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INTRASPECIFIC VARIATION IN THE VENOM ELECTROPHORETIC PROFILE OF RECENTLY CAPTURED *Crotalus durissus terrificus* (LAURENTI, 1768) SNAKES

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ABSTRACT: The aim of this study was to compare individual venom samples of *Crotalus durissus terrificus* recently captured in the wild to evaluate possible differences in venom protein composition. Protein levels were quantified by biochemical method (Biuret) and then submitted to electrophoresis. Electrophoresis studies of native protein were performed in vertical slabs of polyacrylamide gel (PAGE), in an alkaline discontinuous buffer system, with a concentration of 10% in the separation gel. SDS-PAGE was performed in PhastGel® (8-25). Both gels were stained with Coomassie Blue. Gels were analyzed using the VDS-Pharmacia® device. Our results indicate that all analyzed venom samples showed different protein composition, although common protein fractions were detected in some individual samples. Differences were observed between the different individual venom samples and so in the same specimen in relation to the time of collection, for both techniques used. Diet did not influence the variability of venom composition. There is a significant difference in native venom protein composition of males and females.
KEY WORDS: *Crotalus durissus terrificus*, intraspecific variation, protein composition, venom, sex, feeding, electrophoresis.

INTRODUCTION

The *Crotalus* genus has 27 species, which are characterized by a rattle in the tail end and a large number of small scales covering the dorsal region of the head. This genus is found from the south of Canada to Argentina in places below sea level up to four thousand meters of altitude. *Crotalus* snakes are found in a great variety of habitats, and the venom produced by the different species shows great variability of composition and effects (21). In Brazil, there is only one species, *Crotalus durissus*, which is subdivided into six subspecies: *C. d. cascavella, C. d. collilineatus, C. d. marajoensis, C. d. ruruima, C. d. trigonicus,* and *C. d. terrificus*. These snakes are popularly known as "cascavel", "cobra-de-quatro-ventas", "boicininga", "maracá", "boiquira", or "maracabóia" (1,5,29). *Crotalus durissus terrificus* is found in areas of the States of Amazonas, Pará, Mato Grosso, Minas Gerais, Paraná, Santa Catarina, Rio Grande do Sul, and São Paulo. They are relatively big snakes, of about one meter in length; some males occasionally reach one and a half meters. They live in dry and rocky areas, of low vegetation, being rarely found in forests (1,21,29).

Variation in snake venom composition has been associated with factors, such as geographical origin (3,6,9,12,16,23,24), season (14), sex (20), age (17), and diet (7,30). Willemse (34) worked with the venom of six different species (*Bitis arietans, Causus rhombeatus, Naja nivea, Naja haje, Naja nigricollis,* and *Hemachatus*

haemachatus) by analyzing individual venom samples using polyacrylamide gel electrophoresis. Diet, geographical origin, and season were controlled. The author found individual electrophoretic variations within the six species, but he did not find difference in venom sample composition related to sex.

Fiero *et al*. (8) analyzed a pool of *Crotalus viridis viridis* from young snakes and their mothers in different periods, using polyacrylamide gel electrophoresis. Although the venom from young snakes showed higher toxicity, its electrophoretic composition was similar to the adults.

Variation in snake venom composition influenced by geographical origin has been suggested (3,6,9,12,16,23,24). Daltry *et al*. (7) working with *Calloselasma rhodostoma* populations, found that variation in the geographical origin would be directly related to feeding habits.

Crotalus durissus venom is a complex mixture of substances, such as crotamine, gyroxin, PM-toxin, crotoxin (a complex formed by phospholipase A2 and crotapotin), tissue kalikrein-like activity, trombine-like enzyme, phosphodiesterase, 5'-nucleotidase, L-amino oxidase, convulsin, and peptides (4).

Barrio (2) analyzed samples of *Crotalus durissus terrificus* from several parts of Brazil and found three different types of electrophoretic profiles. His study allowed him to divide these snakes according to their origin into northern, central, and southern populations. Barrio (2) reported that the venom of specimens collected in southern Brazil differs from the others, since it has a substance denominated crotamine. Gonçalves and Vieira (13) also showed variation in *Crotalus durissus terrificus* venom composition related to geographical origin.

Electrophoresis is one of the major techniques available to current biochemical investigation. Its application has been greatly expanded over the past few years due to simplified equipment, which permits quick and precise analyses. Soares *et al*. (28) have recently suggested the use of electrophoresis as an auxiliary tool for the taxonomic study on some species of the Elapidae and Viperidae families. In this work, the authors described the venom electrophoretic profile of *Micrurus, Bothrops, Bothriopsis, Crotalus,* and *Lachesis* concluding that electrophoretic characterization of the basic proteins is an efficient and easy method for venom taxonomic studies of the families Elapidae and Viperidae.

Thus, a great variation has been observed in the individual protein composition of snake venom, and electrophoresis has been the most useful technique to study this variation. The objective of this study was to analyze the composition of *Crotalus durissus terrificus* venom in relation to sex and feeding, as there are only a few studies on this subject in literature.

Twenty-four recently captured adult specimens of *Crotalus durissus terrificus* (1,5) were used. The snakes were collected from different regions of São Paulo State and were donated to The Center for the Study of Venoms and Venomous Animals of São Paulo State University (CEVAP/UNESP), Botucatu, SP (Table 1).

Table 1. Origin of *Crotalus durissus terrificus* specimens.

Number	Date of collection	Date of receipt	Place of collection	Sex	Total length (cm)	Tail length (cm)	Weight (g)
275	12/11/97	14/11/97	Itatinga	M	114	12.0	945
279	20/11/97	20/11/97	Botucatu	M	112	10.0	980
280	25/11/97	26/11/97	Botucatu	M	115	12.5	1140
281	25/11/97	26/11/97	Fartura	M	113	12.0	720
283	??	24/11/97	Anhembi	M	102	10.0	610
284	28/11/97	01/12/97	São Pedro	F	100	6.5	680
285	03/12/97	04/12/97	Itatinga	F	81	5.5	470
287	09/12/97	11/12/97	Anhembi	F	83	5.5	375
427	??	06/11/98	Fartura	F	66	6.5	170
434	??/10/98	19/11/98	Assis	F	73	5.2	230
436	??/10/98	19/11/98	Assis	F	98	7.0	970
437	??/10/98	19/11/98	Assis	F	56	4.5	100
440	17/11/98	19/11/98	Anhembi	M	79	7.5	350
441	14/11/98	19/11/98	Anhembi	F	74	4.5	280
577	06/07/99	07/07/99	Itatinga	F	73	4.5	200
578	??	14/07/99	Águas de Santa Bárbara	F	94	6.5	600
579	12/07/99	14/07/99	Botucatu	F	92	5.5	550
580	??	14/07/99	Paulistânia	M	86	10.0	450
581	13/07/99	14/07/99	Paulistânia	F	91	5.0	620
582	??	02/08/99	Santa Maria da Serra	F	91	5.5	440
583	27/07/99	02/08/99	Santa Maria da Serra	M	93	8.5	550
584	12/08/99	13/08/99	Itatinga	M	82	8.5	300
585	24/08/99	27/08/99	Piracicaba	F	99	6.0	780
586	24/08/99	27/08/99	Piracicaba	F	89	5.5	740

? – Data not provided by the collector.

Information about the day of capture and origin were provided by the donor. The animals were weighed and the total and tail length measured. Sexing was performed by the observation of tail length and diameter (Table 1).

After the first venom extraction, the animals were housed in the quarantine room 30 cm h. x 40 cm w. x 60 cm l. propylene cages, with water in aluminum recipients. Light was artificial, there was no humidity control, and temperature was approximately 25 ± 3°C, maintained by an electric heater. The animals were handled depending on hygiene conditions in the cages. Feeding during the quarantine period consisted of mice (*Mus musculus*) from the Central Animal Facility of UNESP, Botucatu. The number of mice each snake ate varied.

Venom was extracted immediately after the animals' arrival without anesthetics, using the technique described by Silva (25). Two lots of venom were collected. The first consisted of 14 samples, one sample from each snake, collected between November 1997 and November 1998. The second lot consisted of 25 samples collected between June and August 1999 immediately after snake arrival and on the 30th and 60th days. All the venom samples were stored at -20°C.

Protein quantity of each venom sample was determined by the Biuret test (33). Venom (100 μl) was collected, mixed to Biuret (5 ml), and warmed for 15 minutes at 32°C. Readings were performed using a Ultrospec 2000® - Pharmacia® spectrophotometer at 540 nm wave length. One hundred fifty μg of each protein sample was applied to the gel.

Polyacrylamide gel electrophoresis (PAGE) was performed in vertical plate for the analysis of native protein, as described by Hames and Rickwood (15) and Gahne *et al.* (10). A potential difference of 100 V was applied for 1 hour, and then increased to 200 V for 5 hours. After the electrophoretic run, the gels were immersed in a staining solution of 0.25% Coomassie Blue for 45 minutes. The plates were then left in a distaining solution for 15 minutes at 45°C, with two repetitions. The gels were placed in water boiled in a microwave oven for 25 minutes (1:200 gel/water).

Protein molecular weight was determined using a PhastSystem® (Pharmacia-Biotech®) and PhastGel® gradient 8-25 (PAGE-SDS), following the manufacturer's instructions. Buffer was added to the venom samples (1:1). This was placed in water at 100°C for 1 minute. A kit of molecular weights was used (LMW Electrophoresis Calibration Kit® and HMW-SDS Calibration Kit®). The results were expressed as kDa.

The plates were analyzed in IMAGE MASTER VDS® (Pharmacia-Biotech®), using an appropriate software. The results obtained with the native protein were expressed as migration rate (Rf).

Comparison of the number of bands in the venom samples from males and females was performed using the "t" test at 5% significance level.

The electrophoretic results are shown in Tables 2 to 7A and 7B, and Figures 1A, 1B, and 2. Gel analysis revealed 11 different protein fractions in the first venom lot (Tables 2 and 3) and 28 in the second lot (Tables 4A, 4B, 5A, and 5B). In contrast, analysis of molecular weight revealed 12 different protein fractions in the first lot and 36 in the second lot (Tables 6A, 6B, 7A, and 7B). A great variation was observed in the individual venom sample composition both in the analysis of native proteins (Tables 2 to 5A, and 5B, Figure 1) and in the analysis of molecular weight (Tables 6A, 6B, 7A, and 7B, Figure 2).

Table 2. PAGE of native proteins from *Crotalus durissus terrificus* venom. Values in migration rate (Rf). Samples collected and stored between November 1997 and November 1998.

Fraction	275	279	280	281	283	284	285	287	427	434	436	437	440	441
F1				0.13	0.13		0.13	0.13		0.13	0.13	0.13	0.13	
F2				0.16				0.16	0.16		0.16	0.16		0.16
F3						0.41	0.41						0.41	0.41
F4						0.46	0.46						0.46	
F5		0.58			0.58	0.58								
F6				0.61			0.61		0.61	0.61	0.61	0.61	0.61	0.61
F7	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66				0.66		0.66
F8				0.69		0.69	0.69		0.69	0.69	0.69	0.69	0.69	
F9	0.75		0.75		0.75	0.75	0.75	0.75	0.75	0.75	0.75		0.75	0.75
F10	0.80		0.80	0.80							0.80			0.80
F11					0.91		0.91	0.91			0.91	0.91		0.91

Table 3. Analysis of the amount of each protein fraction obtained from the determination of migration rate of proteins from *Crotalus durissus terrificus* venom. Percentage. Samples collected and stored between November 1997 and November 1998.

Fraction	275	279	280	281	283	284	285	287	427	434	436	437	440	441
F1				8.35	20.46		23.60	4.77		7.37	6.71	7.49	5.58	
F2				7.52				6.44	28.07		19.10	12.46		6.14
F3						3.14	5.20						1.87	9.60
F4						3.27	0.67						2.19	
F5		29.18			22.56	10.00								
F6				9.90			22.88		29.88	18.88	14.24	23.43	19.41	16.42
F7	24.15	70.80	36.10	28.16	29.76	16.47	5.45	40.95				19.03		31.64
F8				35.80		15.03	24.98		18.06	20.63	9.36	30.68	19.20	
F9	34.73		10.72		24.13	52.07	12.95	45.52	24.00	53.12	29.10		51.75	11.07
F10	41.12		53.18	10.27							16.71			22.62
F11					3.10		4.27	2.33			4.79	6.91		2.52

Table 4A. PAGE of native proteins from *Crotalus durissus terrificus* venom. Values in migration rate (Rf). Samples collected and stored between June and October 1999.

Fractions	577A	577B	577C	578A	578B	578C	579A	579B	579C	580A	580B	580C
F1										0.03		
F2	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05			
F3												
F4	0.11		0.11									
F5	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12			
F6												
F7	0.18	0.18	0.18	0.18	0.18			0.18	0.18			
F8	0.23	0.23	0.23	0.23	0.23		0.23	0.23	0.23			
F9												
F10	0.34	0.34	0.34			0.34					0.34	0.34
F11												
F12											0.40	0.40
F13												
F14								0.47	0.47			0.47
F15												
F16												
F17								0.55	0.55			0.55
F18	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57		0.57		
F19	0.62	0.62	0.62	0.62	0.62	0.62	0.62	0.62		0.62	0.62	
F20												
F21												
F22				0.72	0.72			0.72	0.72	0.72	0.72	
F23	0.75	0.75	0.75		0.75	0.75	0.75	0.75	0.75		0.75	0.75
F24				0.78	0.78		0.78	0.78	0.78			0.78
F25	0.83	0.83	0.83	0.83						0.83	0.83	0.83
F26											0.89	0.89
F27												
F28												

Legend: extraction performed right after snake arrival (A), and on the 30th (B) and 60th days (C).

Table 4B. PAGE of native proteins from *Crotalus durissus terrificus* venom. Values in migration rate (Rf). Samples collected and stored between June and October 1999.

Fractions	581A	581B	582A	582B	582C	583A	583B	583C	584A	584B	585	586A	586B
F1													
F2													
F3										0.08	0.08	0.08	0.08
F4													
F5													
F6									0.14	0.14	0.14	0.14	0.14
F7	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18					
F8	0.23	0.23	0.23	0.23	0.23				0.23	0.23	0.23	0.23	0.23
F9										0.27	0.27	0.27	0.27
F10													
F11										0.37			0.37
F12		0.40											
F13		0.44	0.44	0.44	0.44	0.44			0.44	0.44			0.44
F14		0.47	0.47	0.47	0.47						0.47	0.47	
F15									0.50				
F16										0.52		0.52	0.52
F17											0.55		
F18													
F19	0.62		0.62	0.62	0.62				0.62		0.62	0.62	0.62
F20	0.65		0.65	0.65	0.65		0.65						
F21										0.67	0.67	0.67	0.67
F22									0.72	0.72	0.72	0.72	0.72
F23	0.75	0.75		0.75	0.75	0.75	0.75	0.75					
F24									0.78	0.78	0.78		
F25	0.83		0.83	0.83	0.83		0.83	0.83					
F26			0.89										
F27					0.91					0.91	0.91		
F28									0.94				

Table 5A. Analysis of the amount of each protein fraction obtained from the determination of migration rate of proteins from *Crotalus durissus terrificus* venom. Percentage. Samples collected and stored between June and October 1999.

Fractions	577A	577B	577C	578A	578B	578C	579A	579B	579C	580A	580B	580C
F1										10.66		
F2	5.84	7.08	3.10	3.79	5.15	6.47	3.64	3.98	4.40			
F3												
F4	2.76		2.30									
F5	10.90	2.68	7.57	4.97	3.92	7.06	4.96	2.87	1.04			
F6												
F7	1.97	1.33	2.37	1.30	0.39			9.36	10.12			
F8	3.91	6.55	5.33	6.57	5.11		8.50	11.26	13.41			
F9												
F10	21.57	3.23	19.15			20.73					5.98	13.37
F11												
F12											3.81	8.52
F13												
F14								2.09	16.63			6.89
F15												
F16												
F17								1.75	8.23			27.75
F18	4.98	6.38	4.85	7.99	12.08	13.39	27.99	22.60		17.69		
F19	12.15	10.68	8.86	23.58	15.58	20.39	10.40	3.80		21.56	23.90	
F20												
F21												
F22				32.97	0.81			10.34	11.96	46.21	10.52	
F23	26.40	49.35	35.14		11.63	31.96	26.57	24.07	13.83		46.12	15.29
F24				17.17	45.33		17.95	7.87	20.39			12.62
F25	9.55	12.72	11.33	1.68						3.88	4.45	3.46
F26											5.22	12.09
F27												
F28												

Legend: extraction performed right after snake arrival (A), and on the 30th (B) and 60th days (C).

Table 5B. Analysis of the amount of each protein fraction obtained from the determination of migration rate of proteins from *Crotalus durissus terrificus* venom. Percentage. Samples collected and stored between June and October 1999.

Fractions	581A	581B	582A	582B	582C	583A	583B	583C	584A	584B	585	586A	586B
F1													
F2													
F3										4.18	4.20	4.95	5.93
F4													
F5													
F6									16.15	5.50	3.80	5.00	7.13
F7	8.86	15.99	4.07	4.84	5.69	31.48	15.20	20.03					
F8	14.50	9.21	9.40	11.14	12.50				9.88	6.43	6.08	6.30	7.73
F9										1.91	4.80	7.64	3.63
F10													
F11										4.92			4.53
F12		8.61											
F13		2.74	11.36	13.45	2.77	14.10			40.13	1.93			5.67
F14		23.96	12.79	15.12	2.23						3.14	4.39	
F15									3.89				
F16										14.41		2.47	3.69
F17											3.56		
F18													
F19	14.99		14.77	17.46	2.61				12.98		6.81	12.87	7.67
F20	19.85		4.23	6.62	5.85		15.32						
F21										6.46	15.00	31.92	29.26
F22									10.87	19.27	19.19	24.46	24.66
F23	34.01	39.49		12.84	15.51	54.41	28.82	33.09					
F24									37.54	32.92	31.19		
F25	7.80		13.84	18.52	23.30		40.65	46.88					
F26			29.55										
F27					29.54					2.06	2.22		
F28									4.57				

Legend: extraction performed right after snake arrival (A), and on the 30th (B) and 60th days (C).

Table 6A. Determination of molecular weight of proteins from *Crotalus durissus terrificus* venom by PAGE-SDS. Values in kDa. Samples collected and stored between November 1997 and November 1998 (columns 1 to 3) and samples collected between June and October 1999 (other columns).

Fractions	281	283	434	577A	577B	577C	578A	578B	579	580A	580B	580C
F1												
F2	121.2	121.2	121.2		121.2	121.2	121.2	121.2	121.2			
F3										119.0		
F4											117.5	117.5
F5												
F6												
F6												
F8												
F9	98.8	98.8	98.8	98.8	98.8	98.8	98.8	98.8	98.8			
F10												
F11												83.2
F12		79.3										
F13	77.5		77.5					77.5	77.5			
F14												
F15									70.6			
F16	69.4	69.4	69.4		69.4	69.4	69.4					
F17										68.3	68.3	68.3
F18												
F19	64.6	64.6	64.6			64.6		64.6				
F20												
F21	60.6	60.6	60.6	60.6	60.6	60.6			60.6			
F22	55.4	55.4	55.4	55.4	55.4	55.4	55.4	55.4	55.4			
F23										53.0	53.0	53.0
F24	51.8	51.8	51.8		51.8	51.8	51.8					
F25									51.0			
F26												
F27	48.4	48.4	48.4		48.4	48.4	48.4		48.4			
F28											44.8	44.8
F29												
F30										34.3	34.3	34.3
F31					32.6							
F32	31.7		31.7									
F33							30.9					
F34		30.0							30.0			
F35				29.0								
F36								28.0				
F37						26.0						
F38												

Legend: extraction performed right after snake arrival (A), and on the 30th (B) and 60th days (C).

Table 6B. Determination of molecular weight of proteins from *Crotalus durissus terrificus* venom by PAGE-SDS. Values in kDa. Samples collected and stored between November 1997 and November 1998 (columns 1 to 3) and samples collected between June and October 1999 (other columns).

Fractions	581A	581B	582A	582B	582C	583A	583B	583C	584A	584B	585	586A	586B
F1		123.9								123.9	123.9	123.9	123.9
F2													
F3													
F4													
F5			116.0						116.0				
F6				114.9	114.9								
F6							112.8	112.8					
F8	111.8												
F9													
F10										95.0	95.0	95.0	95.0
F11	83.2		83.2	83.2	83.2		83.2	83.2	83.2				
F12													
F13													
F14										71.8	71.8		
F15								70.6					
F16													
F17	68.3		68.3	68.3	68.3		68.3	68.3	68.3				
F18	67.6		67.6	67.6	67.6		67.6	67.6	67.6				
F19													
F20						61.8				61.8	61.8		61.8
F21													
F22		55.4				55.4				55.4	55.4	55.4	55.4
F23	53.0	53.0	53.0	53.0	53.0	53.0	53.0	53.0	53.0	53.0	53.0		53.0
F24													
F25													
F26	50.1	50.1	50.1	50.1	50.1	50.1	50.1	50.1	50.1	50.1	50.1		
F27				48.4	48.4				48.4				
F28	44.8		44.8	44.8	44.8		44.8						
F29		36.0				36.0				36.0	36.0	36.0	36.0
F30	34.3		34.3	34.3	34.3		34.3	34.3	34.3				
F31													
F32													
F33													
F34													
F35													
F36													
F37													
F38		20.1											

Legend: extraction performed right after snake arrival (A), and on the 30th (B) and 60th days (C).

Table 7A. Analysis of the amount of each protein fraction obtained from the molecular weight determination of proteins from *Crotalus durissus terrificus* venom. Percentage. Samples collected and stored between November 1997 and November 1998 (columns 1 to 3) and samples collected between June and October 1999 (other columns).

Fractions	281	283	434	577A	577B	577C	578A	578B	579	580A	580B	580C
F1												
F2	121.2	121.2	121.2		121.2	121.2	121.2	121.2	121.2			
F3										119.0		
F4											117.5	117.5
F5												
F6												
F6												
F8												
F9	98.8	98.8	98.8	98.8	98.8	98.8	98.8	98.8	98.8			
F10												
F11												83.2
F12		79.3										
F13	77.5		77.5					77.5	77.5			
F14												
F15									70.6			
F16	69.4	69.4	69.4		69.4	69.4	69.4					
F17										68.3	68.3	68.3
F18												
F19	64.6	64.6	64.6			64.6		64.6				
F20												
F21	60.6	60.6	60.6	60.6	60.6	60.6			60.6			
F22	55.4	55.4	55.4	55.4	55.4	55.4	55.4	55.4	55.4			
F23										53.0	53.0	53.0
F24	51.8	51.8	51.8		51.8	51.8	51.8					
F25									51.0			
F26												
F27	48.4	48.4	48.4		48.4	48.4	48.4		48.4			
F28											44.8	44.8
F29												
F30										34.3	34.3	34.3
F31					32.6							
F32	31.7		31.7									
F33							30.9					
F34		30.0							30.0			
F35				29.0								
F36								28.0				
F37						26.0						
F38												

Legend: extraction performed right after snake arrival (A), and on the 30th (B) and 60th days (C).

Table 7B. Analysis of the amount of each protein fraction obtained from the molecular weight determination of proteins from *Crotalus durissus terrificus* venom. Percentage. Samples collected and stored between November 1997 and November 1998 (columns 1 to 3) and samples collected between June and October 1999 (other columns).

Fractions	581A	581B	582A	582B	582C	583A	583B	583C	584A	584B	585	586A	586B
F1		123.9								123.9	123.9	123.9	123.9
F2													
F3													
F4													
F5			116.0						116.0				
F6				114.9	114.9								
F6							112.8	112.8					
F8	111.8												
F9													
F10										95.0	95.0	95.0	95.0
F11	83.2		83.2	83.2	83.2		83.2	83.2	83.2				
F12													
F13													
F14										71.8	71.8		
F15								70.6					
F16													
F17	68.3		68.3	68.3	68.3		68.3	68.3	68.3				
F18	67.6		67.6	67.6	67.6		67.6	67.6	67.6				
F19													
F20						61.8				61.8	61.8		61.8
F21													
F22		55.4				55.4				55.4	55.4	55.4	55.4
F23	53.0	53.0	53.0	53.0	53.0	53.0	53.0	53.0	53.0	53.0	53.0		53.0
F24													
F25													
F26	50.1	50.1	50.1	50.1	50.1	50.1	50.1	50.1	50.1	50.1	50.1		
F27				48.4	48.4				48.4				
F28	44.8		44.8	44.8	44.8		44.8						
F29		36.0				36.0				36.0	36.0	36.0	36.0
F30	34.3		34.3	34.3	34.3		34.3	34.3	34.3				
F31													
F32													
F33													
F34													
F35													
F36													
F37													
F38													

Legend: extraction performed right after snake arrival (A), and on the 30th (B) and 60th days (C).

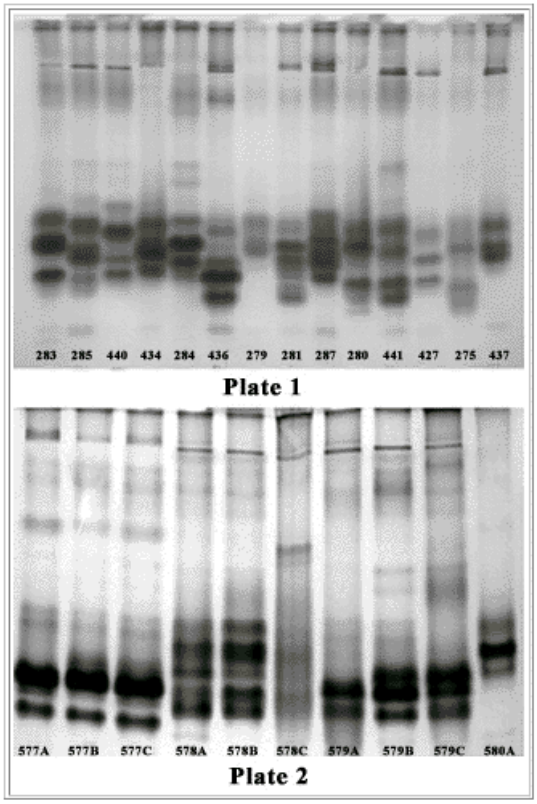

Figure 1A. Electrophoretic runs in polyacrylamide gel (PAGE) of native proteins from *Crotalus durissus terrificus* venom. Extraction performed immediately after snake arrival (A), and on the 30th (B) and 60th days (C).

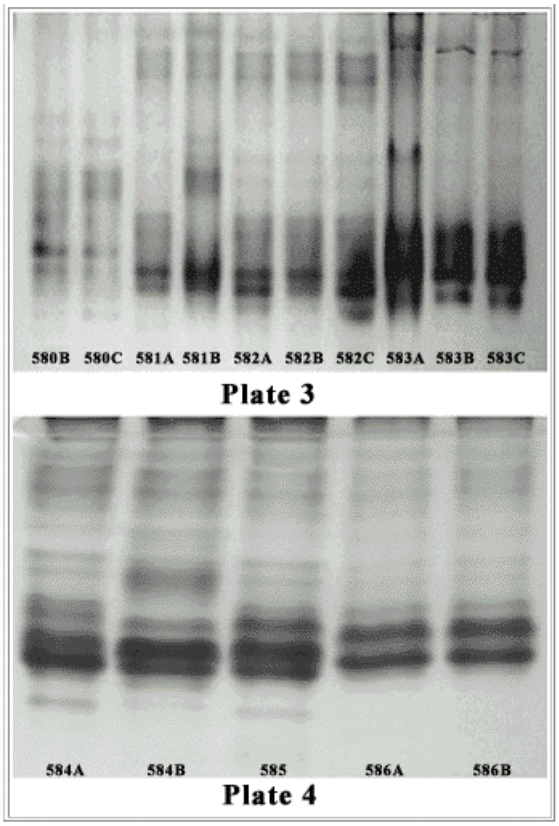

Figure 1B. Electrophoretic runs in polyacrylamide gel (PAGE) of native proteins from *Crotalus durissus terrificus* venom. Extraction performed immediately after snake arrival (A), and on the 30th (B) and 60th days (C).

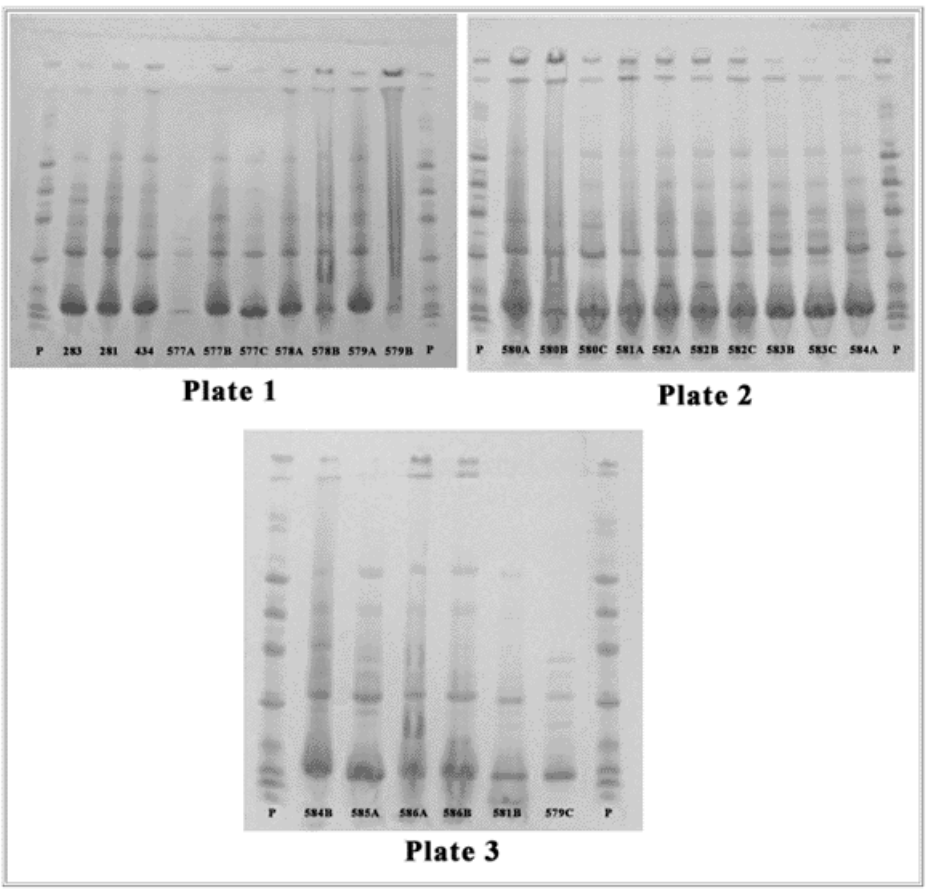

Figure 2. Electrophoretic runs of proteins from *Crotalus durissus terrificus* venom using the PhastSystem® of Pharmacia-Biotech®(PAGE-SDS). Extraction performed immediatelly after snake arrival (A), and on the 30th (B) and 60th days (C). P = molecular weight standard.

The difference between individual native protein was significant. A snake (279) showed two bands, while some others (579, 584, and 585) showed 11 bands (Tables 2, 4A, and 4B). This was also observed in relation to band molecular weight, some samples (577, 580, and 586) with 4 bands, and others (281, 283, and 434) with 10 bands (Tables 6A, and 6B).

With regard to the percentage of each protein fraction in the different venom samples, most of these fractions were shown to have great variability (Tables 3, 5A, 5B, 7A, and 7B). In the analysis of native protein, fraction F1 gave 4.77% (in relation to the total amount of protein present in the sample) for animal 287 and 23.60% for animal 285 (Table 3). This was also observed in relation to molecular weight determination. Fraction F1 ranged from 3.92% (animal 584) to 22.02% (animal 586), and F26 from 1.71% (animal 582) to 26.46% (animal 585) (Tables 7A, and 7B).

Variations were observed between the different venom extractions in relation to the number of fractions in each individual sample. Some animals showed variations in relation to the number of native protein bands. Animal 579 showed 7 bands in a sample and 11 in another sample; animal 577 showed absence of only one band in the second sample (Tables 4A, and 4B). In some animals, although the number of bands had shown little alteration, band values were not similar (animal 583) (Tables 4A, and 4B). The same was observed for the individual samples in relation to molecular weight (Tables 6A, and 6B).

Individual variations in the amount of each protein fraction were also observed on days zero, 30, and 60. Some animals did not show significant quantitative alterations in native proteins. Fraction F18 (animal 577) represented 4.98% and 4.85% of the total fractions on days zero and 60, respectively (Tables 5A, and 5B). On the other hand, other snakes showed an increase or a decrease in protein amount. In animal 578, fraction F18 represented 7.99% of the total fractions on day zero and 13.39% on day 60. In animal 582, fraction F19 showed 14.77% on day zero and 2.61% on day 60 (Tables 5A, and 5B). The same was observed in relation the molecular weight (Tables 7A, and 7B).

The results of this study showed a wide protein variation in individual venom samples of recently captured *Crotalus durissus terrificus*. These data are in agreement with other works that showed individual variation in snake venom composition (3,6,7,9,12,14,16,17,20,23,24,30).

Variation in snake venom composition, even in natural conditions, may be associated with season of the year (14), geographical origin (3,6,9,12,16,23,24), sex (20), age (17), and diet (7,30). However, Willemse (34) demonstrated that, even under experimental conditions, in which these factors were controlled, extrinsic individual variations in venom composition were also observed. This author suggested that variations resulted from intrinsic or genetic factors.

In this study, variables related to geographical origin, season, and age were not controlled. The animals donated to CEVAP/UNESP came from different regions, and the number of animals from each region was small. This did not allow a precise analysis of this variable. In relation to age, all studied animals were adult, being impossible to determine their precise age. In addition, the venom samples were collected on different days, which did not permit to control the effect of seasonality on venom composition. After housing the animals in the quarentine room, they were fed mice (*Mus musculus*). Despite receiving a controlled diet, variations in venom composition were still observed. Daltry *et al*. (7) suggested that variation in venom composition results from natural selection and not from changes in diet. Different preys vary in their susceptibility to venom. Consequently, variation in venom composition would reflect the process of natural selection between prey and predator. Natural selection may have caused different populations to produce venoms appropriate to obtain local food. The diet the animals received in captivity may not have affected their venom composition. This can be corroborated by the observation of the animals that did not eat during the quarantine period, but showed alteration in venom electrophoretic profiles.

The number of protein bands found in the venom of males and females was also compared in this study. The number of native protein bands was significantly higher in females (7.2 ± 2.1) than in males (4.9 ± 2.3). The variation in snake venom protein composition related to sex remains a controversial issue (6). While some authors (6,12,18,24,32,34) did not find any variation in venom composition in relation to sex, two authors reported a possible correlation (20,22). The results of native venom composition analysis are in agreement with the findings of these two latter authors.

However, analysis of molecular weight showed that the difference in the number of bands was not significant between males (8.5 ± 1.4) and females (7.7 ± 1.6). These data are in agreement with the results obtained by Willemse (34), Glenn and Straight (12), Schenberg (24), Latifi (18), Taborska (32), and Chippaux *et al*.(6).

The apparently paradoxical results obtained in this study can be explained by the different electrophoretic methods used in this study. The electrophoretic analysis of native protein was performed studying native venoms without any treatment that could change their physical chemical characteristics. In this method, the proteins migrated due to their liquid electric charge and their molecular weights. There was an interrelation between these two characteristics, determining the position of proteins at the end of electrophoretic run. In contrast, in studies using the PhastSystem® (Pharmacia-Biotech®) to obtain electrophoretic profile based only on the molecular weight of protein bands, venom proteins were submitted to a thermal treatment with SDS and β-Mercaptoethanol. Breaks were caused in the disulfide bridges of these proteins. Most bands obtained by this method were formed by sub-unities. Due to its detergent properties, SDS surrounding the sub-unities gave them a homogeneous electric charge density. Thus, electric charge was eliminated and the proteins migrated only due to their molecular weights. Females are thought to have different venom protein composition from the males when native venoms are compared. However, the sub-unities that compose venoms of males and females are similar.

Due to practical problems, the number of snakes used in this study was small. Then, the results obtained from sex analysis cannot be representative of this species. Further studies including a larger number of specimens may contribute to a better understanding of this issue.

Venom extractions on days zero, 30, and 60 showed difficulties. A possible explanation for small or no venom production by some snakes may be related to the proximity between diet dates and venom extraction, since snakes bite their preys to immobilize them. Absence of fangs was observed in one snake, which made the last

venom extraction impossible. Difficulties in restraining some snakes led to material losses.

In this study, a great variability was seen in *Crotalus durissus terrificus* venom in natural conditions. This fact may be important to the symptomatology of crotalid envenoming. This was also suggested by Daltry *et al*. (7) in a study involving *Calloselasma rhodostoma* snakes. Today, snake venoms have been used in the search of pharmacologically active substances that may be used in human and veterinary medicine. Some authors have shown that venoms of some snake species have analgesic (11,19) and antineoplastic properties (26,27). They have also been used as a biological glue in certain surgical procedures as a substitute for conventional suture (31). Thus, understanding the variability of venom composition may be of major importance in the research of new drugs.

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