

A novel expression profile of the *Loxosceles intermedia* spider venomous gland revealed by transcriptome analysis

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Spiders of the *Loxosceles* genus are cosmopolitan, and their venom components possess remarkable biological properties associated with their ability to act upon different molecules and receptors. Accidents with *Loxosceles intermedia* specimens are recognized as a public health problem in the south of Brazil. To describe the transcriptional profile of the *L. intermedia* venom gland, we generated a wide cDNA library, and its transcripts were functionally and structurally analyzed. After initial analyses, 1843 expressed sequence tags (ESTs) produced readable sequences that were grouped into 538 clusters, 281 of which were singletons. 985 reads (53% of total ESTs) matched to known proteins. Similarity searches showed that toxin-encoding transcripts account for 43% of the total library and comprise a great number of ESTs. The most frequent toxins were from the LiTx family, which are known for their insecticidal activity. Both phospholipase D and astacin-like metalloproteases toxins account for approximately 9% of total transcripts. Toxins components such as serine proteases, hyaluronidases and venom allergens were also found but with minor representation. Almost 10% of the ESTs encode for proteins involved in cellular processes. These data provide an important overview of the *L. intermedia* venom gland expression scenario and revealed significant differences from profiles of other spiders from the *Loxosceles* genus. Furthermore, our results also confirm that this venom constitutes an amazing source of novel compounds with potential agrochemical, industrial and pharmacological applications.

Introduction

The *Loxosceles* genus is one of four groups of spiders capable of causing human fatality.¹ They are distributed worldwide, and specifically in Brazil, only three species have been implicated in envenoming: *L. intermedia*, *L. gaucho* and *L. laeta*. The bites of brown spiders (*Loxosceles* genus) led to several clinical manifestations such as necrotic skin degeneration and gravitational spread at the bite site, renal failure and hematological disturbances.² Loxoscelism is the term used to describe the set of symptoms that commonly appear after accidents with brown spiders. The molecular and physiological mechanisms of loxoscelism are currently under investigation.

Several studies conducted over the past 30 years concerning the structural and biological roles of various venom components have shown the complex nature of these secretions.³ Likewise, the venom of *Loxosceles* spiders is a complex mixture of

protein toxins with a molecular mass profile ranging from 5 to 40 kDa.⁴

Today, it is evident that venom constituents are endowed with remarkable biological properties associated with their ability to act upon a number of specific and non-specific molecules and receptors in order to incapacitate their target organisms.³ Spider venom has received less attention than the venom produced by other animals due to their minimal impact on human health.⁵ Alternatively, spider venom constitutes an amazing putative source of novel compounds with potential agrochemical, industrial and pharmacological applications. Spiders are highly successful and one of the most diversified groups of arthropods, but only a few species have had their venom investigated.⁶

In *Loxosceles* venom, many proteins have been identified and biochemically characterized. Some of the components are not involved in the noxious activities of the venom, and others, such as members of the phospholipase D family (also known as dermonecrotic toxins) can produce deleterious effects that contribute significantly to the typical response after envenoming.⁷ Despite this accumulated knowledge, it is essential to conduct a wider study concerning the nature of venom components and their proportion in the mixture. Today, modern molecular biology techniques for DNA and RNA manipulation allow the construction of vast transcript libraries that further the

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understanding of the behavior of complex biological processes by identifying their molecular constituents. Also, a library will create an overview of the venom and may enable the discovery of new molecules of biotechnological interest.

Various studies of the repertoire of transcripts expressed in venom glands of diverse animals have been published recently.^{8–12} The majority of these studies refer to the venom glands of snakes, whereas only a few studies were conducted concerning the transcripts present in spider venom glands.^{13–16} As mentioned earlier, spider venom has become a valuable resource for the isolation of potentially useful molecules. Also, an expressed sequence tag (EST) analysis approach provides a rapid and reliable method for identifying new components as well as a resource for the analysis of the expression of known and unknown genes.

L. intermedia venom is a focus of different studies performed by our group for the last decade. Various components of the venom were identified and characterized, and some components were produced as active recombinant molecules for isolated studies and biological characterizations.^{2,7,17–22} Up to now, two proteomic analyses of *L. intermedia* venom were performed: one focused on dermonecrotic proteins²³ and one more diverse that identified 39 proteins.²⁴ Therefore, in order to expand the knowledge about *L. intermedia* venom, a cDNA library was generated from venom glands and partial sequencing of cDNAs was performed, which resulted in thousands of ESTs that were subsequently analyzed.

Results and discussion

cDNA library and EST clustering

The length of cloned cDNAs was estimated by agarose gel electrophoresis analysis of PCR amplified products. The results showed a distribution between 200 and 2300 bp, with an average of 700 bp (data not shown). After sequencing and analysis, 1843 ESTs produced readable sequences. Subsequently, all ESTs were submitted to clustering, and 538 clusters were generated, of which 281 were singletons. Clusters including two or more sequences were named “LIC” followed by the number of the cluster (LIC179–LIC438). Analyses resulted in two classes of singletons: some with similar sequences when compared to other clusters (LIS1–LIS178) and others with no similarity with other clusters (LIS179–LIS282).

Cluster identification

Resulting clusters were analyzed against the GenBank database (blastx, blastn and tblastx) and submitted to GenBank dbEST under accession numbers HO002618–HO004460. Of 538 clusters, 246 (45.7%) resulted in significant hits to known sequences. The other 292 clusters, of which 153 were singletons, were similar to proteins with no associated function (hypothetical proteins), were not matched (no hits) or matched against deposited *L. laeta* mRNA sequences that presented no similarity to database sequences (*L. laeta* cDNA). Identified sequences were classified into two main classes according to their general attribution: toxic function (toxins) and proteins involved in cellular processes.

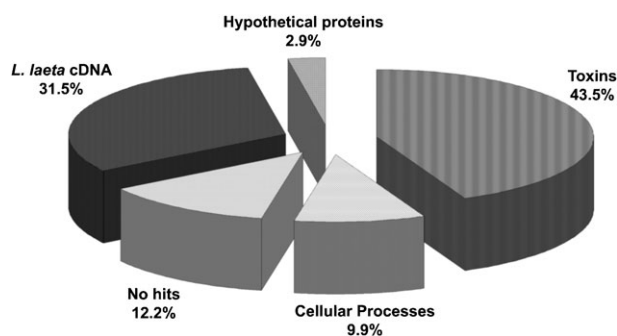


Fig. 1 The annotated *L. intermedia* venom gland transcriptome: relative proportions of toxin-encoding, hypothetical proteins, cellular processes, similar to *L. laeta* cDNA and non-significant hit ESTs.

The overview of the annotated *L. intermedia* venom gland transcriptome is presented in Fig. 1 and in more detail in Table 1. Based on data analysis, the majority of ESTs annotated as toxins are transcripts that were included in contigs (90%). These ratios are similar to other venom gland transcriptome studies^{11,13} and reflect the tissue speciality, particularly during the phase of venom replacement.

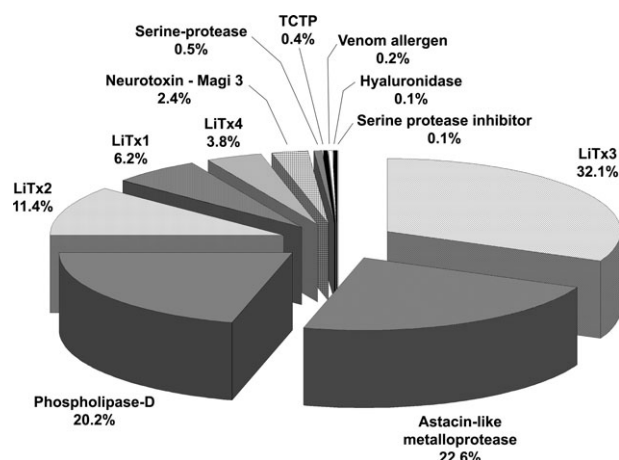
The number of transcripts with no known function is significant (47%). As accounted by Fernandes-Pedrosa,¹⁴ this number is understandable as *Loxosceles* genus sequences deposited in the public databases are still restricted. Furthermore, the complete function and components of the venom gland of *Loxosceles* are not yet fully characterized. Data also reveal that 68% of *L. intermedia* transcripts with no attributed function are similar to *L. laeta* ESTs sequences that do not match with known proteins. This ratio reveals that, as observed with already characterized proteins, unknown venom proteins produced by the two species are very similar. Furthermore, this finding demonstrates that a close evolutionary relationship exists between these species.

Transcripts assigned as toxins represent 43.4% of the total library and comprise the majority of ESTs. There are 801 toxin sequences grouped into 88 clusters and 80 singletons. The redundancy of clustering is in agreement with previous data and is due to the peculiar function of venom gland tissue in producing proteins that function in capturing prey and as a defense mechanism. Toxin clusters were grouped according to the structural and functional features of the protein: phospholipases; knottins LiTx1, 2, 3 or 4 (insecticidal peptides);²⁷ astacin-like metalloproteases; neurotoxins, and others. Fig. 2 shows the proportion of each group of toxins over the total of toxin-encoding transcripts. Table 2 describes the absolute numbers of each group of toxin-encoding messengers and their proportion among all transcriptome ESTs. A list of the putative identities of representative toxin-related clusters is provided in Table 3.

Data reveal that insecticidal peptides, structurally associated to knottins, comprise the majority of expressed toxins in *L. intermedia* venom glands. The proportion of transcripts encoding for phospholipases and astacin-like metalloproteases are next in size and are followed by lower expressed but not less important toxins, like neurotoxins and serine-proteases.

Table 1 Summary statistics after clustering of 1843 EST sequences

	No. of clusters	No. of ESTs	Percentage over total ESTs	Percentage over identified ESTs
Total no. of clusters (reads > 1)	257	1562	84.75	87
Similar to toxins	88	721	39.12	73.2
Similar to cellular processes proteins	30	136	7.38	13.8
No hits	36	135	7.32	—
Similar to <i>L. laeta</i> cDNA	97	530	28.76	—
Hypothetical proteins	6	40	2.17	—
Total no. of singletons	281	281	15.25	13
Similar to toxins	—	80	4.34	8.1
Similar to cellular processes proteins	—	48	2.60	4.9
No hits	—	89	4.83	—
Similar to <i>L. laeta</i> cDNA	—	51	2.78	—
Hypothetical proteins	—	13	0.70	—
Total	538	1843	100	100

**Fig. 2** Relative proportions of each group of toxins over the total toxin-encoding transcripts.

Knottins—insecticidal peptides

Insecticidal peptides isoforms LiTx1, LiTx2 and LiTx3 were initially described in the venom of *L. intermedia* and functionally characterized by de Castro.²⁷ The sequence of a new isoform of this class, LiTx4, was deposited in 2006 under the

accession numbers DQ388598 (nucleotide) and Q27Q53 (amino acid).

Briefly, after submitting *L. intermedia* venom to various chromatography steps, the authors isolated three peptides with insecticidal activity, which were then sequenced by the Edman degradation method. Based on the peptide sequences, degenerated probes were used to screen a cDNA library of *L. intermedia* venom glands and positive clones had their plasmids sequenced. Similarity analysis revealed three isoforms of insecticidal peptides: LiTx1, LiTx2 and LiTx3. These three peptides, and the fourth isoform later described, are small disulfide-rich proteins that have a “disulfide through disulfide knot” that structurally defines the peptides as knottins.^{27,28}

The biologically characterized components are polypeptides with molecular weights ranging from 5.6 to 7.9 kDa, and they presented insecticidal activity against highly destructive pests such as *Spodoptera frugiperda* and *Spodoptera cosmioides*. Sequence similarity analyses indicate that these molecules act on specific ion channels.²⁷

The LiTx1 peptide has a calculated molecular weight of 7431.63 Da and may present *N*-myristoylation, protein kinase C phosphorylation, amidation and casein kinase II phosphorylation sites. Based on sequence similarity, it was not possible to

Table 2 Putative toxin transcripts classes identified in *L. intermedia* venom gland transcriptome

Toxins	No. of clusters	No. of ESTs	% of total
LiTx3 ^a	70	257	13.9
Astacin-like metalloprotease	39	181	9.8
Phospholipase D	27	162	8.8
LiTx2 ^a	1	91	4.9
LiTx1 ^a	11	50	2.7
LiTx4 ^a	5	30	1.6
Neurotoxin ^b	9	19	1
Serine proteinase	2	4	0.3
TCTP ^c	1	3	0.2
Venom allergen	1	2	0.1
Hyaluronidase	1	1	0.05
Serine proteinase inhibitor	1	1	0.05
Total	168	801	43.4

^a LiTx—Knottins/Similar to insecticidal toxins of *L. intermedia*. ^b Neurotoxin—Similar to Neurotoxin Magi 3.⁴⁶ ^c TCTP—Similar to Translationally controlled tumor protein.

Table 3 Catalogue of most representative toxin-encoding transcripts from *L. intermedia* cDNA library

Cluster	No. of ESTs	Bit score (e-value)	BLAST annotation
<i>Knottin/Insecticidal toxin</i>			
<i>LiTx3</i>			
LIC341	4	730 (0.0)	gb AY681977.1 Loxosceles intermedia toxin 3 mRNA, complete cds
LIC430	29	739 (0.0)	gb AY681977.1 Loxosceles intermedia toxin 3 mRNA, complete cds
LIC402	10	243 (2e-60)	gb AY681977.1 Loxosceles intermedia toxin 3 mRNA, complete cds
LIC431	30	211 (5e-51)	gb AY681977.1 Loxosceles intermedia toxin 3 mRNA, complete cds
LIC298	3	69.7 (1e-10)	sp Q6B4T3.1 TX3_LOXIN Toxin 3 (LiTx3) precursor
LIC257	2	73.7 (6e-12)	sp Q6B4T3.1 TX3_LOXIN Toxin 3 (LiTx3) precursor
LIS161	1	68.6 (8e-10)	sp Q6B4T3.1 TX3_LOXIN Toxin 3 (LiTx3) precursor
<i>Phospholipase D</i>			
LIC434	62	2074 (0.0)	gb DQ218155.1 Loxosceles intermedia dermonecrotic toxin isoform 1 mRNA, complete cds
LIC419	15	1978 (0.0)	gb EF535250.1 Loxosceles intermedia loxtox i1 mRNA, complete cds
LIC420	16	1757 (0.0)	gb DQ267927.1 Loxosceles intermedia dermonecrotic toxin isoform 3 mRNA, complete cds
LIC395	9	2241 (0.0)	gb DQ431848.1 Loxosceles intermedia dermonecrotic toxin isoform 4 mRNA, complete cds
LIC336	4	1877 (0.0)	gb DQ431849.1 Loxosceles intermedia dermonecrotic toxin isoform 5 mRNA, complete cds
LIC182	2	765 (0.0)	gb EF474482.1 Loxosceles intermedia dermonecrotic toxin isoform 6 mRNA, complete cds
LIC365	5	1624 (0.0)	gb EF535254.1 Loxosceles intermedia loxtox i5 mRNA, complete cds
LIC423	17	1092 (0.0)	gb FJ171452.1 Loxosceles hirsuta isolate alphaIV2 sphingomyelinase D-like mRNA, partial cds
LIC316	3	365 (5e-99)	gb ACN49022.1 sphingomyelinase D-like protein [Sicarius albospinosus]
LIC203	2	184 (9e-45)	gb ABD91847.1 dermonecrotic toxin isoform 5 [Loxosceles intermedia]
LIS80	1	64.3 (1e-18)	gb AAM21156.1 sphingomyelinase-like protein [Loxosceles laeta]
LIS279	1	265 (2e-69)	sp Q8I914.1 SMD1_LOXLA Sphingomyelin phosphodiesterase D 1 (SMD1) precursor
<i>Astacin-like metalloprotease</i>			
LIC359	5	1580 (0.0)	gb EF028089.1 Loxosceles intermedia astacin-like metalloprotease toxin precursor, mRNA, complete cds
LIC412	12	1463 (0.0)	gb GQ227490.1 Loxosceles intermedia astacin-like metalloprotease toxin 2 precursor, mRNA, complete cds
LIC415	14	1559 (0.0)	gb GQ227491.1 Loxosceles intermedia astacin-like metalloprotease toxin 3 precursor, mRNA, complete cds
LIC369	6	628 (1e-176)	gb GQ227491.1 Loxosceles intermedia astacin-like metalloprotease toxin 3 precursor, mRNA, complete cds
<i>Knottin/Insecticidal toxin</i>			
<i>LiTx2</i>			
LIC436	91	809 (0.0)	gb AY681976.1 Loxosceles intermedia toxin 2 mRNA, complete cds
<i>Knottin/Insecticidal toxin</i>			
<i>LiTx1</i>			
LIC425	18	780 (0.0)	gb AY681975.1 Loxosceles intermedia toxin 1 mRNA, complete cds
LIC385	7	612 (1e-171)	gb AY681975.1 Loxosceles intermedia toxin 1 mRNA, complete cds
<i>Knottin/Insecticidal toxin</i>			
<i>LiTx4</i>			
LIC427	20	580 (4e-162)	gb DQ388598.1 Loxosceles intermedia toxin 4 mRNA, partial cds
<i>Neurotoxin</i>			
LIC327	4	294 (9e-76)	gb EY188583.1 LLAE0211C Loxosceles laeta cDNA 5' similar to Neurotoxin magi-3, mRNA sequence
LIC381	6	267 (1e-67)	gb EY188583.1 LLAE0211C Loxosceles laeta cDNA 5' similar to Neurotoxin magi-3, mRNA sequence
<i>Serine-proteases</i>			
LIC305	4	60.8 (2e-07)	ref XP_001653721.1 serine protease [Aedes aegypti]
<i>TCTP</i>			
LIC317	3	253 (2e-65)	gb AAY66972.1 translationally controlled tumor protein [Ixodes scapularis]
<i>Venom allergen</i>			
LIC179	2	108 (1e-21)	gb ABX75373.1 venom allergen [Lycosa singoriensis]
<i>Hyaluronidase</i>			
LIS222	1	142 (2e-32)	gb EDL77243.1 hyaluronoglucosaminidase 1 [Rattus norvegicus]
<i>Serine-protease inhibitor</i>			
LIS209	1	143 (2e-32)	gb AAH06776.1 Serine (or cysteine) peptidase inhibitor, clade I, member 1 [Mus musculus]

conclude if LiTx1 interacts specifically with Na⁺ or Ca²⁺ channels, like ω -toxins.²⁷ In the *L. intermedia* transcriptome, 50 sequences that grouped together in 11 clusters showed similarity with LiTx1, comprising 2.7% of total transcripts and 6% of toxin-encoding ESTs. By similarity analysis, 31

sequences appear to be LiTx1 itself. The remaining 19 ESTs annotated as LiTx1 are slightly different, with two important amino acid substitutions (data not shown). These substitutions are not located at the post-translational modification sites but still may reflect a distinct isoform of this toxin.

LiTx2 has the same possible post-translational modification regions of LiTx1, except the casein kinase II phosphorylation site. This peptide has a calculated molecular weight of 7920.11 Da, and, like LiTx1, the type of ion channel that LiTx2 interacts with is still unknown.²⁷ Almost 5% of the messengers produced by the *L. intermedia* venom gland correspond to LiTx2, and 11% among toxin-encoding transcripts are similar to this peptide. Interestingly, all 91 ESTs annotated as LiTx2 were grouped in the same cluster and appear to be LiTx2 itself. These data demonstrate with high reliability that LiTx2 is a highly conserved isoform.

The results of our study also reveal a minor expressed peptide annotated as LiTx4. This peptide has not yet been characterized by the authors who deposited the sequence at GenBank (DQ388598). However, there is an important similarity between LiTx2 and LiTx4 sequences (77%) that leads us to conclude that they are structurally and functionally related. It is possible to hypothesize that LiTx4 is also a member of the family of knottins and most likely possesses an ability to interact with ion channels. In the *L. intermedia* transcriptome, 30 transcripts annotated as LiTx4 were grouped in 5 clusters, and, like LiTx2, LiTx4 does not have sequence variability. The current sequence deposited at GenBank (DQ388598) is a partial coding sequence. The sequences clustered by similarity generated a consensus sequence that apparently completes the previous sequence of LiTx4 (data not shown); however, it is important to state that we are dealing with ESTs that are known to be low confidence sequences. Therefore, a more accurate analysis must be done to ensure this information.

The last insecticidal peptide characterized by de Castro²⁷ was named LiTx3. This component has a calculated molecular weight of 5648.49 Da and some post-translationally modified regions: *N*-myristoylation and protein kinase C phosphorylation sites. Based on sequence similarity analyses, the authors hypothesized that LiTx3 may interact with Na⁺ channels. Structurally, LiTx3 can also be defined as a member of the knottin family. Different to the other three characterized peptides, LiTx3 presented significant similarity with metallothioneins, which are a family of small cysteine-rich proteins with the ability to bind heavy metals. According to de Castro,²⁷ metallothioneins in spider venom may act to regulate the toxicity of the venom because several toxic activities of venom proteins depend on divalent metals.

According to our data, transcripts annotated as LiTx3 were the most abundant sequences in the *L. intermedia* transcriptome: 13.9% of all ESTs showed similarity with this peptide. 257 sequences grouped in 70 clusters presented some grade of similarity with LiTx3, comprising 32% of toxin-encoding messengers. A more accurate analysis revealed that the *L. intermedia* venom gland may produce various isoforms of this toxin. Fig. 3 displays seven possible isoforms that differ in varying degrees from LiTx3. Represented by clusters LIC341 and LIC279, 57 transcripts grouped in 12 clusters appear to be LiTx3 itself and are described in Fig. 3 as group 1. Group 2 has only one amino acid substitution compared to LiTx3 (position 29), but it is a significant replacement and may be located in an important site of the peptide; we consider this version to be an isoform. According to our data,

group 4 contains the most expressed transcripts: 74 sequences (clustered in 12 series) formed this group, while group 1 (LiTx3 itself) comprises 57 sequences. Groups 5, 6 and 7 contain sequences with more amino acid substitutions that modify the post-translational modification sites of LiTx3, and the sequences belonging to these groups are less numerous. Nevertheless, cysteine residues are highly conserved in all displayed sequences, as indicated by the asterisks in Fig. 3. The function of these molecules is not well understood; therefore, we cannot hypothesize how these differences in the primary structure of these peptides can modify the conformation and subsequently their activity and affinity. However, the number of isoforms certainly explains the abundance of transcripts annotated as the LiTx3 peptide.

Our data reveal a prevalence of peptides with insecticidal activities among the transcripts produced by the *L. intermedia* venom gland. The electrophoretic protein profile of *L. intermedia* venom (Fig. 4) agrees with this aspect of the transcriptome, as the intensity of bands located at the expected size of the peptides are clearly superior to the intensity of other bands (5–8 kDa). Also, the abundance of these neurotoxic components is not surprising because the primary role of brown spider venom, as in all arachnids, is to paralyze or kill envenomed prey.

Previous studies analyzed the protein electrophoretic separation of the venom of *L. intermedia* and *L. laeta*.²⁹ As demonstrated here, low molecular mass polypeptides (< 14 kDa) appear in *L. intermedia*, as well as in the *L. laeta* profile, although in a minor concentration. It is intriguing that none of the peptides described in the *L. intermedia* venom were described in the *L. laeta* venom gland transcriptome.¹⁴

Arachnids, together with cone snails, are particularly remarkable because of the incredible diversity of toxic peptides produced by their venom glands. Some types of venom contain more than 1000 unique peptides.³⁰ The knowledge about the structural and pharmacological diversity of these peptides is limited despite the enormous number of these peptides. This diversification appears to be based on subtle variations on their conformational shapes and resultant surfaces, which may be caused by single residue modifications. These variations lead not only to different selectivity but also to diverse modes of interaction between the peptides and their receptors.⁵ Here, we describe not only the high abundance of this class of molecules but also a great diversity of isoforms, confirming the tendency observed in other spider venom. However, our knowledge about these toxic peptides in *L. intermedia* venom is very limited, and this subject undoubtedly creates a lot of questions to be further investigated.

Most of these toxic peptides appear to target very specific subtypes of ion channels, receptors and transporters in the peripheral or central nervous system.³¹ This activity justifies the increasing interest regarding the potential of some venom as sources of novel compounds with potential agrochemical or pharmacological applications. For example, insect ion channels are significantly different from their vertebrate counterparts, but the development of specific pharmacological reagents for studying these channels has not occurred.³⁰ Also, the higher specificity of spider peptides for insect receptors has led to the

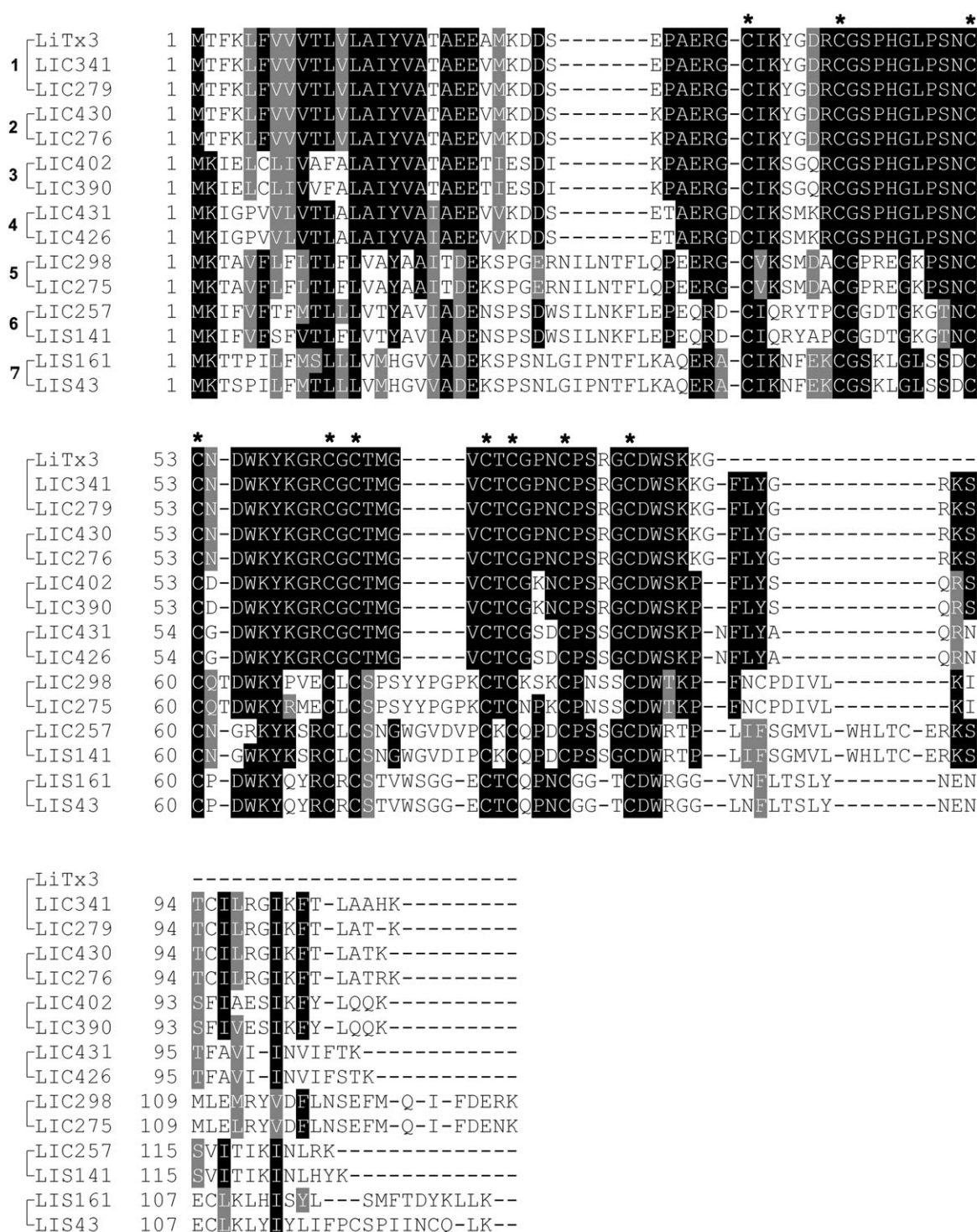


Fig. 3 Multiple alignment analysis of predicted amino acid sequences of LiTx3 and its novel presumable isoforms. Residues are numbered according to the aligned peptide sequences. Amino acid identities are shaded in black and conservative substitutions are in gray. The conserved cysteine residues are indicated by asterisks. The GenBank accession number for LiTx3 sequence is Q6B4T3.

proposal of using the peptides for defining novel insecticidal targets or for pioneering new biopesticides.³¹

Phospholipase D

Among all the toxins found in *Loxosceles* spider venom, dermonecrotic toxin is undoubtedly the most investigated

and characterized component. A dermonecrotic toxin is able to reproduce the major biological effects triggered by whole venom such as dermonecrosis, massive inflammatory response, lysis of red blood cells, acute renal failure, among other effects. Currently, the term phospholipase D (PLD) is more accurate than sphingomyelinase D, having a broader spectrum

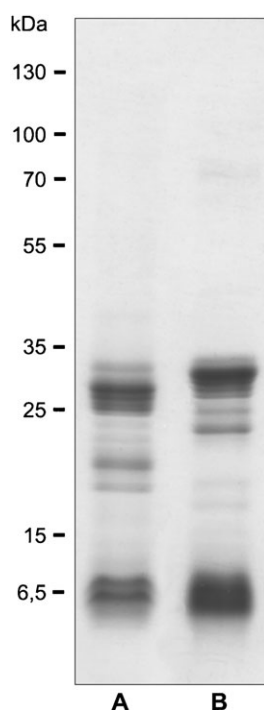


Fig. 4 SDS-polyacrylamide gel electrophoretic separation of *L. intermedia* venom. (A) Non-reduced sample. (B) Reduced sample. Numbers on the left correspond to positions of molecular weight markers.

of targets as it hydrolyzes sphingophospholipids and lysoglycerophospholipids.³²

Our work revealed that 9% of all analyzed transcripts correspond to phospholipase D, comprising 162 sequences grouped in 27 clusters. These phospholipase D messenger RNAs are 20.2% of all toxin-encoding ESTs, which is a very significant proportion of the majority of toxins.

Dermonecrotic toxin comprises a family of toxins with different related isoforms that have biological, amino acid and immunological similarities, and which are found in diverse *Loxosceles* species. Various isoforms of phospholipase D were already reported for different species.³³ For example, Machado and colleagues²³ described eleven isoforms of phospholipase D toxins in the venom of *L. gaucho*. Many isoforms of dermonecrotic toxin were also characterized in the venom of other species such as *L. reclusa* and *L. laeta*.^{34,35} In *L. intermedia* venom, several isoforms were described, and nine isoforms were already expressed as recombinant proteins.^{33,34–36}

In light of this, we performed a more accurate analysis of phospholipase D sequences to study the profile of the different isoforms of this molecule in the *L. intermedia* venom gland. Gunning and colleagues³⁷ consider protein isoforms to be highly similar at the level of amino acids sequence and to perform highly related functions. Functional diversity in higher organisms is due to the description of several numbers isoforms families. In addition, the presence of protein families that retain sufficient similarity among members may compensate for the elimination of one isoform. A great number of isoforms can be created by single nucleotide polymorphisms.^{38,39} In the case of the *Loxosceles* genus phospholipase D isoforms, it has

been proposed that multiple paralogous genes and alleles may explain this phenomenon. For example, the cDNA sequences of the LiRecDT2, Loxtox i1 and i3 isoforms are identical, except for a few nucleotides located in the 3' untranslated region (3'UTR) of the mRNA, and are, therefore, most likely, alleles.³⁶ Based on a phylogenetic analysis of putative mature PLD toxins, Kalapothakis and colleagues³⁶ proposed six possible major groups of this family of toxins in *L. intermedia* venom. Herein, we employed these categories to describe the profile of PLD isoforms expression in the *L. intermedia* venom gland.

Our results confirmed the presence of dermonecrotic toxin variants (Table 4). 69 clones were characterized as LiRecDT1 itself,¹⁸ LiD1,⁴⁰ Loxtox i4³⁶ or P1,³⁵ proteins that share 99% of identity and were placed in the Loxtox group 2. These sequences add up to 41.3% of phospholipase D-encoding transcripts of the *L. intermedia* venom gland, which may explain why this phospholipase D isoform was the first to be described in *L. intermedia* venom.

Transcripts annotated as Loxtox groups 1 and 6 also presented an important proportion among phospholipase D-encoding ESTs: 14.2%. Loxtox group 6 includes LiRecDT3, which does not show dermonecrotic activity and has low sphingomyelinase activity.⁴¹ This toxin has a different profile concerning its secondary structure when compared to other dermonecrotic phospholipase D proteins, which may explain its different biological and biochemical behavior. Nevertheless, different isoforms of the same toxins can account for the natural properties of whole venom and its activities.²¹

All other Loxtox groups were identified among *L. intermedia* venom gland transcripts, each one in its particular proportion. Dermonecrotic LiRecDT6 toxin was recently identified and characterized by Appel²² and was also recognized among messengers (Table 4).

Five clusters annotated as phospholipase D proteins were also submitted to similarity analyses against all characterized *L. intermedia* phospholipase D molecules. However, despite having the amino acid residues typical of the catalytic domain of phospholipase D toxins, these sequences exhibit significant differences in the remaining residues. For example, LIC423 and LIS9 clusters could not be identified as any of the characterized *L. intermedia* phospholipase D toxins because the highest similarity level—observed with LiRecDT6—was 70%. These two clusters represent approximately 11% of all phospholipase D transcripts and may be considered as an important new isoform of this family of toxins produced by *L. intermedia*. Other clusters (LIC316, LIC203, LIS80 and LIS279) have the same profile and may also be different isoforms of phospholipase D not yet characterized (Table 4).

A minor proportion of phospholipase D-encoding transcripts were observed in our work when compared to the *L. laeta* venom gland transcriptome.¹⁴ It is already known that *L. intermedia* female venom has higher concentrations of phospholipase D toxins compared to male.⁴² *L. laeta* venom gland EST analysis was performed using only female specimens, whereas the *L. intermedia* venom gland transcriptome used specimens of both sexes. This finding may in part explain the significant difference between the proportions of the transcripts analyzed.

Table 4 Representation of phospholipase D ESTs in the venom gland of *L. intermedia*

Phospholipase-like toxins	No. of clusters	No. of ESTs	% Over total phospholipases
Loxtox group 1 ^a	4	23	14.2
Loxtox group 2 ^a	2	67	41.3
Loxtox group 3 ^a	2	9	5.6
Loxtox group 4 ^a	1	5	3.1
Loxtox group 5 ^a	1	4	2.5
Loxtox group 6 ^a	4	23	14.2
LiRecDT6 ^b	3	5	3.1
LIC423/LIS9	2	17	10.5
LIC316	1	3	1.8
LIC203	1	2	1.2
LIS80	1	1	0.6
LIS279	1	1	0.6

^a Phospholipase D protein families proposed by Kalapothakis.³⁶ ^b GenBank accession number gb|EF474482.1.

Astacin-like metalloproteases

Astacin-like molecules were first described as a component of animal venom in a study that identified, cloned and characterized a metalloprotease from the *L. intermedia* venom gland.¹⁹ This toxin was named LALP (*Loxosceles* astacin-like proteinase). Recently, Trevisan-Silva and co-workers⁴³ identified two different isoforms of this molecule in *L. intermedia* venom, designating the three isoforms LALP1 (previously named LALP), LALP2 and LALP3. This work also identified astacin-like metalloproteases in the venom of *L. laeta* and *L. gaucho*, named LALP4 and LALP5, respectively.

The astacin family of metalloproteases is formed by zinc endopeptidases. The family members are multifunctional proteases that present a consensus sequence, HEXXHXXGXXHE (zinc-binding motif) and a Met-turn (MXY), both essential elements for these molecules.⁴⁴

In our study, astacin-like metalloproteases (LALPs) encoding transcripts represent 9.8% of all ESTs, *i.e.*, 181 messengers grouped in 39 clusters. Among toxin-encoding transcripts, LALP messengers comprise more than 22% of ESTs, which is a very significant ratio.

These molecules are homologous to digestive astacin-like metalloproteases from *Astacus astacus*. This fact may indicate that LALPs possess a digestive function used to initiate the degradation of prey molecules, facilitating the posterior ingestion process.⁴³ Furthermore, LALPs in *L. intermedia* venom may play an important role in the pathogenesis observed in loxoscelism, particularly at the local hemorrhage sites and difficulties during wound healing. Likewise, these metalloproteases can also render tissue structures more permeable, facilitating other noxious toxins to spread throughout the body of victims.¹⁹ The putative functions attributed to this class of toxins legitimize the abundance of LALP-encoding transcripts in *L. intermedia* venom may.

As described earlier, three isoforms of LALPs were already described in *L. intermedia* venom. In the present work, transcripts encoding for LALP1, 2 and 3 were identified. Our data suggest that they are produced in different proportions and point to the predominance of LALP2-encoding transcripts, followed by LALP3 and then LALP1 (data not shown). These apparent proportions cannot be justified because the possible differences concerning biological activities of the three isoforms are still not elucidated.

A more accurate analysis showed that some clusters cannot be identified as any of the three characterized astacin-like metalloproteases because amino acid residues differ significantly from other LALPs residues. As shown in Fig. 5, these clusters (represented by LIC 313, LIC369 and LIC421) are more similar to LALP3, but it is possible to observe some important substitutions. For example, a phenylalanine replaces a leucine or a methionine at the third residue of the consensus sequence of astacin family members, which contains the zinc-binding motif. Interestingly the sequence of LIC421 shows significant differences from the sequences of LIC313 and LIC369, particularly in the initial and final regions. These findings support the hypothesis that two new isoforms of astacin-like metalloproteases are present in *L. intermedia* venom.

Neurotoxins similar to Magi 3

Over time, venomous animals were gradually equipped with a complex arsenal of neurotoxins that act against cellular receptors with a singular selectivity and sensibility. Among all venomous animals, spiders are poorly explored in this respect, despite their particular potential concerning the discovery of new ligands. The spiders' venom contains diverse polypeptide toxins that are highly reticulated by disulfide bridges.⁴⁵

Previously in this work, peptides that selectively bind to ion channels were described as components of *L. intermedia* venom (LiTx peptides). However, our data revealed that some transcripts present some grade of similarity with another class of ion channel binding peptides. In total, 19 ESTs grouped in 9 clusters were annotated as similar to Neurotoxin Magi 3. In 2003, this toxin was isolated from the venom of the *Macrothele gigas* spider by Corzo and colleagues.⁴⁶ They characterized Magi 3 as a peptide with a molecular mass of 5222.8 Da that shows specific inhibitory effects on site 3 of the insect sodium channel. This property is associated with its ability to cause paralytic effects to insects, although it is not possible to confirm whether Magi 3 is specific only for insect sodium channels or if it can also act upon calcium channels.⁴⁶

Fig. 6 shows an alignment of representative clusters that were annotated as Neurotoxin Magi 3 and the peptide sequence itself. As observed, there is a particular region (KCVVCYCH) with amino acid residues extremely conserved among all sequences. Moreover, this analysis suggests that two

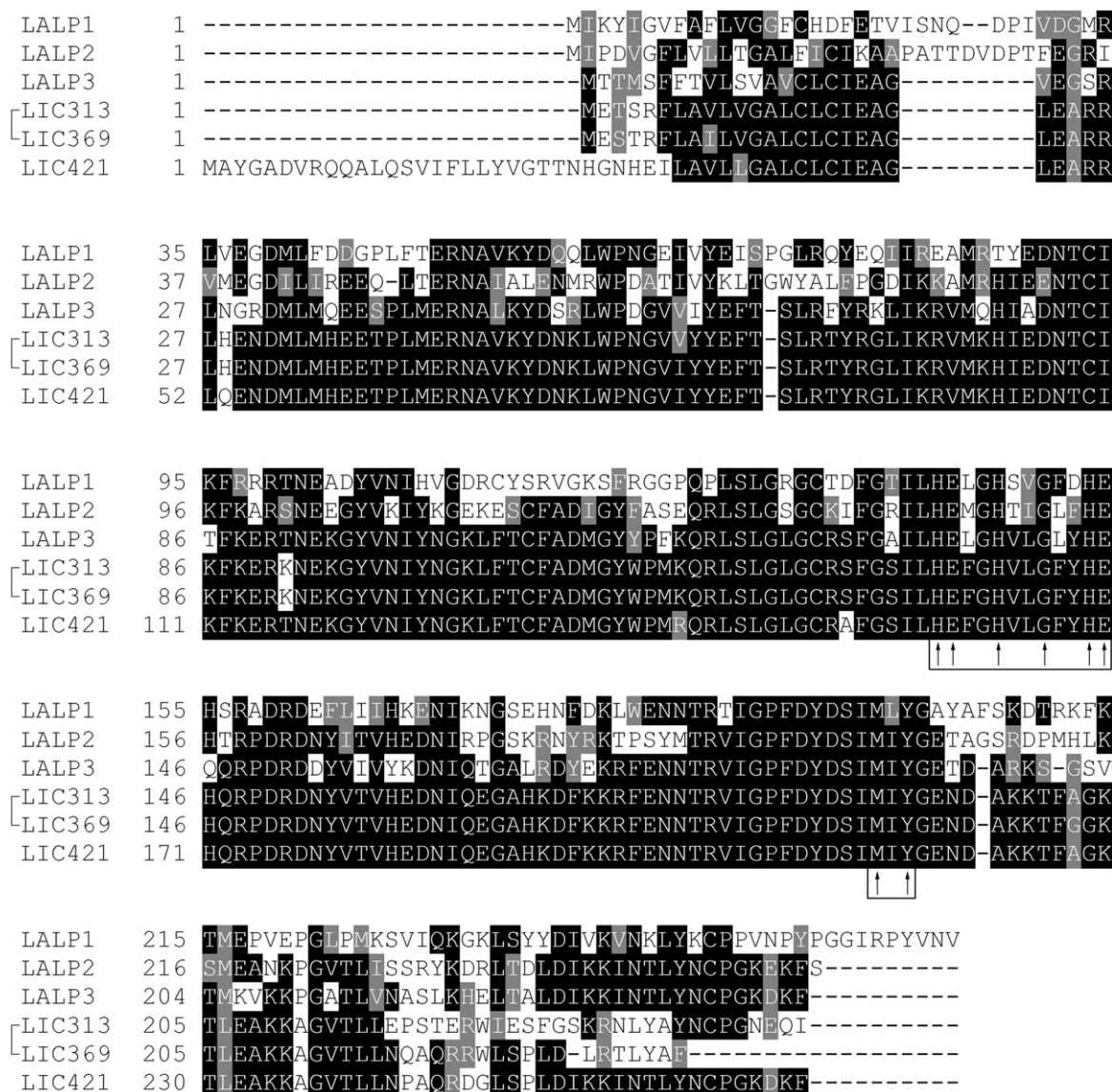


Fig. 5 Multiple alignment analysis of predicted amino acid sequences of *L. intermedia* astacin-like metalloproteases (LALPs) and the novel presumable isoforms of the toxin. Residues are numbered according to the aligned peptide sequences. Amino acid identities are shaded in black and conservative substitutions are in gray. Arrows in the box point to the astacin signature sequence and Met-turn. The GenBank accession numbers for the LALP1, 2 and 3 sequences are A0FKN6, ACV52010, ACV52011, respectively.

isoforms of these peptides may be present in *L. intermedia* venom, which increases the diversity of this class of toxins in the venom. Nevertheless, all cysteine residues present in the sequence of Neurotoxin Magi 3 are highly conserved in all *L. intermedia* sequences displayed in Fig. 6.

Magi 3, like all other *L. intermedia* peptides described here, is most likely a knottin due to the presence of various disulfide bonds in its structure.⁴⁶ Although it shares some important characteristics with the LiTx class of toxins, it is more likely that Magi 3 presents a different specificity when interacting with certain sites of cellular receptors. Thus, as observed in all spider venoms studied in this respect, we believe that *L. intermedia* venom also possesses a complex arsenal of neurotoxins that interact with different cellular targets. This diversity extends the variability of prey susceptible to the toxins, representing a great evolutionary process for a predator. Furthermore, this diversity

represents an incredibly rich source of novel molecular tools with potential pharmacological and biotechnological applications.⁴⁵

Serine proteases

Previous work from our group has already described two gelatinolytic proteases characterized as serine-type proteases.⁴⁷ This study also featured the gelatinolytic proteases as high molecular weight serine proteases (85 and 95 kDa), which are synthesized as zymogens and depend on the proteolytic cleavage of the pro-peptide domain by other proteases such as trypsin. Venom serine proteases, in addition to their contribution to the digestion of the prey, can play an important role in local tissue destruction and interfere in blood coagulation and fibrinolysis.^{47–49}

Veiga⁴⁷ noted that characterized serine proteases from *L. intermedia* venom are apparently poorly expressed. Analyses



Fig. 6 Multiple alignment analysis of predicted amino acid sequences of Neurotoxin Magi 3 (GenBank accession number P83559) and its novel presumable isoforms. Residues are numbered according to the aligned peptide sequences. Amino acid identities are shaded in black and conservative substitutions are in gray. The conserved cysteine residues are indicated by asterisks.

of the *L. intermedia* venom gland transcriptome revealed five transcripts (0.3% of total ESTs) that displayed similarity with members of the serine protease database. These messengers were grouped into two clusters (LIC321 and LIS237) with no sequence similarity among each other. LIC321 significantly aligned with a serine protease reference sequence from *Aedes aegypti*, a trypsin-like serine protease probably synthesized as an inactive precursor zymogen because a cleavage site was identified.⁵⁰ Also, LIS237 presented similarity with a serine protease sequence from the recently published *Lycosa sigoriensis* venom gland transcriptome.¹⁶

Serine protease molecules are quite diverse molecules concerning their structures, targets, functions and modes of action.⁵¹ Furthermore, as previously described, *L. intermedia* venom presented serine proteolytic activity in two high molecular weights, suggesting that two or more molecules in the venom exhibit these particular characteristics.⁴⁷ Thus, in our study, the presence of two different serine proteases corroborates these findings. The presence of different types of molecules from the same class leads to a highly efficient synergism of toxins acting in tissues of the prey or predator.

Venom allergen

Arthropod allergens are potent molecules capable of causing sensitization, and they provoke anaphylaxis. Several allergens have been described in various animals, and these molecules induce similar immune responses at the cellular level. The clinical signs vary depending on whether the allergens enter the body through mucus membranes, skin, or other ways.⁵²

Data from our work show that some messengers encode for venom allergens that are cysteine-rich molecules. These messengers are poorly expressed, and two ESTs are grouped in one cluster. They make 0.2% of toxin-encoding transcripts. These transcripts encode for allergens that showed major similarity with this class of molecules described in another spider genus (*Lycosa sigoriensis*) and with a scorpion species (*Opisthacanthus cayaporum*). Significant similarities were also found with some mite allergens (*Ixodes scapularis* and *Argas monolakensis*). A *L. intermedia* venom proteomic study have also reported the possible occurrence of an allergenic protein similar to a mite allergen.²⁴ Fernandes-Pedrosa¹⁴ described a venom allergen in the *L. laeta* venom gland transcriptome,

which presents an important similarity with the *L. intermedia* venom allergen.

Allergic symptoms such as tissue swelling, hypotension, itching and dermatitis have been reported in a few cases of incidents involving spiders of the *Loxosceles* genus.⁵³ However, venom allergens like the allergen described in this work are likely to contribute to the development of these specific responses.

We also identified transcripts (LIC207) that presented significant similarity with troponin C. These transcripts were included in the group "cellular processes genes" due to the function of troponin C in muscle contraction. However, the annotation of this messenger comprises a troponin C sequence described in the *Blattella germanica* cockroach where it was featured as an allergen with a calcium dependent IgE reactivity, named Bla g 6.0101.⁵⁴ Another transcript (LIS263) coding for a myosin light chain protein also presented significant similarity with a *B. germanica* calcium binding allergen (Bla g 8) not yet fully characterized (GI|88657350). It is known that excreted materials, cast-overs from skin-molting and dead debris are sources of allergens that can sensitize genetically predisposed individuals and elicit allergic disorders.⁵⁵ Homologous allergens may occur among all arthropods and cause co-sensitization or allergenic cross-reactivity.⁵⁴

Translationally controlled tumor protein

The translationally controlled tumor protein (TCTP) was first identified approximately 25 years ago by some groups interested in translationally regulated genes. Over time, the understanding of TCTP biological functions is a growing issue because it possesses diverse properties and its role in different processes is not yet clear.^{56,57}

The TCTP protein is highly conserved in the animal kingdom: nearly 50% of all amino acid residues are preserved. Among species from the same genus, TCTPs are completely conserved. This fact can be clearly seen when comparing the TCTP sequence found in the *L. intermedia* venom gland transcriptome with the one described in the venom gland of *L. laeta*:¹⁴ 97% of positivity is observed among predicted amino acid sequences (the low quality of ESTs should be considered to justify the subtle differences). *L. intermedia* TCTP also presented important similarities with the same protein from

other arthropods such as mites (*Ixodes scapularis*, *Amblyomma americanum*, etc.).^{58,59}

It is already known that TCTP is essential for embryonic development and cell proliferation in mice and *Drosophila*. Moreover, the protein has calcium-binding activity and is capable of stabilizing microtubules, a property that may be related to a possible role of TCTP in cell cycle control.⁵⁶

Extracellular activities of TCTP were also described,⁵⁶ and based on these data, we considered this transcript as a toxin-coding messenger. Studies demonstrate that the protein triggers histamine release in basophile leukocytes by means that may be dependent on or independent of the presence of IgE.⁵⁶ It is believed that a specific TCTP receptor may participate in the process, leading to mast cell activation. Mulenga and colleagues⁶⁰ showed that TCTP is expressed in diverse tissues from the *Dermacentor variabilis* tick, including its salivary gland. TCTP from the salivary gland was cloned and expressed as a recombinant protein and was able to release histamine from a basophilic cell lineage. *D. variabilis* TCTP is very similar to the *L. intermedia* TCTP described here. Thus, based on these data, it is possible that *L. intermedia* TCTP may also act as a histamine release factor. This hypothesis is reinforced by recent results from Paludo and colleagues⁶¹ that revealed a component present in *L. intermedia* venom related to the histaminergic activity of venom.

However, TCTP mRNAs, including the transcript expressed in the *L. intermedia* venom gland, do not code for a signal sequence. Thus, TCTP may be secreted by a non-classical secretory pathway based on exosomes or, in the case of the *Loxosceles* venom gland, via holocrine secretion.

Hyaluronidases

Hyaluronidases are a class of enzymes that degrade hyaluronic acid, which is a major component of the extracellular matrix of vertebrates. In *L. intermedia* venom, two hyaluronidases (41 and 43 kDa) were already identified and characterized as endo- β -N-acetyl-D-hexosaminidases hydrolases.²⁰ Hyaluronidase precursor-like proteins were also described in a proteomic analysis of *L. intermedia* venom.²⁴ In accidents involving spiders of *Loxosceles* genus, hyaluronidases may act as spreading factors that increase the diffusion of other toxins and the gravitational spreading of the dermonecrotic lesion.²

Hyaluronidases were also described in the venom of other spiders of the *Loxosceles* genus.^{62–64} A transcript with similarity to hyaluronoglucosaminidase 1 from *Rattus norvegicus* (gb|EDL77243.1) was found in the *L. intermedia* transcriptome. In the *L. laeta* transcriptome, only 0.1% of transcripts encode for hyaluronidases,¹⁴ a proportion that matches our results showing the very low expression level of this enzyme. This fact may explain why we were able to describe only one of the hyaluronidases previously described in *L. intermedia* venom.

The identified hyaluronidase-coding sequence exhibits a significant alignment (e-Value of $4e^{-32}$) with a hyaluronidase from *Bos Taurus* (NP_001008413.2), a known sperm adhesion molecule that binds to the pellucid zone. This property explains the role of hyaluronidases in the fertilization of eggs by mammalian sperm and suggests one of the potential

biotechnological applications of these enzymes as tools for *in vitro* fertilization. Biotechnological applications of hyaluronidases are quite diverse as exposed by Senff-Ribeiro and co-workers.⁷

Serine protease inhibitors

Serine protease inhibitors are the largest and most broadly distributed superfamily of protease inhibitors. These molecules were already described in various venomous animals such as snakes, cone snails, ticks and spiders.^{65–68} The reasons for the presence of protease inhibitors in animal venom and the physiological targets of these molecules are still not fully understood. It has been proposed that one of the putative roles of these protease inhibitors is to protect their venom protein toxins from prey proteases.⁶⁹ We identified one transcript expressed by the *L. intermedia* venom gland that presented significant similarity with serine proteases from mammals such as *Mus musculus* and *Pan troglodytes*. Important similarities were also found with a serine protease identified in the *Ambliomma americanum* tick.

Textilin-1 is a serine protease inhibitor isolated from the venom of the *Pseudonaja textilis* snake.⁷⁰ This molecule binds to and blocks the activity of serine proteases such as plasmin and trypsin. Textilin-1 is an experimental drug that is being investigated as an alternative intravenous treatment to aprotinin (Trasylol®), which has been used to reduce blood loss during cardiopulmonary surgery.⁷⁰ This fact demonstrates the biotechnological potential that venom serine proteases inhibitors present.

ESTs relevant to cellular functions

Transcripts encoding for proteins involved in cellular processes and ribosomal RNA account for almost 10% of the total sequences. This relative low proportion is due to the fact that RNA extraction was made when the venom gland was mobilized to restore its toxin content. Thus, except for certain cellular transcripts, most of the ESTs classified as housekeeping sequences were grouped in singletons or clusters with 2 or 3 sequences.

As shown in Fig. 7, the majority of housekeeping messengers were classified as membrane transporters, particularly NaPi co-transporters. This class of transporters imports a phosphate ion coupled with the internalization of a sodium ion.⁷¹ The transporters are common in epithelial cells from the human digestive tract and the kidney. Furthermore, these transporters are mainly responsible for absorption and re-absorption of the phosphate ion. Although this transporter appears to be primordial for *L. intermedia* venom gland machinery, we cannot attribute any specific function to the protein in this context, and more information is needed to underlie this hypothesis.

Transcripts related to structural functions of cells were also found to encode proteins such as actin, tubulin and histones. The motor protein myosin and troponin coding messengers were the most abundant of the class (15 and 11 ESTs, respectively), which could be due to the contraction ability of the gland in order to release the venom to the fangs.

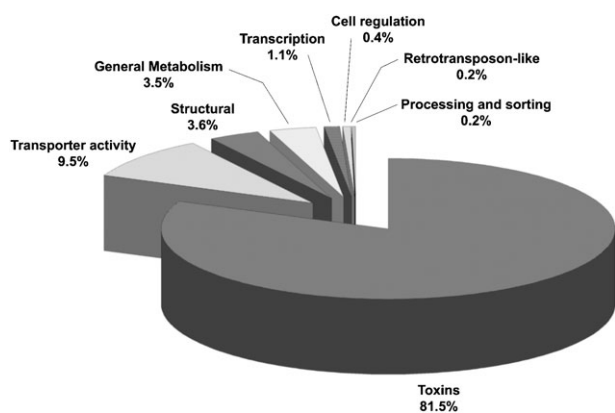


Fig. 7 Graphic showing toxin and cellular processes transcripts proportions in the *L. intermedia* venom gland.

General metabolism transcripts account for 3.5% of cellular processes encoding ESTs and include messengers such as NADH dehydrogenase and cytochrome C oxidase enzymes. ESTs related to transcription and translation functions, like ribosomal proteins, account for 1.1% of housekeeping messengers.

Sequences showing homology with retrotransposable elements were also detected by our analyses. Retrotransposable elements are mobile DNA sequences that can induce genomic rearrangements, such as gene duplication, acting as driving forces of genome evolution.⁷² It is reasonable to assume that the finding of retrotransposable elements sequences in *L. intermedia* library is due to insights which hypothesized that gene families encoding spider venom toxins are evolving *via* mechanisms of rapid duplication. Those studies also suggest that the rapid duplication rates in these gene families can be assumed based on large numbers of paralogs expressed in animal venoms.^{5,13,33,73,74}

As observed in the *L. laeta* transcriptome,¹⁴ the *L. intermedia* venom gland also produces the processing enzyme, protein disulfide isomerase. Gowd and colleagues⁷⁵ have described protein disulfide isomerase in a protein extract from the venom duct of the marine snail *Conus amadis*. Conotoxins are the major components of the mixture of peptides contained in the *Conus* venom. These molecules are short peptides containing multiple disulfide bonds and are heavily post-translationally modified. Thus, disulfide isomerases would be essential in the formation of the native state cross-links. In the same logic, the presence of disulfide isomerases in the *L. intermedia* venom gland extract might be justified, since a large number of sequences encoding for peptides containing multiple disulfide bonds were also described in this work.

Transcripts related to *L. laeta* ESTs

As described above, 68% of *L. intermedia* transcripts with no attributed function are similar to *L. laeta* ESTs sequences that also have no match with known proteins.¹⁴ Studies in cross-neutralization of lethal activity of different *Loxosceles* venom using immunized rabbit serum revealed that the anti-*L. laeta* serum was almost completely efficient against *L. intermedia* venom. Anti-*L. intermedia* serum was very efficient at neutralizing the lethal activity of *L. laeta* venom.⁷⁶ These data revealed many common antigenic components, suggesting a

similar composition of the two venoms. Then, it is not surprising that a great part of functionally characterized and non-characterized ESTs from our work are similar to the ESTs from the *L. laeta* transcriptome (data not shown).

However, important differences among *Loxosceles* spider venom were also found in some studies. Machado and co-workers,²³ by means of proteomics analysis, reported some variations in the distribution of phospholipase D isoforms focused along the isoelectric point gradient, describing a larger number of basic isoforms in *L. intermedia* venom and a rather acidic major group of isoforms in *L. laeta* venom. This study also showed that the *L. intermedia* proteome exhibited more low molecular mass proteins (14–25 kDa) than observed in *L. laeta* and *L. gaucho*.

Furthermore, studies of LD50 using mice show that *L. intermedia* venom is more lethal than the venom of *L. laeta* and *L. gaucho*, although no difference in the dermonecrotic activity was reported.²⁹ Thus, it is possible that venom components that are not involved only in dermonecrotic activity may be present, probably in higher concentrations, in *L. intermedia* venom.

Important differences among the proportions of similar transcripts or even the presence of distinct molecules were observed between the *L. intermedia* and *L. laeta* transcriptomes; therefore, these inferences are in agreement with results presented in this work.

Finally, this work enabled us to have an overview of the *L. intermedia* venom gland expression scenario but, as we generated a non-normalized cDNA library, it is likely that some rare transcripts may have escaped from our analysis. According to Bogdanova and colleagues,⁷⁷ sequencing of a complete eukaryotic transcriptome may require the analysis of about $\sim 10^8$ clones from cDNA library to identify rare sequences, whereas transcripts of medium and high abundance are sequenced several times. The same authors describe an interesting normalization procedure, named DSN-normalization. The efficiency of this method has been confirmed in many EST sequencing projects. Thus, further studies using this kind of procedure extract must be done to find rare messages present in *L. intermedia* venom gland. Indeed, our work described some low expressed messengers RNAs. This work also enabled an overview of the expression profile of *L. intermedia* venom gland, and clarified some aspects concerning the mechanisms of envenoming, toxins' function and expression pattern.

Experimental

cDNA library construction

Venom glands of 350 *L. intermedia* spider specimens (male and female) were collected 5 days after venom extraction because transcription is highly intense at this time. After dissection, venom glands were immediately frozen in dry ice and kept in a -80°C ultra low temperature (ULT) freezer until used. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and quality and quantity were checked by denaturing agarose gel electrophoresis and spectrophotometrically using the ratio of absorbances at 260 and 280 nm. mRNA was purified from total RNA using the magnetic separation kit,

PolyATtract[®] mRNA Isolation System III (Promega Corporation, Madison, WI, USA). A directional *L. intermedia* venom gland cDNA library was constructed using the Creator SMART cDNA Library Construction Kit (BD Biosciences Clontech, Mountain View, CA, USA). The first strand of cDNA was synthesized from purified mRNA, and the second strand was obtained by long distance PCR (LD-PCR), following the manufacturer's instructions. The cDNA was size fractionated *via* chromatography to avoid contaminating the library with short length sequences. Competent *Escherichia coli* DH5 α cells were transformed with the cDNA library plasmids to amplify the cDNA. Transformants were selected on LB (Luria-Bertani) agar plates containing 34 $\mu\text{g mL}^{-1}$ chloramphenicol, and more than 20 000 colonies were obtained.

DNA sequencing and bioinformatics analysis

From the resulting colonies, 2400 clones were randomly picked and placed in permanent culture plates with 96 wells and grown for 20 h in LB medium (34 $\mu\text{g mL}^{-1}$ chloramphenicol). Next, the DNA was extracted by alkaline lysis. The DNA was sequenced with MegaBace[™] 1000 DNA Analyzing Systems (Amersham Biosciences, Pittsburgh, PA, USA), with the DYEnamic ET terminator Cycle Sequencing Kit (GE Healthcare, Piscataway, NJ, EUA). The forward primer was designed to anneal in the insert flanking region of the vector pDNR-LIB (TCGACGGTACCGGACATA). Base-calling was performed using PHRED. CrossMatch software was used to process the sequences to remove the vector and *E. coli* sequences. ESTs were assembled in clusters of contiguous sequences using the CAP3 program. Processed sequences were compared to GenBank sequences using blastx, blastn (*E* values < 1e–05) and tblastx (*E* values < 1e–10) algorithms. Afterwards, ESTs were manually inspected for functional classification. Selected sequences were submitted for further alignment analyses using CLUSTAL W2 Software (<http://www.ebi.ac.uk/clustal>).²⁵ Protein isoforms are highly similar at the level of amino acid sequence and perform highly related functions; therefore, we identified potential isoforms among the toxin-coding messengers of transcriptome.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Loxosceles intermedia venom was analyzed by SDS-PAGE (8–18% acrylamide resolution gels) under reducing and non-reducing conditions using the Laemmli method.²⁶ Prior to electrophoresis, venom samples (10 μg) were mixed with sample buffer. Aprotinin from bovine lung (6511 Da) (Sigma-Aldrich, St. Louis, MO, USA) and the PageRuler[™] Plus Prestained Protein Ladder (Fermentas, Burlington, Canada) were used as molecular weight markers.

Conclusion

Different components of *Loxosceles intermedia* venom were already identified, characterized and even produced as recombinant toxins. However, it is known that this venom is a complex mixture of protein toxins and only a few of these proteins were already featured. Herein, by using the Expressed Sequence Tag strategy, it was possible to obtain an overview of the expression profile of the *L. intermedia* venom gland. Furthermore, we identified components not yet described in

the venom. Data confirmed the highly specialized nature of the spider venom gland on producing toxins and allowed a broad description of these components. Results revealed that the most abundant transcripts code for insecticidal peptides, which agree with the fact that the primary role of the venom is to paralyze or kill envenomed prey. Components with notorious activity in mammal envenoming such as phospholipase D proteins and metalloproteases were also found as highly expressed toxins. Fewer expressed, but not less important toxins were also described; they include components like serine-proteases and hyaluronidases that were already identified in *L. intermedia* venom, and they may have a putative role in envenoming. In addition, molecules not yet identified in this venom were described here, such as a serine protease inhibitor, venom allergen, certain neurotoxins and the translationally controlled tumor protein (TCTP). More accurate analyses allowed the identification of probable new isoforms of some components. Data presented here revealed significant differences from the profiles of other *Loxosceles* spiders, such as *L. laeta* and *L. gaucho*, reinforcing previous data that denoted distinct behaviors among venoms from distinct *Loxosceles* species. Finally, the results obtained and presented here are an initial approach to the characterization of promissory biotechnologically relevant agents.

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References

- 1 G. K. Isbister and J. White, *Toxicon*, 2004, **43**, 477–492.
- 2 P. H. da Silva, R. B. da Silveira, M. H. Appel, O. C. Mangili, W. Gremski and S. S. Veiga, *Toxicon*, 2004, **44**, 693–709.
- 3 P. Escoubas, L. Quinton and G. M. Nicholson, *J. Mass Spectrom.*, 2008, **43**, 279–295.
- 4 J. Kusma, O. M. Chaim, A. C. Wille, V. P. Ferrer, Y. B. Sade, L. Donatti, W. Gremski, O. C. Mangili and S. S. Veiga, *Biochimie*, 2008, **90**, 1722–1736.
- 5 P. Escoubas, *Mol. Diversity*, 2006, **10**, 545–554.
- 6 P. Escoubas and G. F. King, *Expert Rev. Proteomics*, 2009, **6**, 221–224.
- 7 A. Senff-Ribeiro, P. Henrique da Silva, O. M. Chaim, L. H. Gremski, K. S. Paludo, R. Bertoni da Silveira, W. Gremski, O. C. Mangili and S. S. Veiga, *Biotechnol. Adv.*, 2008, **26**, 210–218.
- 8 M. Neiva, F. B. Arraes, J. V. de Souza, G. Radis-Baptista, A. R. Prieto da Silva, M. E. Walter, M. Brígido Mde, T. Yamane, J. L. Lopez-Lozano and S. Astolfi-Filho, *Toxicon*, 2009, **53**, 427–436.
- 9 J. Boldrini-França, R. S. Rodrigues, F. P. Fonseca, D. L. Menaldo, F. B. Ferreira, F. Henrique-Silva, A. M. Soares, A. Hamaguchi, V. M. Rodrigues, A. R. Otaviano and M. I. Homs-Brandeburgo, *Biochimie*, 2009, **91**, 586–595.
- 10 C. M. Whittington, J. M. Koh, W. C. Warren, A. T. Papenfuss, A. M. Torres, P. W. Kuchel and K. Belov, *J. Proteomics*, 2009, **72**, 155–164.
- 11 S. Pahari, S. P. Mackessy and R. M. Kini, *BMC Mol. Biol.*, 2007, **8**, 115.
- 12 E. F. Schwartz, E. Diego-Garcia, R. C. Rodriguez de la Vega and L. D. Possani, *BMC Genomics*, 2007, **8**, 119.
- 13 L. Jiang, L. Peng, J. Chen, Y. Zhang, X. Xiong and S. Liang, *Toxicon*, 2008, **51**, 1479–1489.
- 14 F. M. de Fernandes-Pedrosa, I. de L. M. Junqueira-de-Azevedo, R. M. Gonçalves-de-Andrade, L. S. Kobashi, D. D. Almeida, P. L. Ho and D. V. Tambourgi, *BMC Genomics*, 2008, **9**, 279.

- 15 J. Chen, L. Zhao, L. Jiang, E. Meng, Y. Zhang, X. Xiong and S. Liang, *Toxicol.*, 2008, **52**, 794–806.
- 16 Y. Zhang, J. Chen, X. Tang, F. Wang, L. Jiang, X. Xiong, M. Wang, M. Rong, Z. Liu and S. Liang, *Zoology (Jena)*, 2010, **113**, 10–18.
- 17 L. Feitosa, W. Gremski, S. S. Veiga, M. C. Elias, E. Graner, O. C. Mangili and R. R. Brentani, *Toxicol.*, 1998, **36**, 1039–1051.
- 18 O. M. Chaim, Y. B. Sade, R. B. da Silveira, L. Toma, E. Kalapothakis, C. Chavez-Olortegui, O. C. Mangili, W. Gremski, C. P. von Dietrich, H. B. Nader and S. Sanches Veiga, *Toxicol. Appl. Pharmacol.*, 2006, **211**, 64–77.
- 19 R. B. da Silveira, A. C. Wille, O. M. Chaim, M. H. Appel, D. T. Silva, C. R. Franco, L. Toma, O. C. Mangili, W. Gremski, C. P. Dietrich, H. B. Nader and S. S. Veiga, *Biochem. J.*, 2007, **406**, 355–363.
- 20 R. B. da Silveira, O. M. Chaim, O. C. Mangili, W. Gremski, C. P. Dietrich, H. B. Nader and S. S. Veiga, *Toxicol.*, 2007, **49**, 758–768.
- 21 R. O. Ribeiro, O. M. Chaim, R. B. da Silveira, L. H. Gremski, Y. B. Sade, K. S. Paludo, A. Senff-Ribeiro, J. de Moura, C. Chavez-Olortegui, W. Gremski, H. B. Nader and S. S. Veiga, *Toxicol.*, 2007, **50**, 1162–1174.
- 22 M. H. Appel, R. B. da Silveira, O. M. Chaim, K. S. Paludo, D. T. Silva, D. M. Chaves, P. H. da Silva, O. C. Mangili, A. Senff-Ribeiro, W. Gremski, H. B. Nader and S. S. Veiga, *Biochim. Biophys. Acta, Gen. Subj.*, 2008, **1780**, 167–178.
- 23 L. F. Machado, S. Laugesen, E. D. Botelho, C. A. Ricart, W. Fontes, K. C. Barbaro, P. Roepstorff and M. V. Sousa, *Proteomics*, 2005, **5**, 2167–2176.
- 24 L. D. dos Santos, N. B. Dias, J. R. A. S. Pinto and M. S. Palma, *Protein Pept. Lett.*, 2009, **16**, 933–943.
- 25 J. D. Thompson, D. G. Higgins and T. J. Gibson, *Nucleic Acids Res.*, 1994, **22**, 4673–4680.
- 26 U. K. Laemmli, *Nature*, 1970, **227**, 680–685.
- 27 C. S. de Castro, F. G. Silvestre, S. C. Araujo, M. Y. Gabriel de, O. C. Mangili, I. Cruz, C. Chavez-Olortegui and E. Kalapothakis, *Toxicol.*, 2004, **44**, 273–280.
- 28 S. Cheek, S. S. Krishna and N. V. Grishin, *J. Mol. Biol.*, 2006, **359**, 215–237.
- 29 K. C. Barbaro, M. L. Ferreira, D. F. Cardoso, V. R. Eickstedt and I. Mota, *Braz. J. Med. Biol. Res.*, 1996, **29**, 1491–1497.
- 30 G. F. King, P. Escoubas and G. M. Nicholson, *Channels (Austin)*, 2008, **2**, 110–116.
- 31 G. Estrada, E. Villegas and G. Corzo, *Nat. Prod. Rep.*, 2007, **24**, 145–161.
- 32 D. Chaves-Moreira, O. M. Chaim, Y. B. Sade, K. S. Paludo, L. H. Gremski, L. Donatti, J. de Moura, O. C. Mangili, W. Gremski, R. B. da Silveira, A. Senff-Ribeiro and S. S. Veiga, *J. Cell. Biochem.*, 2009, **107**, 655–666.
- 33 G. J. Binford, M. R. Bodner, M. H. Cordes, K. L. Baldwin, M. R. Rynerson, S. N. Burns and P. A. Zobel-Thropp, *Mol. Biol. Evol.*, 2009, **26**, 547–566.
- 34 B. Ramos-Cerrillo, A. Olvera, G. V. Odell, F. Zamudio, J. Paniagua-Solis, A. Alagon and R. P. Stock, *Toxicol.*, 2004, **44**, 507–514.
- 35 D. V. Tambourgi, F. F. P. M. de, C. W. van den Berg, R. M. Goncalves-de-Andrade, M. Ferracini, D. Paixao-Cavalcante, B. P. Morgan and N. K. Rushmere, *Mol. Immunol.*, 2004, **41**, 831–840.
- 36 E. Kalapothakis, M. Chatzaki, H. Goncalves-Dornelas, C. S. de Castro, F. G. Silvestre, F. V. Laborne, J. F. de Moura, S. S. Veiga, C. Chavez-Olortegui, C. Granier and K. C. Barbaro, *Toxicol.*, 2007, **50**, 938–946.
- 37 P. Gunning, R. Weinberger, P. Jeffrey and E. Hardeman, *Annu. Rev. Cell Dev. Biol.*, 1998, **14**, 339–372.
- 38 T. Kobayakawa, S. Yamada, A. Mizuno, Y. Ohara-Nemoto, T. T. Baba and T. K. Nemoto, *Protein J.*, 2009, **28**, 24–28.
- 39 N. Ueffing, K. K. Singh, A. Christians, C. Thorns, A. C. Feller, F. Nagl, F. Fend, S. Heikhaus, A. Marx, R. B. Zotz, J. Brade, W. A. Schulz, K. Schulze-Osthoff, I. Schmitz and C. Schwert, *Blood*, 2009, **114**, 572–579.
- 40 E. Kalapothakis, S. C. Araujo, C. S. de Castro, T. M. Mendes, M. V. Gomez, O. C. Mangili, I. C. Gubert and C. Chavez-Olortegui, *Toxicol.*, 2002, **40**, 1691–1699.
- 41 R. B. da Silveira, R. B. Pigozzo, O. M. Chaim, M. H. Appel, J. L. Dreyfuss, L. Toma, O. C. Mangili, W. Gremski, C. P. Dietrich, H. B. Nader and S. S. Veiga, *Biochimie*, 2006, **88**, 1241–1253.
- 42 K. C. de Oliveira, R. M. Goncalves de Andrade, A. L. Giusti, W. Dias da Silva and D. V. Tambourgi, *Toxicol.*, 1999, **37**, 217–221.
- 43 D. Trevisan-Silva, L. H. Gremski, O. M. Chaim, R. B. da Silveira, G. O. Meissner, O. C. Mangili, K. C. Barbaro, W. Gremski, S. S. Veiga and A. Senff-Ribeiro, *Biochimie*, 2010, **92**, 21–32.
- 44 W. Bode, F. X. Gomis-Ruth and W. Stockler, *FEBS Lett.*, 1993, **331**, 134–140.
- 45 P. Escoubas, S. Diochot and G. Corzo, *Biochimie*, 2000, **82**, 893–907.
- 46 G. Corzo, N. Gilles, H. Satake, E. Villegas, L. Dai, T. Nakajima and J. Haupt, *FEBS Lett.*, 2003, **547**, 43–50.
- 47 S. S. Veiga, R. B. da Silveira, J. L. Dreyfus, J. Haoach, A. M. Pereira, O. C. Mangili and W. Gremski, *Toxicol.*, 2000, **38**, 825–839.
- 48 R. M. Kini, *Pathophysiol. Haemostasis Thromb.*, 2005, **34**, 200–204.
- 49 S. Devaraja, S. Nagaraju, Y. H. Mahadeswaraswamy, K. S. Girish and K. Kemparaju, *Toxicol.*, 2008, **52**, 130–138.
- 50 V. Nene, J. R. Wortman, D. Lawson, B. Haas, C. Kodira, Z. J. Tu, B. Loftus, Z. Xi, K. Megy, M. Grabherr, Q. Ren, E. M. Zdobnov, N. F. Lobo, K. S. Campbell and S. E. Brown, *et al.*, *Science*, 2007, **316**, 1718–1723.
- 51 E. Di Cera, *IUBMB Life*, 2009, **61**, 510–515.
- 52 L. G. Arlian, *Annu. Rev. Entomol.*, 2002, **47**, 395–433.
- 53 U. Pippirs, H. Mehlhorn, A. S. Antal, K. W. Schulte and B. Homey, *Br. J. Dermatol.*, 2009, **161**, 208–209.
- 54 J. Hindley, S. Wunschmann, S. M. Satinover, J. A. Woodfolk, F. T. Chew, M. D. Chapman and A. Pomes, *J. Allergy Clin. Immunol.*, 2006, **117**, 1389–1395.
- 55 K. Y. Jeong, C. S. Hong and T. S. Yong, *Protein Pept. Lett.*, 2007, **14**, 934–942.
- 56 U. A. Bommer and B. J. Thiele, *Int. J. Biochem. Cell Biol.*, 2004, **36**, 379–385.
- 57 K. W. Choi and Y. C. Hsu, *Cell Adhes. Migr.*, 2007, **1**, 129–130.
- 58 J. M. Ribeiro, F. Alarcon-Chaidez, I. M. Francischetti, B. J. Mans, T. N. Mather, J. G. Valenzuela and S. K. Wikel, *Insect Biochem. Mol. Biol.*, 2006, **36**, 111–129.
- 59 A. Mulenga and A. F. Azad, *Exp. Appl. Acarol.*, 2005, **37**, 215–229.
- 60 A. Mulenga, K. R. Macaluso, J. A. Simser and A. F. Azad, *Insect Biochem. Mol. Biol.*, 2003, **33**, 911–919.
- 61 K. S. Paludo, S. M. Biscaia, O. M. Chaim, M. F. Otuki, K. Naliwaiko, P. A. Dombrowski, C. R. Franco and S. S. Veiga, *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.*, 2009, **149**, 323–333.
- 62 R. P. Wright, K. D. Elgert, B. J. Campbell and J. T. Barrett, *Arch. Biochem. Biophys.*, 1973, **159**, 415–426.
- 63 A. R. Young and S. J. Pincus, *Toxicol.*, 2001, **39**, 391–400.
- 64 K. C. Barbaro, I. Knysak, R. Martins, C. Hogan and K. Winkel, *Toxicol.*, 2005, **45**, 489–499.
- 65 D. J. Strydom, *Nat. New Biol.*, 1973, **243**, 88–89.
- 66 C. Y. Dy, P. Buczek, J. S. Imperial, G. Bulaj and M. P. Horvath, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2006, **62**, 980–990.
- 67 A. Mulenga, R. Khumthong and M. A. Blandon, *J. Exp. Biol.*, 2007, **210**, 3188–3198.
- 68 C. H. Yuan, Q. Y. He, K. Peng, J. B. Diao, L. P. Jiang, X. Tang and S. P. Liang, *PLoS One*, 2008, **3**, e3414.
- 69 V. Župunski, D. Kordis and F. Gubensek, *FEBS Lett.*, 2003, **547**, 131–136.
- 70 E. K. Millers, M. Trabi, P. P. Masci, M. F. Lavin, J. de Jersey and L. W. Guddat, *FEBS J.*, 2009, **276**, 3163–3175.
- 71 I. C. Forster, N. Hernando, J. Biber and H. Murer, *Kidney Int.*, 2006, **70**, 1548–1559.
- 72 A. Böhne, F. Brunet, D. Galiana-Arnoux, C. Schultheis and J. N. Volff, *Chromosome Res.*, 2008, **16**, 203–215.
- 73 G. F. King, H. W. Teford and F. Maggio, *J. Toxicol., Toxin Rev.*, 2002, **21**, 359–389.
- 74 B. L. Sollod, D. Wilson, O. Zhaxybayeva, J. P. Gogarten, R. Drinkwater and G. F. King, *Peptides*, 2005, **26**, 131–139.
- 75 K. H. Gowd, K. S. Krishnan and P. Balaram, *Mol. Biosyst.*, 2007, **3**, 554–566.
- 76 K. C. Barbaro, V. R. Eickstedt and I. Mota, *Toxicol.*, 1994, **32**, 113–120.
- 77 E. A. Bogdanova, D. A. Shagin and S. A. Lukyanov, *Mol. Biosyst.*, 2008, **4**, 205–212.