existence of LTSS in the female.

4.3. Fractionation procedure and peptidase activities

The pronounced LDH activity (cytosolic marker) in SF of all samples confirmed the efficiency of the employed procedure. The evaluation of the distribution of peptidase activities in SF and MF allows the association of these activities with intra and extracellular peptides. In the overall, peptides involved in intracellular signaling are primarily hydrolyzed outside the cell, mainly by membrane-bound peptidases (O'Cuinn, 1998). These peptidases have, generally, the active sites in the extracellular side and interact with released peptides (O'Cuinn, 1998). Soluble peptidases act mainly in intracellular processes, but can also be exocytosed or act in recaptured peptides, which are internalized as part of the peptide–receptor complex, as occurs with peptide hormones in the target cells (Gibson et al., 1989). The extracellular substrate can also be efficiently processed by cytosolic enzymes, after enzyme translocation to the cell surface which occurs under stimulation (Sumitani et al., 1997). In the present study, APB, PIP and POP were detected only in SF, while the other peptidase activities were

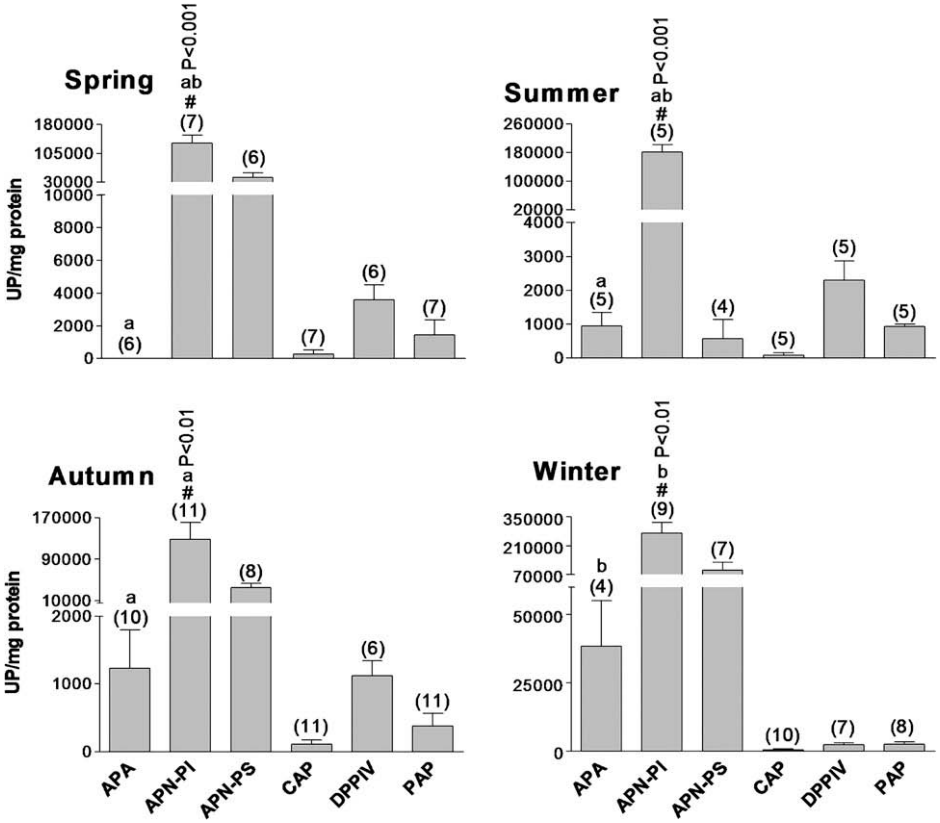

Fig. 4. Variations of activity levels (means ± SEM) of acid (APA), puromycin-insensitive (APN-PI) and -sensitive neutral (APN-PS), cystyl (CAP), dipeptidyl-peptidase IV (DPPIV) and type-I pyroglutamyl (PAP) in solubilized membrane-bound fraction of the uterus of *C. d. terrificus* in the wet (spring/summer) and dry (autumn/winter) seasons. Number of animals is given in parentheses. UP = picomoles of hydrolyzed substrate/min. Different letters indicate seasonal variation of a peptidase activity (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni $P < 0.05$). # Differences, in the same season, related to all activities (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni: P is indicated above each bar).

detected in SF and MF of all tissues, in a pattern similar to that in the majority of mammalian tissues (Irazusta et al., 2001, 2004; Varona et al., 2003).

4.4. Reproductive cycle and peptidase activities

The activity levels of APB, APN-PI and APN-PS were prominent in the majority of tissues and seasons. Overall, seasonal differences in most of the peptidase activities had a common pattern of increase during the dry season (austral autumn/winter). The relationships of peptidase activities with sex steroid levels, superior integrity of spermatozoa in the uterus during autumn compared to winter (time of LTSS in the female), and the fertilization capacity in the vagina during autumn relatively to spring (period of fertilization) or winter are suggested by the results shown in the present study. For instance, the decrease of APN-PI (MF) in the uterus and APN-PS (SF) in the vagina, observed in autumn compared to winter, coincides with the decrease of progesterone and increase of estradiol. In *C. d. terrificus*, the estradiol increases in autumn (mating), peaks in winter and has an expressive drop off in spring (fertilization); while progesterone decreases in autumn, coincidently with the beginning of secondary vitellogenesis, increases in spring and its level is maintained during the pregnancy (Almeida-Santos et al., 2004a). APN is known to have an extracellular catalytic site in the ovary and endometrium and, when inhibited by bestatin, murine follicular growth, porcine steroidogenesis and decidualization induced by progesterone in human endometrium are altered (Fujiwara et al., 1999). In pregnant women, there is an increase of APN activity in the endometrium (Olsen et al., 1995). In ovariectomized rabbits, APN activity in the vagina diminished (Acartürk et al., 2001) and it is influenced by sexual hormones (Olsen et al., 1995). It was also noteworthy that the higher CAP (SF) activity in the vagina of *C. d. terrificus* in winter, relatively to spring and summer, coincides with the muscular relaxation (possible non-retention and/or the transport of spermatozoa from the vagina to the uterus during the LTSS in the female). Furthermore, relatively to other seasons, occurred an increase of PAP-I (SF) activity in the vagina during the winter (post-mating period and LTSS in the female). During the austral summer (pre-mating period and peak of spermatogenesis), PAP-I (SF) in the ductus deferens was higher than in spring and winter. During the winter, the lowest level of PAP-I (SF) in the ductus deferens coincided with its highest level in the vagina, and the opposite occurs in summer. FPP-like peptides, which are substrates of PAP-I (Siviter and Cockle, 1995), are known for their capacity to stimulate *in vitro* the fertilization capacity of sperm from rat epididymis (Cockle et al., 1994) and human ejaculated sperm and, also, for the inhibition of spontaneous loss of acrosome of rat sperm (Fraser et al., 1997a,b), suggesting a possible modulation of different aspects of seminal physiology (Cockle et al., 1994; Fraser et al., 1997a,b; Green et al., 1996a,b). In this sense, simultaneous variations of PAP-I (SF) activity of the ductus deferens and vagina are in agreement to the necessity to enhance or inhibit the fertilization capacity of spermatozoa of rattlesnake, respectively, to the peak of spermatogenesis and LTSS in the female, by altering the rate of hydrolysis of FPP-like peptides and modulating the action of these peptides in the spermatozoa of the rattlesnake. In human semen, PAP-I and POP had increased activity in necrozoospermia (Valdivia et al., 2004). Comparing the levels of peptidase activities between autumn and winter in the tissue where occurs the spermatozoa storage in the male (ductus deferens), APN-PS (MF) and DPPIV (MF) were lower in winter than in autumn (mating). Moreover, DPPIV

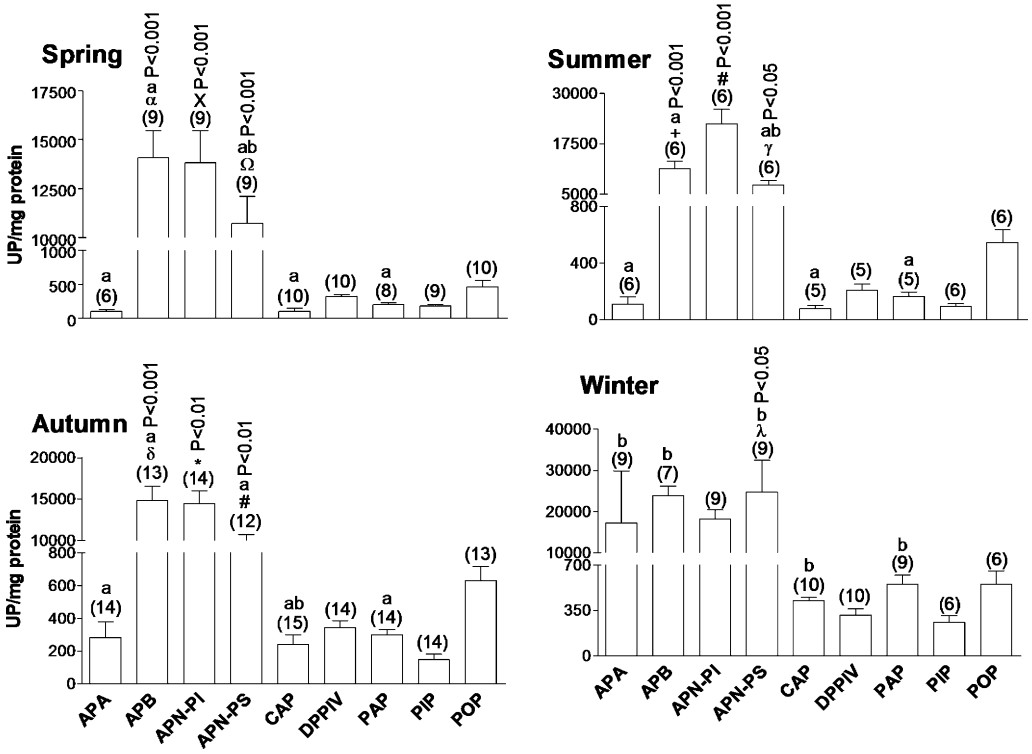

Fig. 5. Variations of activity levels (means ± SEM) of acid (APA), basic (APB), puromycin-insensitive (APN-PI) and -sensitive neutral (APN-PS), cystyl (CAP), dipeptidyl-peptidase IV (DPPIV), type-I pyroglutamyl (PAP), prolyl-imino (PIP) and prolyl oligopeptidase (POP) in soluble fraction of the vagina of *C. d. terrificus* in the wet (spring/summer) and dry (autumn/winter) seasons. Number of animals is given in parentheses. UP = picomoles of hydrolyzed substrate/min. Different letters indicate seasonal variation of a peptidase activity (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni $P < 0.05$). Differences, in the same season, related to: #, all activities; ∗, all activities, except APB; δ, all activities, except APN-PI; +, all activities, except APN-PS; Ω, all activities, except APB and APN-PI; γ, all activities, except APB, DPPIV, PAP and POP; λ, DPPIV and CAP; α, all activities, except APN-PI, APN-PS; X, all activities, except APB, APN-PS (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni: P is indicated above each bar).

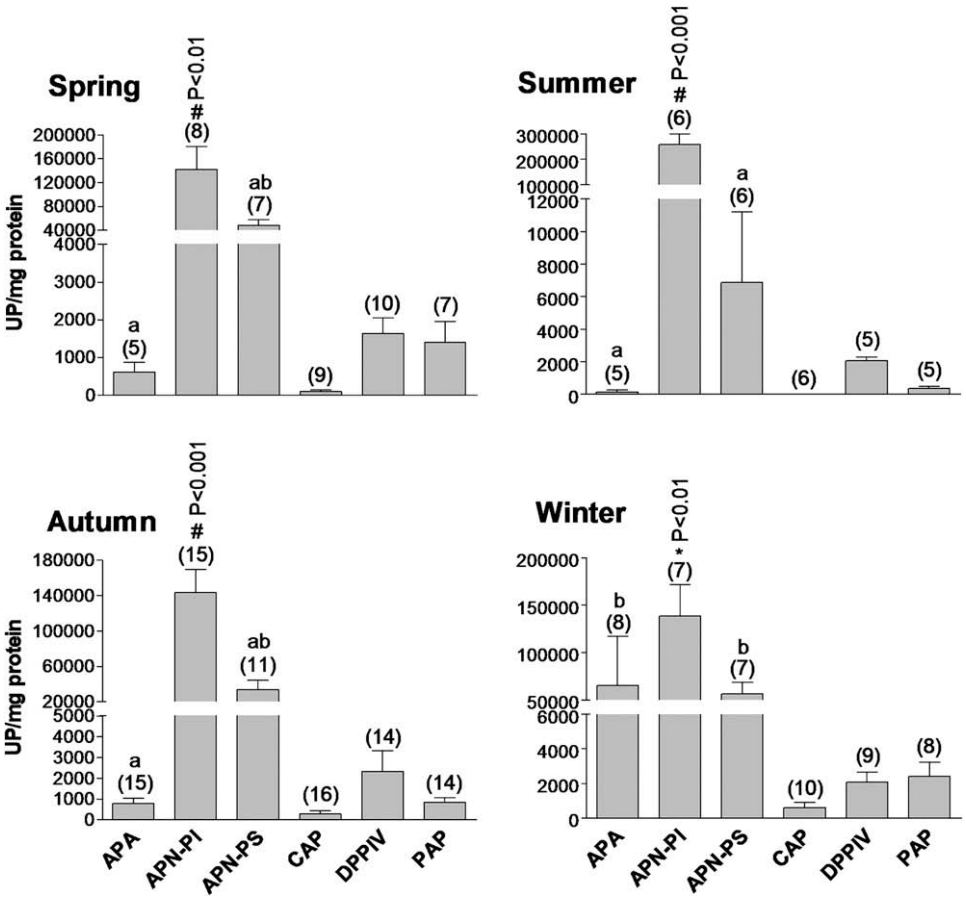

Fig. 6. Variations of activity levels (means ± SEM) of acid (APA), puromycin-insensitive (APN-PI) and -sensitive neutral (APN-PS), cystyl (CAP), dipeptidyl-peptidase IV (DPPIV) and type-I pyroglutamyl (PAP) in solubilized membrane-bound fraction of the vagina of *C. d. terrificus* in the wet (spring/summer) and dry (autumn/winter) seasons. Number of animals is given in parentheses. UP = picomoles of hydrolyzed substrate/min. Different letters indicate seasonal variation of a peptidase activity (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni $P < 0.05$). Differences, in the same season, related to: #, all activities; ∗, DPPIV, PAP and CAP (one-way analysis of variance ANOVA $P < 0.05$, Bonferroni: P is indicated above each bar).

(MF) in the ductus deferens was also lower in spring/summer than in autumn. The absence of APN-PS has been proposed to be related to the infertility or subfertility (Osada et al., 2001), while the increase of APN-PI activity would be related to defects in sperm motility and viability, and it is found in asthenozoospermia (reduction of sperm motility) and/or necrozoospermia (dead sperm) (Irazusta et al., 2004). In addition, DPPIV hydrolyses substance P, which has a role in the release of seminal fluid from the epididymis and in the initiation of sperm motility in rats (Sastry et al., 1991). Then, in *C. d. terrificus*, under an increase of DPPIV in the ductus deferens during the autumn, the ejaculated spermatozoa would have lower levels of motility by the decrease of substance P.

In conclusion, the present work demonstrates for the first time the relationship between levels of aminopeptidases activities and seasonality in snakes, which are distinct in a specific-tissue way in *C. d. terrificus*. The following characteristics were marked: (i) activities of APA, APB, APN-PS, APN-PI, CAP, DPPIV, PAP-I, POP and PIP were detected both in soluble and/or membrane-bound fractions of the examined tissues; (ii) APN-PS, APN-PI and APB had more expressive activities in all tissues, suggesting a great physiological importance; (iii) the evaluated peptidases had a common pattern of increment during the dry season (autumn/winter), simultaneously with the mating (autumn) and LTSS in the female (winter), as well as with the reduction of spermatozoon motility and maintenance of its fertilization capacity; (iv) the pronounced CAP activity in soluble fraction of vaginal tissue, in winter compared to spring (ovulation and fertilization) and summer (parturition) coincides with the relaxation of this tissue; (v) lower levels of PAP-I activity in the ductus deferens (lower hydrolysis of FPP-like peptides) coincide with its higher levels (pronounced hydrolysis of FPP-like peptides) in the vagina in winter (spermatic defects and LTSS in the female), the inverse occurs in summer (peak of spermatogenesis and parturition).

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