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Stinging caterpillars from the genera *Podalia*, *Leucanella* and *Lonomia* in Misiones, Argentina: A preliminary comparative approach to understand their toxicity

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ABSTRACT

Dermal contact with Lepidoptera specimens at their larval stage (caterpillar) may cause systemic and/or local envenomation. There are multiple venomous species of them in Argentina, but their overall venom composition is poorly known. Lately, several cases of envenomation have been reported in the Misiones province, Northeastern Argentina. Thus, this work aimed to compare the protein composition, and the enzymatic properties of bristle extracts from caterpillars belonging to the families Megalopygidae (*Podalia* ca. *fuscescens*) and Saturniidae (*Leucanella memusae* and *Lonomia obliqua*) - the most common causative agents of accidents in Misiones -, and additionally to test their cross-reactivity with the *L. obliqua* antivenom produced in Brazil. Saturniidae venoms exhibited striking similarity in both their electrophoretic protein profile, and antigenic cross-reactivity. All venoms degraded azocasein – with the highest proteolytic activity observed in the *P.* ca. *fuscescens* bristle extract –, and hyaluronic acid, but the latter at low levels. *Lonomia obliqua* venom exhibited the highest level of phospholipase A_2 activity. Bristle extracts from *P.* ca. *fuscescens* and *L. obliqua* both degraded human fibrin(ogen) and shortened the clotting time triggered by calcium, while *L. memusae* venom inhibited plasma coagulation. Proteins related to the coagulation disturbance were identified by mass spectrometry in all samples. Altogether, our findings show for the first time a comparative biotoxinological analysis of three genera of caterpillars with medical relevance. Moreover, this study provides relevant information about the pathophysiological mechanisms whereby these caterpillar bristle extracts can induce toxicity on human beings, and gives insight into future directions for research on them.

1. Introduction

The order Lepidoptera comprises approximately 160.000 species of moths and butterflies worldwide (Gullan and Cranston, 2008), particularly in temperate and tropical climate zones (French and Brillhart, 2015). At their larval stage, when they are also called caterpillars, some of them display stinging or hairy bristles, which are chitinous evaginations of the cuticule. Usually, accidents with caterpillars occur when the victim inadvertently leans against the bristles, penetrating into the subcutaneous tissue and allowing the release of toxins – e.g. proteolytic enzymes, histamine and other pro-inflammatory substances – from the caterpillar body into the skin (Haddad and Lastoria, 2014).

Cutaneous reactions, such as excruciating pain, edema and erythema are frequent local manifestations of caterpillar envenomation. These reactions are typically mild and self-limited, but the contact with *Lonomia* caterpillars can cause a potentially fatal hemorrhagic syndrome (Hossler, 2010). Species of medical interest in Argentina are those related to Erebidae, Notodontidae, Limacodidae, Megalopygidae and Saturniidae families (de Roodt et al., 2000; Specht et al., 2008). Caterpillars from the last two families are the most common causative

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agents of accidents in the Misiones province, Northeastern Argentina, and *Lonomia obliqua* (Saturniidae) induces the most severe cases of envenomation (Sánchez et al., 2015). Caterpillars belonging to both families can be easily differentiated by the shape of their bristles: Megalopygidae caterpillars exhibit fine setae throughout the body, whereas Saturniidae ones show setae in small pine tree format (Haddad and Lastoria, 2014).

With the exception of detailed studies of the bristle extract from Brazilian *Lonomia obliqua* (Chudzinski-Tavassi et al., 2013), other bristle extracts from common caterpillars have not been investigated yet, and therefore their properties and overall venom composition are poorly known. For this reason, we initiated a comparative study of bristle extracts of caterpillars belonging to the families Megalopygidae (*Podalia* ca. *fuscescens*[1]) and Saturniidae (*Leucanella memusae* and *Lonomia obliqua*), which have caused several cases of human envenomation in Misiones. Besides shedding light on the pathophysiological mechanisms following envenomation, we aimed to determine the extent to which individual toxin families occur in common among the venoms of these caterpillars. Furthermore, in order to support the use of a specific treatment in case of *Lonomia* envenomation in Argentina, we investigated whether the specific antivenom produced by Instituto Butantan in Brazil recognizes toxic components present in *L. obliqua* bristle extract from Argentina. On the whole, this study aims to provide relevant information concerning the venom composition and enzymatic activity of bristle extracts of caterpillars from Argentina and nearby countries, and their possible neutralization by the sole antivenom available worldwide for caterpillar envenomation, and to give insight into future directions for research on these venoms.

2. Material and methods

Living larvae of *Lonomia obliqua*, *Leucanella memusae* and *Podalia* ca. *fuscescens* caterpillars were collected in the Misiones province (authorized by the Ministry of Ecology and Natural Renewable Resources of this province, authorization number 046), transported and maintained in the Insectarium of the Instituto Nacional de Medicina Tropical (INMeT) (Argentina).

Specimens of *L. obliqua* from the Southern region in Brazil were collected and sent to Instituto Butantan to obtain the antigen for antivenom production and quality control. From each batch of *Lonomia* venom, protein content and *in vitro* biological activity are determined to establish a reference standard for the bristle extract. As part of this characterization, one sample from Santa Catarina state (produced on February 2015) was used herein to compare the *Lonomia* venom from Argentina with the one used for the production of the *Lonomia* antivenom produced by Instituto Butantan.

For this study, samples were named as: Lo A: *Lonomia obliqua* from Argentina; Lo B: *Lonomia obliqua* from Brazil; Lm: *Leucanella memusae*; and Pf: *Podalia* ca. *fuscescens*. *Lonomia* heterologous antivenom (batch 1304060, expiration date: 03-2016) was kindly donated from the Instituto Butantan.

2.1. Morphology of the scoli and spines

Caterpillar scoli and spines were studied by scanning electron microscopy (SEM). The median dorsal region of caterpillar bodies were dissected and isolated, cleaned, critical-point dried and coated with a thin layer of gold (Denton Vacuum Desk II). Preparations were examined using a JEOL 5800LV scanning electron microscope at an acceleration voltage of 15 kV.

[1] This is equivalent to *Podalia* sp. (near *P. fuscescens*).

2.2. Caterpillar venoms

Preparation of bristle extracts (venoms) was carried out by manually removing the bristles, homogenizing them in cold phosphate-buffered saline (PBS), pH 7.4, and then the suspension was centrifuged and filtered to remove insoluble material (Da Silva et al., 1996). The protein content of bristle extracts was determined by fluorometry using the Qubit 2.0 (Life Technologies, USA) and/or the bicinchoninic acid (BCA) assay (Pierce, USA), and thereafter aliquots were stored at − 20 °C until use.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

In order to evaluate and compare the protein profile of caterpillar bristle extracts, samples were electrophoresed on 12% polyacrylamide slab gels (Laemmli, 1970) under reducing (with 2-mercaptoethanol) and non-reducing (without 2-mercaptoethanol) conditions, and then silver stained (Blum et al., 1987). Each lane was loaded with 4 μg of protein.

2.4. Cross-reaction among caterpillar components

The presence of components reacting with the *Lonomia* antivenom produced by Instituto Butantan was tested by Western Blotting. Proteins separated by one-dimensional electrophoresis (see above) were transferred to 0.2-μm nitrocellulose membranes in a tank transfer system (Hoeffer mini VE, Amersham Biosciences) at 25 V for 1.5 h. Membranes were then blocked with 5% nonfat dry milk, and incubated with the *Lonomia* antivenom diluted 1:500, and subsequently with 1:10,000 peroxidase-conjugated anti-horse IgG (Sigma A9292). The reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma D5637) as reported elsewhere (Antunes et al., 2010).

In order to determine antibody titers against the *L. obliqua* bristle extracts from Argentina and Brazil, an ELISA assay was carried out as described previously (Antunes et al., 2010), except that microplates were incubated at 37 °C for 1 h with the *Lonomia* antivenom diluted 1:4,000, and subsequently with 1:10,000 peroxidase-conjugated anti-horse IgG (Sigma A9292).

2.5. Proteomic assay

Proteomic analyses were performed using bristle extracts in solution. Briefly, samples were treated with 8 M urea for 15 min at 80 °C, 100 mM dithiothreitol (DTT) for 30 min at 60 °C and then 200 mM iodoacetamide (IAA) for 30 min at room temperature, for destruction of 3D structures and reduction and alkylation of disulfide bridges. Then, the samples were hydrolyzed with 20 ng/mL ultrapure trypsin solution (Sigma T8658) diluted in 50 mM ammonium bicarbonate. The reaction was carried out at 37 °C overnight and stopped with 5% formic (FA) acid. The peptides were dried and then dissolved into 0.5% FA for LC-MS/MS analysis, in an IT-TOF mass spectrometer system (Shimadzu, Japan). Sample aliquots were injected in a C18 column (Phenomenex 2.1 × 50 mm, 300 Å) and eluted with a linear gradient of B over A, from 5 to 40% in 25 min, under a constant flow rate of 0.2 mL min^{-1}. The solvents were A = 0.5% FA in ultrapure water and B = 90% acetonitrile containing 0.5% FA in ultrapure water. Instrument control and data acquisition were performed by the LCMS Solution (Shimadzu, Japan). The MGF-converted MS2 profiles were analyzed by MS/MS ion search algorithms by PEAKS studio 7.0 for matches with known proteins sequences deposited on the public UniProt database. The MS and MS/MS tolerances were fixed as 0.1 Da (Zhang et al., 2012).

2.6. Quantitative enzyme assays

Caseinolytic activity was determined as reported previously

(Antunes et al., 2010). One unit of enzymatic activity was defined as the amount of protein that causes an increase of 0.005 units of absorbance per min at 450 nm, and the specific activity was expressed as U/mg protein. Phospholipase A_2 (PLA_2) activity was determined by the method of Antunes et al. (2010), using soybean lecithin as substrate. One unit of PLA_2 activity was defined as the amount of protein that causes a decrease of one unit of absorbance per min at 558 nm, and the specific activity was then expressed as U/mg protein. Hyaluronidase activity was assayed by a previously reported method (Sánchez et al., 2015). Specific activity was expressed as degraded hyaluronic acid/min/mg protein. All enzyme assays were performed in triplicate. Negative controls were also performed in triplicate.

2.7. Fibrin(ogen)olytic activity

Specific cleavage of fibrinogen by the bristle extracts was determined by SDS-PAGE using 12% polyacrylamide gels, as described elsewhere (Peichoto et al., 2007). For the fibrinogenolytic activity, 200 μL of 2 mg/mL human fibrinogen dissolved in 50 mM Tris-HCl buffer (pH 7.4) were incubated at 37 °C with bristle extracts (4 μg of protein). At various time intervals, aliquots were withdrawn from the digestion mixture, and then denatured and reduced by boiling for 7 min with denaturing solution (4% SDS, 20% glycerol and 20% 2-mercaptoethanol) for SDS-PAGE. For the fibrinolytic activity, fibrinogen aliquots (20 μL) were coagulated by adding bovine thrombin (5 U/mL, final concentration, Sigma) prior to the addition of the bristle extract. At various time intervals, 20 μL of denaturing solution were added to the reaction mixture.

2.8. Clotting assays

A plasma coagulation assay adapted to the SpectraMax microplate reader was used as previously described (Veiga et al., 2003). The procedure permits measurement of clot formation and to use kinetics parameters for the coagulation process. Briefly, 160-μL reactions containing 50 μL of normal citrated human plasma were incubated for 5 min with or without varied amounts of bristle extracts. Coagulation was triggered by adding $CaCl_2$ to a final concentration of 10 mM, and clot formation was monitored at 37 °C in the SpectraMax system at 650 nm.

For the extracts that demonstrated procoagulant activity in the previous assay, the minimum coagulant dose (MCD) was determined in citrated human plasma according to the method described by Gene et al. (1989). One hundred microliters of plasma, previously incubated at 37 °C, were mixed with 50 μL of several dilutions of bristle extracts in saline solution and 50 μL of 25 mM $CaCl_2$. The clotting time (CT) in seconds (s) was determined in a semi-automatic coagulation analyzer Start (Stago). The venom concentration was plotted against the CT to determine the clotting activity. The MCD was defined as the concentration of extract (in μg/mL by BCA) which induces coagulation of plasma in 60 s at 37 °C under the described conditions.

For the extracts that demonstrated non-procoagulant activity in the coagulation assay in microplate reader, their effect on the activated partial thromboplastin time (APTT) test was evaluated using the Labtest kit according to the manufacturer's instructions. Several serial dilutions of bristle extract were mixed with plasma, in a proportion of 1 volume of bristle extract to 4 volumes of plasma, and incubated at 37 °C for 5 min. Thereafter, 100 μL of plasma pre-incubated with venom were mixed with 100 μL of rabbit brain phospholipid and incubated for 2 min at 37 °C. Coagulation was initiated by adding 100 μL of 25 mM $CaCl_2$, and the CT in seconds was determined in a semi-automatic coagulation analyzer Start (Stago). The venom concentration was plotted against the CT to determine the effect on coagulation.

2.9. Statistical analyses

Where appropriate, the results were expressed as mean ± standard deviation (SD). Differences between groups were compared using one-way analysis of variance (ANOVA) followed by Dunnett's test. Statistical analyses were performed using the software GraphPad InStat, version 3.01. A value of $p < 0.05$ indicated statistical significance.

3. Results and discussion

Stinging caterpillars produce toxic secretion to protect themselves against predators. When human beings lean against caterpillar bristles accidentally, they may be envenomed by the venom that enters the human skin through the caterpillar spines. The structures of these spines and the composition of the venom vary among diverse caterpillar species, however in most cases they are not yet well known (Fernandes-Pedrosa et al., 2013).

In recent years, accidents by different species of caterpillars have become more common among people living in the Misiones province, Northeastern Argentina. Herein, we conducted a morphological study of the spines of the most frequently encountered and dangerous species involved in those accidents (Fig. 1-A), and a systematic biochemical analysis of their bristle extracts.

It is already known that *L. obliqua* caterpillars from Brazil in the 5th or 6th instar stage possess scoli with spines that bear a hollow canal which can be observed when the tip is broken off (Veiga et al., 2001; Spadacci-Morena et al., 2016). Furthermore, Spadacci-Morena et al. (2016) demonstrated that some spines possess a circular groove similar to a ring-like structure, within which the apical portion of the spine fits perfectly, and spines that lack a groove (even in the same scolus). Accordingly, we found these two types of spines in *L. obliqua* caterpillars from Argentina; however, we could only observe spines without grooves (Fig. 1-B and C) in *L. memusae*. Taking into account that accidents caused by *L. memusae* are not as severe as those by *L. obliqua*, whose envenomation is frequently characterized by systemic hemorrhage, we reinforced the hypothesis of Spadacci-Morena et al. (2016), who assumed that the spines with a groove in *L. obliqua* are associated with the venom glands that produce characteristic and unique toxic substances.

Podalia ca. *fuscescens* caterpillars exhibited structures similar to those described for *Megalopyge opercularis* (Eagleman, 2007), showing long hairs with transverse striations and spicules, and short and hollow spines (Fig. 1-B and C). The latter penetrate skin and discharge venom upon contact. Each hollow spine is connected to a venom sac at its base (Eagleman, 2007).

Because *Lonomia* is considered the most toxic caterpillar genus in South America, its venom has been largely studied, mainly in Brazil and Venezuela, where *L. obliqua* and *L. achelous* have provoked systemic hemorrhagic manifestations in humans (Arocha-Piñango et al., 2011). However, very little is known about venoms of other caterpillar species and even of *Lonomia* from other geographical regions. In this study, one-dimensional electrophoretic profiles showed many protein band similarities among the Saturniidae bristle extracts (Fig. 2-A); the most striking feature of both *Lonomia* venoms was the presence of a protein band of ~20 kDa, which is probably related to the prothrombin activator called Lopap (Chudzinski-Tavassi et al., 2013). However, the protein bands in *Podalia* bristle extract (Fig. 2-B) were more diffuse (under both reducing and non-reducing conditions) and the number of proteins was lower, compared to Saturniidae, which is in line with the fact that the bristle extract consisted of a mixture of both long hairs and short spines (Fig. 1-B and C). Noteworthy, we used the same method for the preparation of the bristle extract (venom) for all caterpillar species investigated in this study. The average yield was 4.99; 5.27; and 4.06 μg of protein (venom) obtained per mg of dry bristles of *L. obliqua*, *L. memusae*, and *P.* ca. *fuscescens*, respectively.

According to Table 1, where the proteins identified by mass

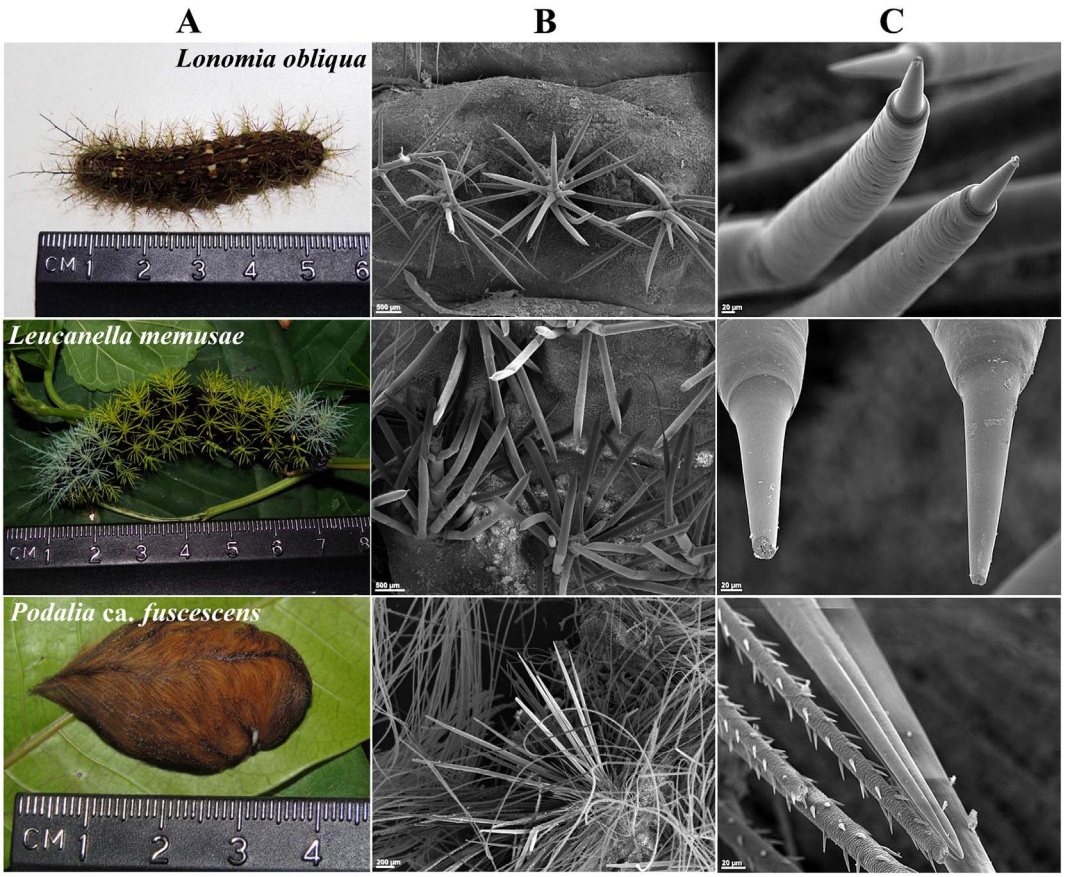

Fig. 1. Micrographs of the three species of caterpillars involved in this study. A - General view. B - SEM micrographs showing some details of scoli from the median dorsal region. C - SEM micrographs of some spines from dorsal scoli.

spectrometry are listed, the bristle extracts of Saturniidae species are more complex, e.g., there are more proteins than in *P.* ca. *fuscescens*, as observed in the SDS-PAGE. Proteins related to the coagulation disturbance, such as serpins, serine proteases and serine protease inhibitors (Veiga et al., 2005) were identified in all samples. Among Saturniidae species, besides some differences in terms of presence and absence of proteins, there were also quantitative differences between the same proteins, as observed by SDS-PAGE. One example is achelase-2, an anticoagulant protein of 22.7 kDa, whose band is more intense in *L. obliqua* than in *L. memusae*. Another difference is serine protease 1, a 57-kDa protein, present in a region where it is possible to notice more intense bands in *L. obliqua* than in *L. memusae*.

Taking into account that enzymes are one of the most common and toxic components found out in caterpillars by now (Fernandes-Pedrosa et al., 2013), three potential enzyme activities present in animal venoms involved in the pathophysiology of envenomation were tested in

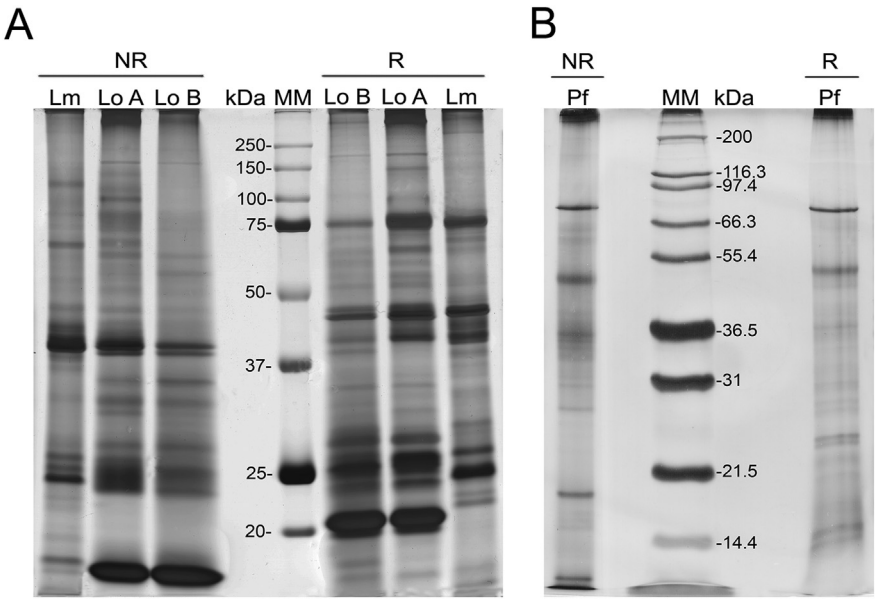

Fig. 2. Electrophoretic profiles of Saturniidae (A) and Megalopygidae (B) caterpillar bristle extracts in SDS-PAGE (12%), under reducing (R) and non-reducing (NR) conditions. Each lane was loaded with 4 μg of protein. Proteins were silver stained. Lo A: *Lonomia obliqua* from Argentina; Lo B: *Lonomia obliqua* from Brazil; Lm: *Leucanella memusae*; Pf: *Podalia* ca. *fuscescens*; MM: molecular mass markers.

Table 1
Proteins identified by mass spectrometry of Argentinian caterpillar bristle extracts.

Lonomia obliqua		*Leucanella memusae*		*Podalia* ca. *fuscescens*	
Entry	Protein name	Entry	Protein name	Entry	Protein name
Q5MGD1	60S ribosomal protein L17	P23605	Achelase-2	Q5MGP4	Elongation factor 1-alpha
P23605	Achelase-2		Cuticle protein 1	Q5MGP0	Heat shock protein 1
Q5MGP9	Defense protein 2	Q5MGP9	Defense protein 2	Q5MGH3	Protease inhibitor 3
Q5MGD5	Heat shock protein 4 heat shock cognate 70 protein	Q5MGP0	Heat shock protein 1	Q5MGK3	Ribosomal protein 25
Q1HLC3	Hemolin	Q5MGD5	Heat shock protein 4 heat shock cognate 70 protein	Q5MGH2	Serpin 1
Q5ECE3	Lopap	Q1HLC3	Hemolin		
Q5MGE2	Prophenoloxidase activating factor 1	Q5MGG4	Lectin 1		
Q5MGH3	Protease inhibitor 3	Q5MGF0	Lectin 3		
Q5MGE9	Protease inhibitor 9	B3TDS9	Putative CAD trifunctional protein		
B3TEY2	Protein Wnt	Q5MGL4	Ribosomal protein L1		
B3TDS9	Putative CAD trifunctional protein	Q5MGK5	Ribosomal protein 23		
Q5MGK5	Ribosomal protein 23	Q5MGK4	Ribosomal protein 24		
Q5MGK0	Ribosomal protein 28	Q5MGK0	Ribosomal protein 28		
Q5MGG7	Serine protease	Q5MGD2	Ribosomal protein 30		
Q5MGG8	Serine protease 1	Q5MGG8	Serine protease 1		
Q5MGE3	Serine protease 6	Q5MGH0	Serine protease inhibitor 3/4		
Q5MGH0	Serine protease inhibitor 3/4	Q5MGH2	Serpin 1		
Q5MGM6	Translationally-controlled tumor protein homolog	Q5MGH1	Serpin 2		
Q5MGI6	Tropomyosin 1	Q5MGI6	Tropomyosin 1		
Q5MGN1	Uncharacterized protein (4)	Q5MGM3	Uncharacterized protein		
Q5MGG3	Uncharacterized protein	Q5MGG3	Uncharacterized protein		
Q5MGN6	Uncharacterized protein	Q5MGN7	Uncharacterized protein		
Q5MGN7	Uncharacterized protein	Q5MGN6	Uncharacterized protein		
P85251	Unknown protein 6	Q5MGN1	Uncharacterized protein		
		P85252	Unknown protein 7		

Table 2
Specific activities of bristle extracts of Argentinian caterpillars toward some substrates.

Bristle extracts of:	Caseinolytic activity (U/mg protein)	Phospholipase A_2 activity (U/mg protein)	Hyaluronidase activity (μg of hydrolyzed hyaluronic acid/min/mg protein)
Podalia ca. *fuscescens*	15.12 ± 3.39	4.36 ± 2.71	2.42 ± 1.08
Leucanella memusae	5.40 ± 2.79	3.80 ± 1.81	0.55 ± 0.35
Lonomia obliqua	7.79 ± 1.23	27.81 ± 15.87	1.63 ± 0.37

All values are mean values for three individual extracts, assayed in triplicate, ± standard deviation.

this study (Table 2). All three venoms degraded azocasein, and the venom of *P.* ca. *fuscescens* showed the highest protease levels. *Lonomia obliqua* venom exhibited the highest level of PLA_2 activity. However, all venoms hydrolyzed hyaluronic acid at low levels.

To analyze the effect of bristle extracts upon clotting, human citrated plasma was incubated with them prior to the induction of coagulation with calcium. As clearly shown in Fig. 3-A and B, bristle extracts of *L. obliqua* and *P.* ca. *fuscescens* caterpillars displayed procoagulant effects, in virtue of the shortening of the clotting time of human plasma. While the control plasma (with no sample added) took over 6 min to initiate coagulation after the addition of calcium, plasma incubated with *L. obliqua* and *P.* ca. *fuscescens* bristle extracts took around 1.5 and 4 min respectively to start coagulating. Normal plasma did clot when neither bristle extract nor calcium were added (data not shown). However, when the procoagulant activity upon plasma was quantified, it was only possible to determine the MCD of *L. obliqua* venom (2.33 μg/mL), since the *P.* ca. *fuscescens* venom showed a CT > 300 s, even with the highest concentration assayed (104.55 μg/mL by BCA). It is possible that *P.* ca. *fuscescens* contains a weak coagulant component whose action can only be revealed when there is enough time to act on plasma proteins.

Regarding the *L. memusae* bristle extract, it showed an inhibitory effect on the coagulation induced by calcium, as it prolonged the APTT of normal human plasma in a concentration-dependent manner (Fig. 3-C and D). This action may be mediated by proteolysis of coagulation factor(s). However, we showed that whereas *L. memusae* bristle extract did not degrade human fibrinogen, both *L. obliqua* and *P.* ca. *fuscescens* bristle extracts hydrolyzed this molecule (Fig. 4) and fibrin (Fig. 5) with similar rates. Both *L. obliqua* and *P.* ca. *fuscescens* venomous secretions showed specificity directed preferentially toward the α chain of both fibrinogen and fibrin, thus allowing us to identify typical α-fibrinogenase enzymes therein, similar to those found in venoms of the Venezuelan *L. achelous* (Arocha-Piñango et al., 1981), the Brazilian *L. obliqua* (Veiga et al., 2003), and also to those found in some snake venoms (Markland, 1998).

It is known that the procoagulant activity of crude bristle extract of Brazilian *L. obliqua* is mainly due to the following two components that are abundant in this venom: Lopap, a prothrombin activator, and Losac (which is identified as hemolin in Table 1), a factor X activator. These procoagulant toxins cause *in vivo* activation of the coagulation system, which result in a consumptive coagulopathy (Chudzinski-Tavassi et al., 2013). Moreover, this venom promotes fibrin(ogen)olytic activity, which in concert with procoagulant toxins, leads to the hemorrhagic syndrome commonly observed in patients envenomed by *L. obliqua* (Veiga et al., 2003). Accordingly, in all recently reported cases of *L. obliqua* envenomation in Argentina, patients clinically manifested some hemorrhagic disturbances (Sánchez et al., 2015). Furthermore, in this study we could identify both Lopap and Losac in the Argentinian *L. obliqua* venom, but what is most important to highlight is that Losac was also detected in *Leucanella* venom (see Table 1, where Losac is identified as hemolin).

Even though both *L. memusae* and *P.* ca. *fuscescens* bristle extracts showed activity on the coagulation system and components able to act on this system, any clinical manifestation of hemostatic disturbance has not been reported by contact with caterpillars of both genera (Espindula et al., 2009) by now. It is probably that hemorrhagic manifestations can occur only when a person touches or smashes many specimens of these caterpillars simultaneously.

It is important to notice that *L. obliqua* accidents frequently involve

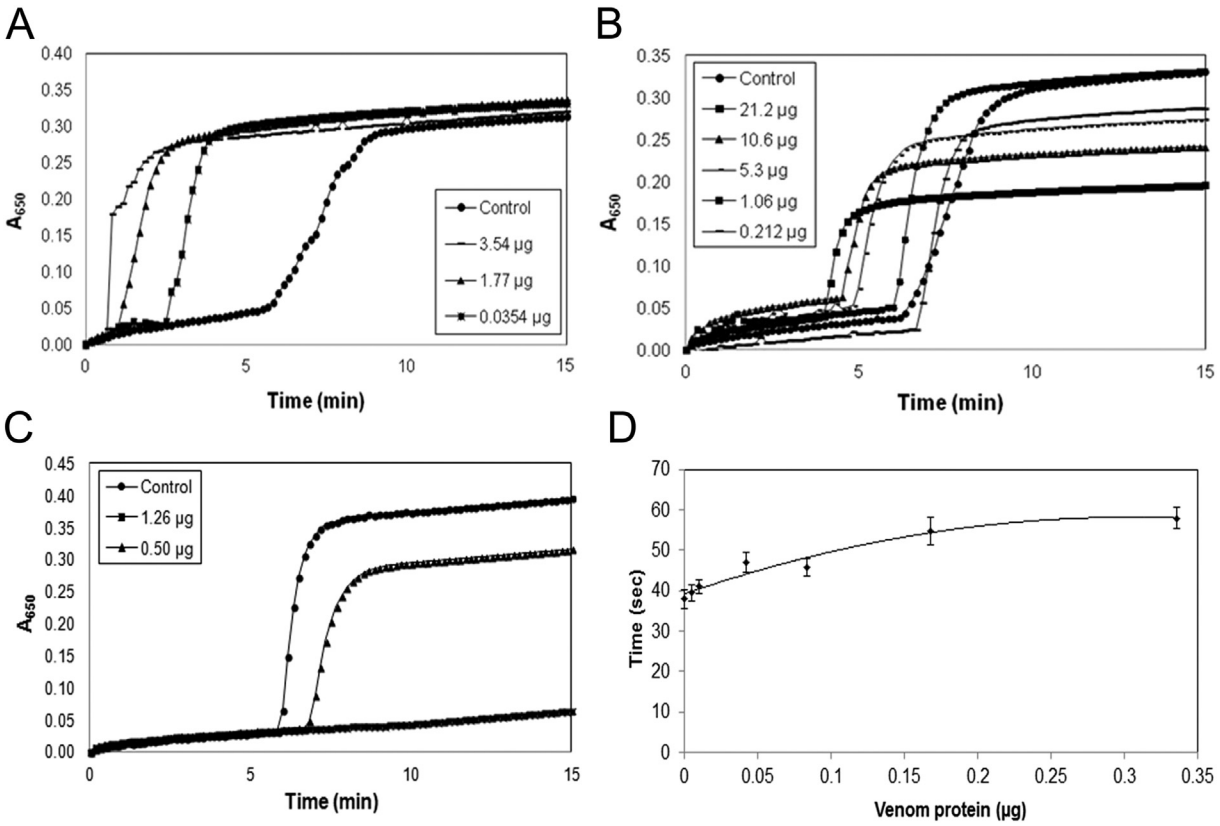

Fig. 3. Effect of Argentinian caterpillar bristle extracts upon coagulation of human plasma: *Lonomia obliqua* from Argentina (A); *Podalia* ca. *fuscescens* (B); *Leucanella memusae* (C). In D, we observed the effect of *L. memusae* extract on the clotting time of APTT in human plasma.

contact with colonies containing dozens or hundreds of caterpillars, which is related with their gregarious habits (Gamborgi et al., 2006). However, accidents by *Leucanella* or *Podalia* are mainly caused by contact with only one specimen (Espindula et al., 2009; Specht et al., 2009). Probably these behavioral features have a strong relationship with the clinical outcome, complicating the prognosis of the envenomation when more caterpillars are involved in the accident.

The specific treatment of *Lonomia* envenomation consists of the use of the specific antivenom, which is only produced by the Instituto Butantan from Brazil. In an attempt to identify whether high concentrations of antibodies (1:500) found in the Brazilian *Lonomia* antivenom could identify protein bands of Argentinian *L. obliqua* venom, a Western blotting was completed (Fig. 6). Apparently, all bands present in both venoms were recognized by the antivenom. This result is in accordance with the fact that the protein profile of both venoms showed to be very similar. *Lonomia* antivenom displayed similar antibody titers by ELISA to both *L. obliqua* venoms (256,000 for the Brazilian venom, and 512,000 for the Argentinian venom), demonstrating that the antivenom produced by Instituto Butantan effectively recognized Argentinian *Lonomia* venom proteins. This result was expected, since caterpillars coming from Southern regions of Brazil are employed to compose the *L. obliqua* venom pool used to immunize horses for obtaining the *Lonomia* antivenom.

Even though *Leucanella* caterpillars have not been involved in severe cases of envenomation, and consequently there has never been a need to apply a specific treatment in accidents caused by these caterpillars, we showed here that the *Lonomia* antivenom recognizes most of the proteins present in *L. memusae* venom. Furthermore, it is important to highlight that both *L. memusae* and *L. obliqua* bristle extracts share a high degree of antigenic similarity, which is in line with the fact that both genera belong to the Saturniidae family. However, few components in *P.* ca. *fuscescens* venom – mainly under non-reducing condition – were recognized by Western Blotting.

It is important to emphasize that this study opens the possibility of understanding the intriguing way by which only representatives of the genus *Lonomia* causes severe hemorrhagic pictures in human beings.

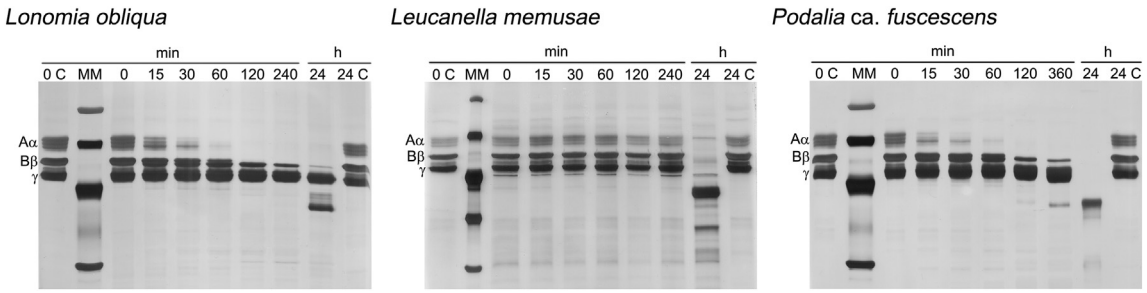

Fig. 4. Effect of Argentinian caterpillar bristle extracts on human fibrinogen. Fibrinogen hydrolysis was evaluated by SDS-PAGE (12% running gels, under reducing conditions), and thereafter gels were silver stained. On the left, the polypeptide chains of human fibrinogen are indicated: Aα (63 kDa), Bβ (56 kDa), and γ (47 kDa). MM: molecular mass markers. C: Control of fibrinogen incubated in the absence of extract.

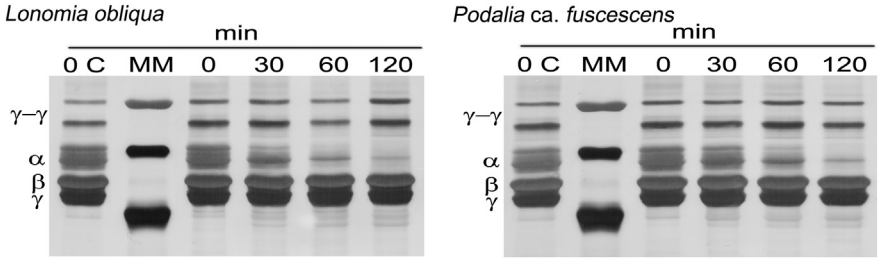

Fig. 5. Effect of Argentinian caterpillar bristle extracts on human fibrin. Fibrin hydrolysis was evaluated by SDS-PAGE (12% running gels, under reducing conditions), and thereafter gels were silver stained. On the left, the polypeptide chains of human fibrin are indicated: γ-γ dimer, α-monomer, β-monomer, and γ-monomer. MM: molecular mass markers. C: Control of fibrin incubated in the absence of extract.

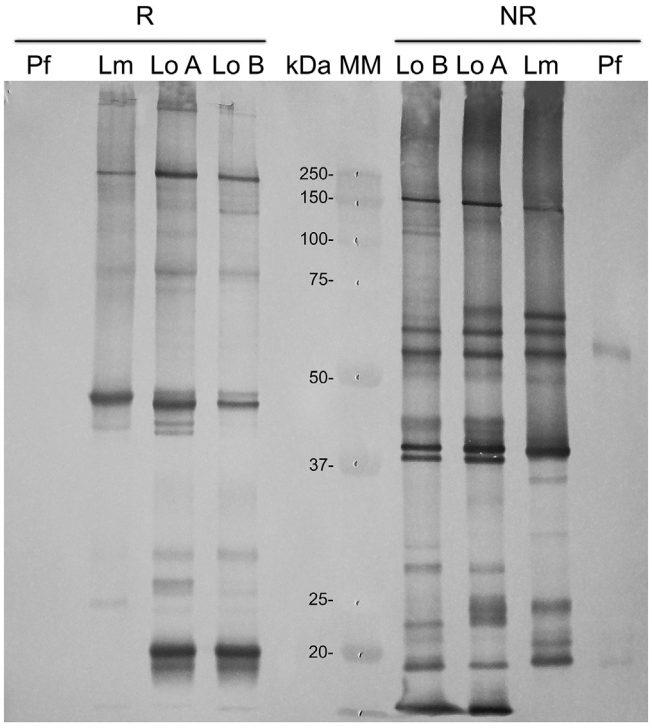

Fig. 6. Western blotting of Saturniidae and Megalopygidae caterpillar bristle extracts. Venom proteins were subjected to SDS-PAGE, and blotted onto a nitrocellulose membrane. Membranes were then sequentially incubated with *Lonomia* antivenom and peroxidase-conjugated anti-horse IgG. The reaction was developed with DAB. Lo A: *Lonomia obliqua* from Argentina; Lo B: *Lonomia obliqua* from Brazil; Lm: *Leucanella memusae*; Pf: *Podalia* ca. *fuscescens*; MM: molecular mass markers.

However, significant efforts need to be placed in understanding the biological aspects that contribute to the venom composition of these caterpillars, particularly because *Leucanella* and other Saturniidae caterpillars share feeding habits in the same host plant species (Pastrana, 2004; Specht et al., 2008).

In conclusion, our findings show for the first time a comparative biotoxinological analysis of three genera of caterpillars, and provide relevant information about the pathophysiological mechanisms whereby these caterpillar bristle extracts can induce toxicity on human beings. Our results also highlight the recognition of most toxins present in *L. obliqua* from Argentina with the *Lonomia* antivenom produced by Brazil, which support the recommendation to strengthen collaborative links among South American countries – where *Lonomia* envenomation is a public health problem - to manufacture, share and conveniently distribute this immunobiological product that proved to be efficient to treat victims of *Lonomia* caterpillars. As shown herein, the toxicity of *L. memusae* and *P.* ca. *fuscescens* caterpillar bristle extracts should not be underestimated by physicians, and any medically significant accident needs to be promptly reviewed by a qualified health professional, who should be able to recognize the envenomation and provide the correct treatment to victims. Altogether, this study reveals that there are proteins acting on the hemostatic system in all caterpillar bristle extracts investigated herein, paving the way to further investigation.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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