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Assessing the stability of historical and desiccated snake venoms from a medically important Ecuadorian collection

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ABSTRACT

Bothrops asper and *Bothrops atrox* are important venomous snakes from Ecuador responsible for the most of ophidic accidents, which in the past were treated with a national polyvant antivenom. For years, the venom pools were collected and stored at room temperature in a laboratory. Taking into account the controversial ability of desiccated samples to retain their biological effects and enzymatic activities, we investigated the biochemical and toxicological properties of venoms after years of storage. The proteomic profiles of historical venoms analyzed by high-performance liquid chromatography and electrophoresis are very similar. The fresh batches of venom were more lethal than those stored for years, just as the initial and current LD_{50} values of these samples changed. Significant differences were showed in the myotoxic and hemorrhagic activity of some venom pools, while no significant statistical differences were found for the edema activity. The enzymatic assays revealed a variation in proteolytic activity on azocasein and phospholipase A_2 activity, and low differences were reported for thrombin-like serine protease activity. The maintenance of the proteomic profile and certain toxicological activities convert this venom library in a valuable source for research purposes. Nonetheless, the significative reduction of toxicological activities, such as hemorrhagic activity not feasible using these samples for the antivenom production.

1. Introduction

Snake venoms are natural chemical catalogues composed mainly of proteins and peptides, which are generally known by their lethal effects both on prey and humans, but that ambiguously present relevant therapeutic, diagnostic and biotechnological value (Simoes-Silva et al., 2018; Mohamed Abd El-Aziz et al., 2019). Several biomedical studies have proven the huge potential of the snake molecules to inspire new discoveries, synthesis of bioactive compounds and development of research tools and therapies to some types of diseases (Li et al., 2018; Mendes et al., 2019). Among the medicines generated from the venom researches are captopril and batroxobin (SB50), all approved by the Food and Drug Administration and commonly used as antihypertensive and defibrinogenating hemostatic agents, respectively (Almeida et al., 2017). Both commercial drugs are inspired by bothropic venom compounds, a snake genus also widely distributed in Ecuador.

Contradictory to the understanding and recognition of the utility of venom proteins, many of snake species are facing a high risk of extinction (Böhm et al., 2013). Therefore, it is necessary besides important conservation policies as well as a race against the clock to know and understanding the biochemical secrets, structure, enzymology, pharmacology and applications of novel snake toxins. An interesting strategy is the development of venom banks of different species of animals for scientific and pharmaceutical purposes. One example of historical collection is the list of venom pools maintained by George Brittten Halford at the University of Melbourne for a large periodic time

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(Pearn and Winkel, 2006; Jesupret et al., 2014). A venom bank is also an important tool to understanding the temporal change of ecological, evolution and geographical of snakes, development of natural product-based pharmaceutical tools and innovative immunotherapy strategies as well as can be an important source in future of complex samples that cannot be studied by current methods and technology.

Ecuador is a country famous for its high-biodiversity wilderness areas with a richness of venomous snakes. Yañez-Arenas et al. (2018) have recently demonstrated that ophidian accidents in Ecuador are concentrated in rural areas, with *B. atrox* in the Amazon region and *B. asper* in the coast provinces. At present, for treatment of ophidian accidents, the government imports the snake antivenom immunoglobulins from other Latin American countries, such as Costa Rica, however it was not always so. The Instituto Nacional de Higiene y Medicina Tropical "Dr. Leopoldo Izquieta Pérez", (Guayaquil, Ecuador), at the moment known as Instituto Nacional de Investigación en Salud Pública (INSPI) produced until 2012 a polyvalent antivenom to neutralize the main Ecuadorian medically-important snake venoms (*Bothrops atrox* and *Bothrops asper*). In this circumstance for the national manufacturing and control of polyspecific heterologous immunoglobulins, venom samples from both species were collected from wild snakes in different locations, particularly in the provinces with the highest snakebite reporting rates. The remaining venom samples from these batches were stored at room temperature in the laboratory of this public institution, as desiccated venom, similarly to vintage collection described above and maintained at the University of Melbourne.

The quality of storage conditions in historical venom collections have not extensively evaluated and the published data about stability of desiccated snake venom throughout short and longer periodic are contradictory (Foote and MacMahon, 1977; Egen and Russell, 1984; Jesupret et al., 2014). Some studies have demonstrated the ability of desiccated samples to retain the lethality and toxicological characteristics after storage (Munekiyo and Mackessy, 1998), however other scientific works have described the influence of temperature, luminosity, vacuum, storage time as well as other factors such as family, genus, species and venom phenotype in the toxicological and enzymatic activities of samples (Russell et al., 1960).

Currently, there is scarce information about the toxicological, proteomic and enzymatic from Ecuadorian *Bothrops asper* and *Bothrops atrox* snake venoms, despite the many studies with these snake species from Brazil, Peru and Costa Rica (Nunez et al., 2009; Kohlhoff et al., 2012; Laines et al., 2014). Interestingly, Gutierrez et al. (2010) and Sousa et al. (2017), respectively have reported the intraspecific snake variability in the venom protein composition of *Bothrops asper* and *Bothrops atrox* from distinct sites, highlighting the relevance to investigate the basic biochemical, proteomic and toxicological aspects of the physiopathology induced by *B. atrox* and *B. asper* from this country, which are useful to establish efficient immunotherapy strategies for ophidian accidents, as suggested by Terán and Lomonte (2016).

In the light of storage conditions, stability and variability of snake venom composition, the present work aimed to compare the proteomic profiles, enzymatic and toxicological activities from a historical collection of Ecuadorian desiccated venoms composed by batches of the species responsible by the majority of snakebites in the country.

2. Material and methods

2.1. Historical and medically important Ecuadorian venom samples

The *Bothrops asper* and *Bothrops atrox* venoms from Ecuadorian snakes were manually milked in distinct years by inducing the healthy adult snakes to bite a parafilm stretched over the glass vessel placed in a bath of crushed ice according to methodology described by Klauber (1957). The venom batches contaminated with blood were rejected. The Fig. 1 shows the date and locality of collection for the snake venom samples investigated in our work. After the collection, the venoms from the same snake species were pooled, dried in a vacuum desiccators over calcium carbonate and kept in small Eppendorfs at room temperature and stored at Laboratory of INSPI as a historical bank of desiccated snake venom. Currently, this laboratory is regulated by the Ministry of Environment and has the operating permit (No. FAU-001-2019-MAE/DPA).

2.2. Proteomic profiling

The proteomic profiling of each snake venom samples of the historical collection were individually analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions for evaluate of proteomic stability of these desiccated samples.

2.2.1. RP-HPLC fractionation and SDS-PAGE

For RP-HPLC analyses, 5 mg desiccated snake venoms were dissolved in 120 µl of 0.1% (v:v) trifluoroacetic acid (TFA) and 80 µl of 1 M ammonium bicarbonate buffer and homogenized using a vortex. Insoluble material was removed by centrifugation at 8000 rpm for 3 min at room temperature and then the supernatant were loaded on a Discovery Bio Wide Pore C5 HPLC Column (4.6 mm × 25 cm, 5 µm particle size) using a HPLC-PDA 991 (Waters), equipped with two Waters Model 510/B pumps, and an UK6 automatic injector sample. The protein elution was monitored at 280 nm under a constant flow of 1 ml/min using a linear gradient from 0 to 100% of solvent B (66% (*v/v*) acetonitrile, 0.1% (v/v) TFA), at a flow rate of 1 ml/min, for 60 min. The fractions obtained from elution of fresh snake venoms of *B. asper* and *B. atrox* (2016) were manually collected and dried in a vacuum centrifuge concentrator (SpeedVac, Thermo).

The desiccated Ecuadorian venoms were dissolved in loading buffer (0.075 M Tris-HCl, pH 6.8, 10% (v:v) glycerol, 4% (w:v) SDS, 0.001% (*w/v*) bromophenol blue) and fractioned by SDS-PAGE using a concentration gel of 5% and a running gel of 12.5% under reducing conditions, in line with the methodology described by Laemmli (1970). The electrophoretic separation was performed at 30 mA at room temperature, and the protein bands stained in 0.2% (w:v) Comassie Blue brilliant blue R-250; the excess dye was removed with a solution of 7% (v:v) acetic acid. The same procedure was repeated for the RP-HPLC fractions from fresh venoms (pools from 2016) of *B. asper* and *B. atrox* aiming to identify proteins family present in these snake venoms and most abundant peaks visualized in liquid chromatography. The molecular mass of snake toxins was estimated using Rainbow™ Molecular Weight Markers (GE Healthcare).

2.3. Enzymatic activities of Ecuadorian historical snake venoms

Phospholipase A_2, metalloproteinase and trombin-like serine protease activities were performed in triplicate, spectrophotometrically monitored at correct wavelength using the specific chromogenic substrates (4-nitro-3-octanoyloxybenzoic acid, azocasein, and Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride, respectively). The enzymatic results were expressed as percentage and calculated based on the venom batch with highest activity considered as 100%.

2.3.1. Phospholipase A_2 activity

Determination of phospholipase (PLA_2) activity of each sample from the historical snake venom collection was measured using the chromogenic substrate 4-nitro-3-octanoyloxybenzoic acid (NOBA) following the aqueous assay system described by Holzer and Mackessy (1996) and modified for use 96-well microplate in accordance with methodology used by Resende et al. (2017). Initially, the PLA_2 substrate solution was prepared by dissolving 3.1 mg of synthetic substrate (Sigma-Aldrich) in a solution 1:10 (v:v) acetonitrile: buffer (10 mM Tris-HCl, 1 M $CaCl_2$, 10 mM NaCl, pH 7.8) and 1 mg of each Ecuadorian venom sample was

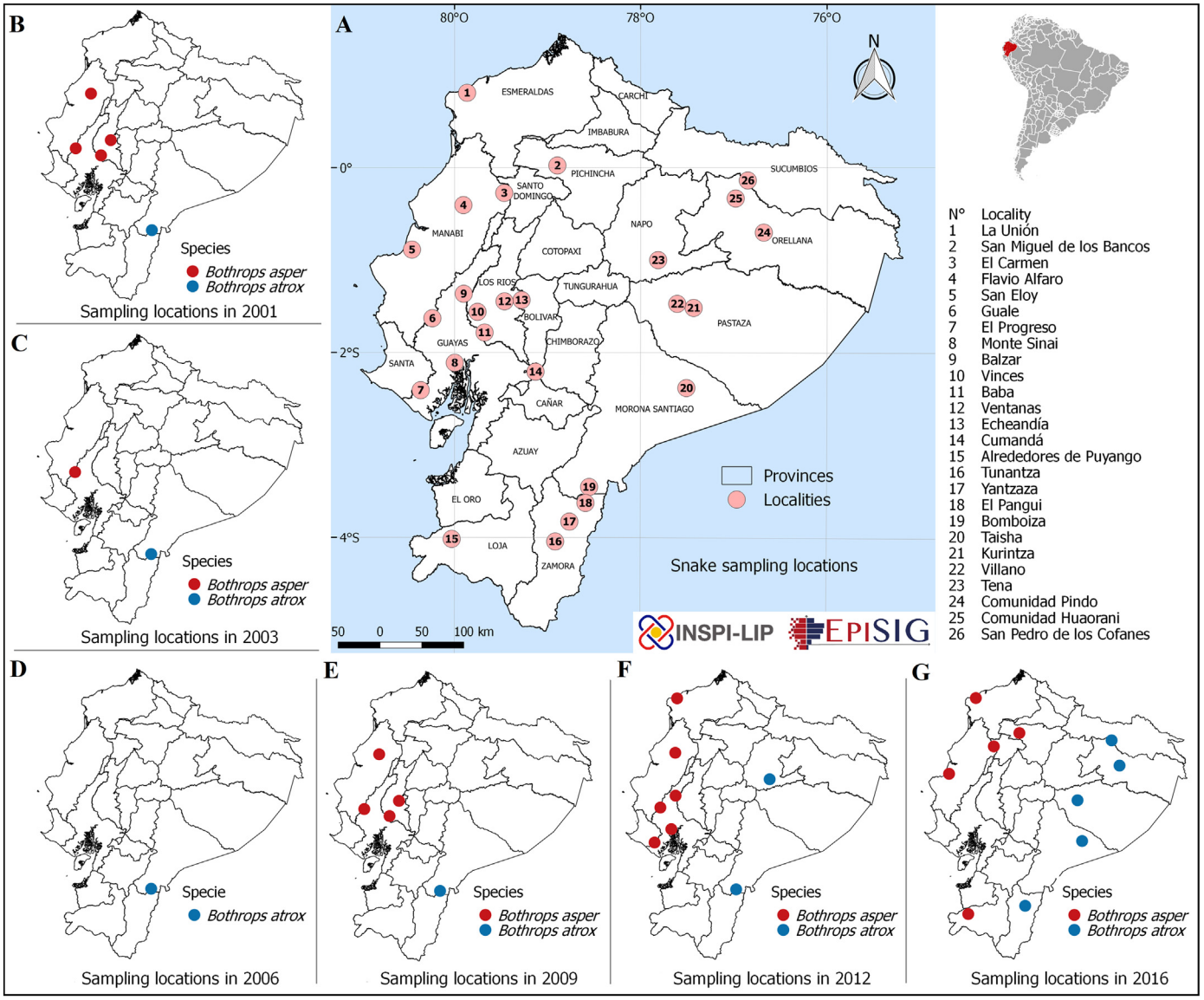

Fig. 1. Historical Ecuadorian snake venom samples. (A) Map of Ecuador marking the sites where *Bothrops asper* (red) and *Bothrops atrox* (blue) snake venom were collected. The venom batches studied were collected at different years (B) 2001, (C) 2003, (D) 2006, (E) 2009, (F) 2012 and (G) 2016. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dissolved in 1 ml 0.15 M NaCl. After this, the standard assay mixture were done mixing 220 µl of substrate solution, 20 µl of test sample and 20 µl of distilled water in 96-well-microplates, followed by incubation at 37 ˚C for absorbance reading. The PLA_2 activity was determined based on chromophore absorbance after 20 min at 425 nm using a Versamax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA). A blank composed by 220 µl of substrate solution and 40 µl of distilled water was used to correct the absorbance readings.

2.3.2. Trombin-like serine protease activity

The Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BapNa) was used as chromogenic substrate to assay samples for serine proteases, similar to methodology used by Munekiyo and Mackessy (1998) and Ghorbanpur et al. (2010). The snake venoms were individually dissolved in a solution 1:1 (*w/v*) of 10 mM buffer A (Tris-HCl, 1 M $CaCl_2$, 10 mM NaCl, pH 7.8) and the substrate solution was done using 2.18 mg of BapNa (Sigma-Aldrich) dissolved in 50 µl of dimethyl sulfoxide with 5 ml of buffer A. The amidolytic activity was performed by mixing 20 µl of stored snake venoms with 200 µl of substrate solution and 50 µl of buffer A, and monitored at 410 nm using a Versamax 190 multiwell plate reader (Molecular Devices, Sunnyvale, CA). The enzymatic activity was measured based on reaction's products formed at 37 ˚C after 30 min of sampling incubation. A blank obtained by reacting the 200 µl of substrate solution and 70 µl of buffer A was used to correct the absorbance values.

2.3.3. Proteolytic activity

The proteolytic activity on azocasein was evaluated by a spectrophotometric microassay previously reported by Lomonte and Gutierrez (1983), using 5 mg of azocasein (Sigma-Aldrich) dissolved in 1 ml of 50 mM Tris-HCl, pH 8.0 and modified for 96-well-microplates (Cabral et al., 2004). The historical snake samples were dissolved in 50 mM Tris-HCl, pH 8.0, to a final concentration of 1 mg/ml. Proteolytic activity was determined by mixing 10 µl of each snake venom with 90 µl of casein solution, followed by incubation for 90 min at 37 ˚C. Posteriorly, 200 µl of 5% (v:v) trichloroacetic acid were added to mixture for stop the hydrolysis reaction. The resultant mixtures were kept at room temperature for 5 min and centrifuged at 8000 rpm for 5 min. Reactions products in supernatant solution (150 µl) were neutralized by the addition of 150 µl of 0.5 M of NaOH and monitored at 440 nm for

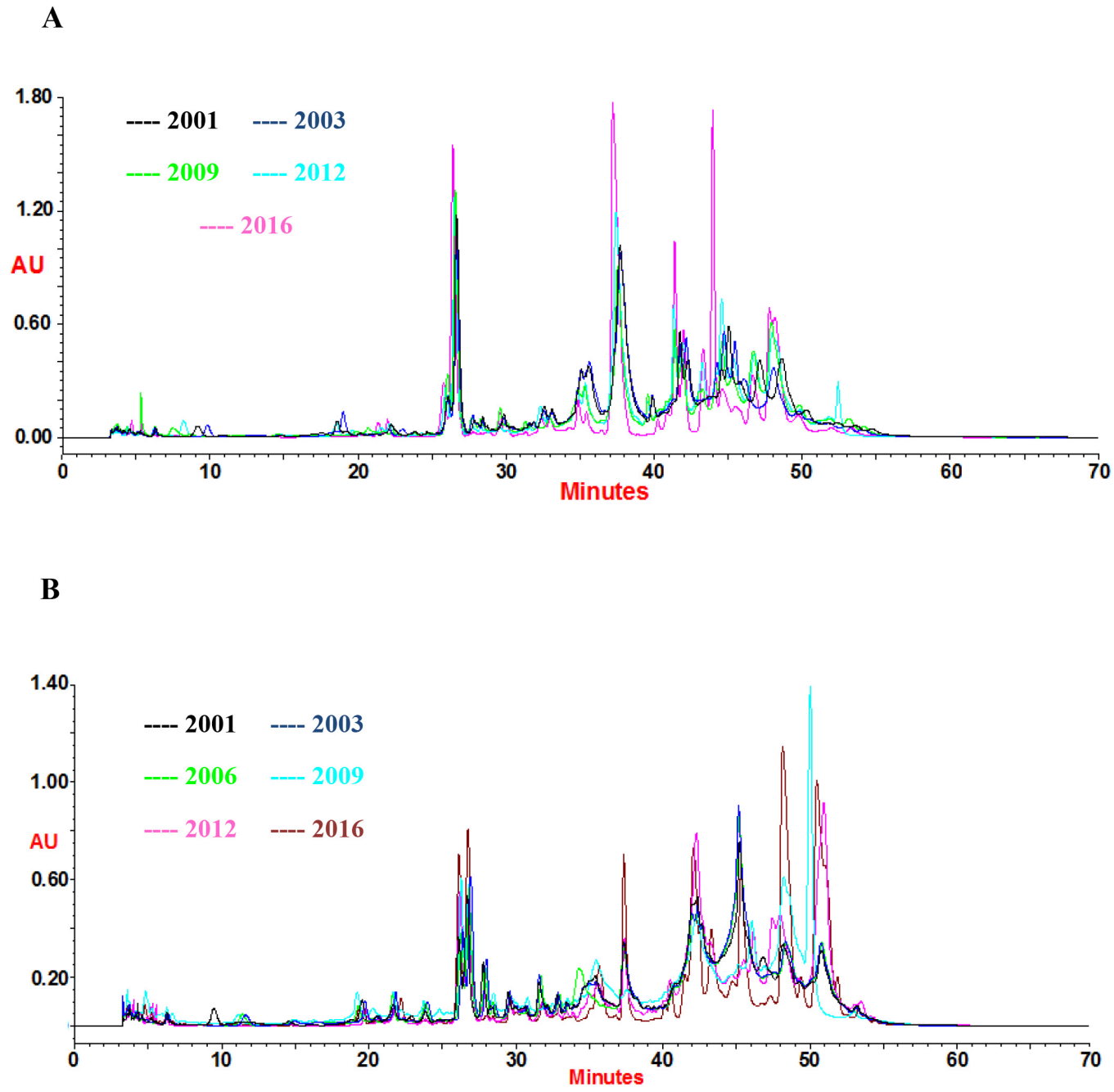

Fig. 2. Chromatographic separation profiles of historical snakes venom from *Bothrops asper* and *Bothrops atrox*. Desiccated snake venoms from *Bothrops asper* (A) and *Bothrops atrox* (B) of varying ages were eluted on a Discovery Bio Wide Pore C5 HPLC Column using a HPLC-PDA 991. The protein fractionation was monitored at 280 nm and the chromatogram data were superimposed. Each color represents the snake venom collected at the determined year and can be identified according to the legend. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

determination of caseinolytic activity. The absorbance readings were corrected using a blank assay using the substrate solution, which followed the same experimental methodology described but without snake venom addition.

2.4. Toxicological assays of Ecuadorian historical snake venoms

The edema, myotoxicity, hemorrhagic activity and lethality were assayed using CD-1 male mice (18–20 g; 3 months) purchased from the INSPI, Ecuador. The animal models used in this toxicological characterization were maintained at 24–28 °C with food and water *ad libitum*. The *in vivo* assays were performed in accordance with protocols of the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010). The toxicological activities were determined as percentage based on the venom batch with highest activity considered as 100%.

2.4.1. Median lethal dose determinations

The median lethal dose (LD_{50}) of each stored venom was assayed by intraperitoneal injection (100 μl) using groups of 6 male mice (18–20 g) for each dose evaluated in the animal experiment (60, 100, 140, 180 and 220 μg), as described by Sanchez et al. (1992). The negative animal controls received an intraperitoneal injection of PBS with the same volume. Mice deaths were scored over a 24 h period and the LD_{50} calculated according to Probit method. These experiments were performed with snake venom immediately after milking and dry process,

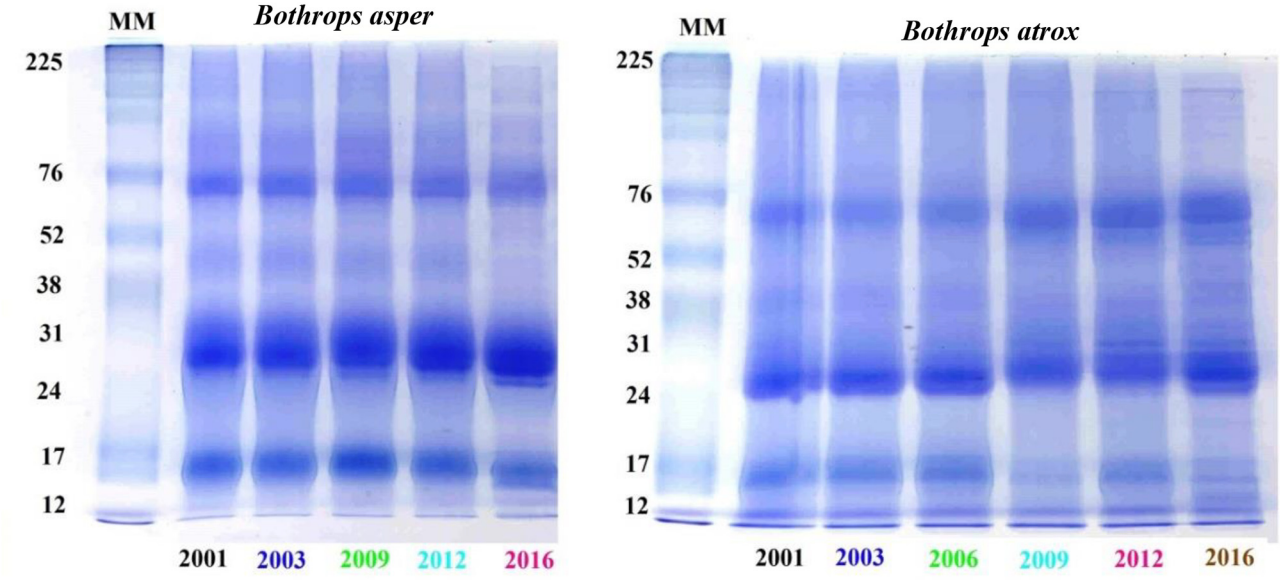

Fig. 3. Electrophoretic mobility of desiccated snake venoms from an Ecuadorian historical collection on one-dimensional gels. The snake proteins from the historical and desiccated samples from *Bothrops asper* (A) and *Bothrops atrox* (B) venoms were separated by one-dimensional SDS-PAGE gels (12.5%) under reduction conductions. The dates of snake milking were represented by distinct colors in line with the colors used in the chromatographic profile to represent the separation of each historical snake venom. Molecular mass markers (MM) are indicated at the side of each gel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and repeated using the same methodology in 2017 after stored for years as desiccated snake venom.

2.4.2. Hemorrhagic activity

Assessment of hemorrhagic activity induced by historical snake venoms was performed by a quantitative and qualitative method previously reported by Kondo et al. (1960), with some adaptations. Groups of four male CD-1 mice (18–20 g), previously anesthetized with a xylamine (10 mg/kg i.p.)/ketamine (80 mg/kg i.p.) solution (1:1), were injected intradermally in the shaved dorsal skin with 100 µl of a solution with 30 µg of snake venom, while controls only received saline buffer with the same volume. After 3 h, the mice were euthanized in an overdose of halothane and their dorsal skin was removed. The total area of the hemorrhagic spots were recorded photographically and measured with an electronic caliper (Truper).

2.4.3. Inflammatory activity

For evaluate the edema-forming activity triggered by desiccated venom of varying ages, groups of four mice (18–10 g) received injections into a hind paw of 50 µg of samples dissolved in 50 µl of PBS, while the PBS alone was injected in the contralateral paw. The volume increase of paws was measured using a low-pressure digital caliper (Truper) 3 h later of the subcutaneous injection of dissolved venom or PBS. The inflammatory activity was quantified based on the difference between the values measured for both paws and reported as percentage, as described by Lomonte et al. (1993).

2.4.4. Myotoxic activity

Groups of four male CD-1 mice (18–20 g) received intramuscular injections in the right thigh muscle, with 50 µg of desiccated snake samples dissolved in 50 µl of PBS. The control group of animals was injected intramuscularly with the same volume of PBS alone. In this assay, the amount of creatine kinase (CK) in the plasma was used as biomarker for determination of ability of desiccated snake venoms to induce local muscular damage. After 3 h, tail blood samples were collected into heparinized capillaries and the plasma CK activity measured using a biochemical kit and colorimetric assay, CK-NAC UV UniTest kit (Wiener lab).

2.5. Statistical analyses

All assays have been performed at least three times and the data are represented as the means ± standard deviations (SD). The statistical significance was calculated using one-way ANOVA followed by post-hoc analysis. The examination of the data was conducted in the Origin Pro 8 Software (OriginLab Corporation). Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Proteomic profiling

RP-HPLC revealed little differences between the chromatographic profiles of the historical desiccated venoms from the same snake species, indicating no significant differences in protein composition. The Fig. 2 shows the chromatographic profile of different batches of desiccated *Bothrops asper* (A) and *Bothrops atrox* (B) snake venoms. Little differences were observed in the absorbance of certain fractions from venom batches. Generally, the fresher venom samples presented some fractions with higher peak area (absorbance).

The SDS-PAGE separation of each sample under reducing conditions revealed a highly similar pattern between the venom batches. The protein profile analysis by SDS-PAGE revealed band patterns of three characteristic ranges of molecular masses: 13–17 kDa, 24–38 kDa, and 70–90 kDa in both Ecuadorian snake venoms (Fig. 3A and B). Gel electrophoresis reveals small variations in the bands between 70 and 90 kDa molecular mass.

From the fractionation of the venom batches from 2016 by RP-HPLC (Fig. 4), we can see 18 main fractions (Fig. 4A and B), which were collected manually, concentrated and analyzed by SDS-PAGE. The chromatographic profile of *B. asper* venom shows peaks with greater absorbance compared to *B. atrox* venom. The analysis of the RP-HPLC fractions by SDS-PAGE shows bands in 4 ranges approximately: 13–17 kDa (PLA_2s and CTL's: C-type lectins), 24–31 kDa (PI-SVMPs: snake venom metalloproteinases), 31–38 kDa (SVSPs: snake venom serine proteases) and 70–90 kDa (PIII-SVMPs). Overall, in *B. asper* venom (Fig. 4A insert) the PLA_2s are the major components in peaks 7

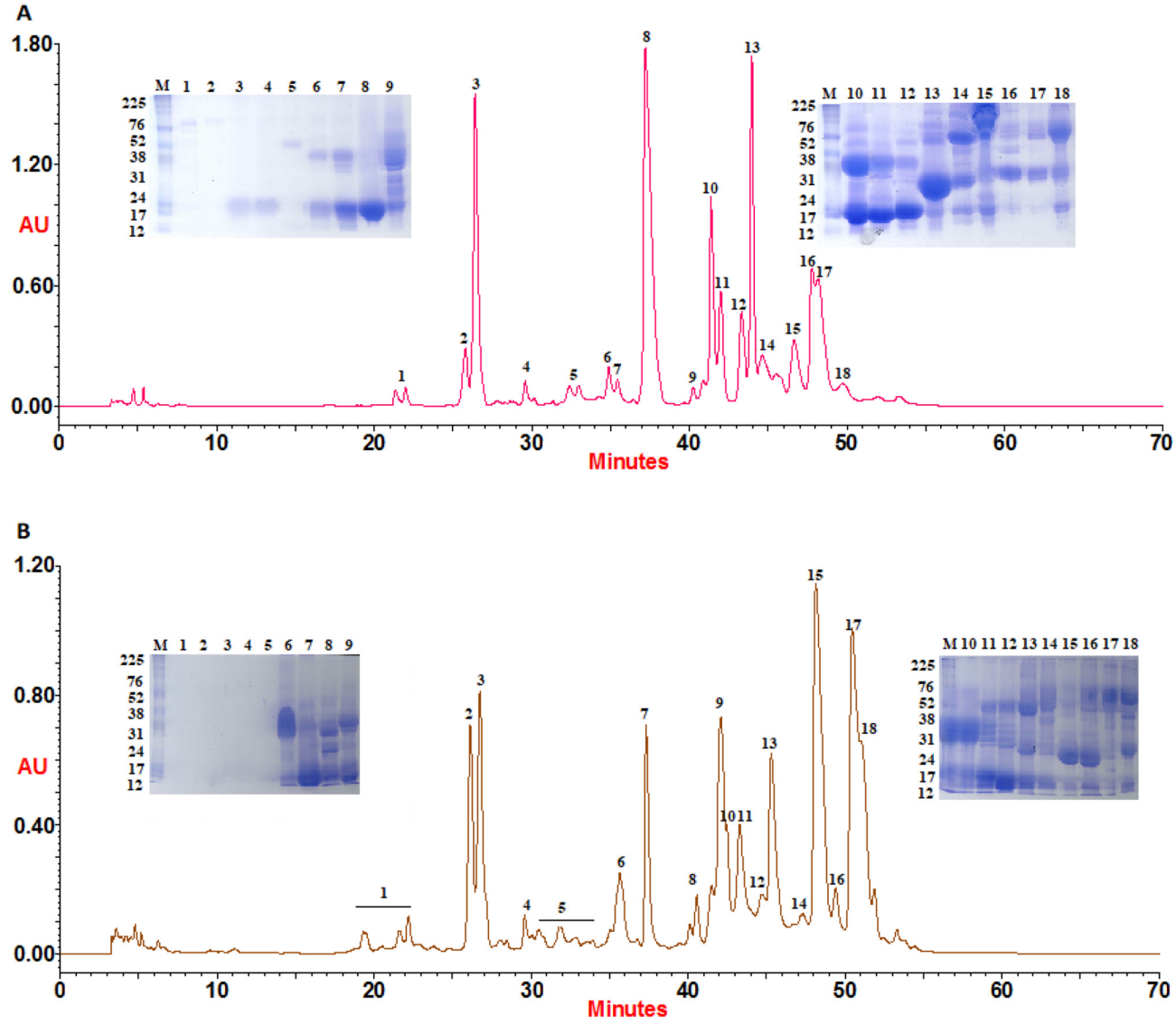

Fig. 4. Proteomic characterization of fresh venoms (pools from 2016) of two medically important Ecuadorian snake species. Venom proteins from *Bothrops asper* (A) and *Bothrops atrox* (B) were fractioned by RP-HPLC using a gradient of 66% acetonitrile in TFA and monitored at 280 nm. Eluted fractions were manually collected, dried using a vacuum concentrator and submitted to a SDS-PAGE (12.5%) analysis performed under reduced conditions. Molecular mass markers (M) are indicated at the side of each gel.

and 8 and the PI-SVMPs of peaks 13 and 14. Peak 10 is predominantly composed of SVSPs. The PIII-SVMPs are the most abundant component in peaks 17 and 18. In *B. atrox* sample (Fig. 4B insert) PLA_2s are the major components in peaks 7, 8 and 12. PI-SVMPs predominantly appear in peaks 15 and 16. While in the peaks 10 and 11 the most abundant component are SVSPs. Finally, PIII-SVMPs have a greater presence at peaks 17 and 18.

3.2. Enzymatic characterization of Ecuadorian historical snake venoms

Enzymatic variability due to storage time was assessed through tests with synthetic substrates. The enzymatic assays revealed differences in phospholipase A_2 activity on NOBA chromogenic substrate. However, the results do not indicate that this activity has been affected by storage time. This is observed in *B. asper* assays (Fig. 5A), analyzing the results of the samples from 2003 and 2012, with similar activities. In the case of *B. atrox* (Fig. 5B), the pool from 2001 has an enzymatic activity very close to that of batch from 2012.

The results of thrombin-like activity, induced mainly by serine proteases, cannot also be associated with storage time, especially as observed for *B. atrox* (Fig. 5C and D). The assays show that the venom batch from 2001 has an activity greater than venom pools from 2003 and 2006, and similar to the most recent venom samples. In the case of caseinolytic activity, for the two species the most recent venom samples are those with greatest enzymatic activity (Fig. 5E and F).

3.3. Toxicological characterization of Ecuadorian historical snake venoms

The Fig. 6 shows the LD_{50} of each historical snake venom determined at the year of collect (initial LD_{50}) and in 2017 (current LD_{50}) by an intraperitoneal injection according to Probit analysis. No statistically significant results were observed in the initial values of LD_{50} determined in year of venom extraction; however, these values are significantly different after up years of storage. The LD_{50} from older snake venoms presented major loss of lethal activity compared to fresh venoms.

The induction of hemorrhage, myotoxicity and inflammation by the venom collection were also evaluated. Significant variations are observed between samples in hemorrhagic and myotoxic activities. The newer venoms of *Bothrops asper* (Fig. 7A) and *Bothrops atrox* (Fig. 7B)

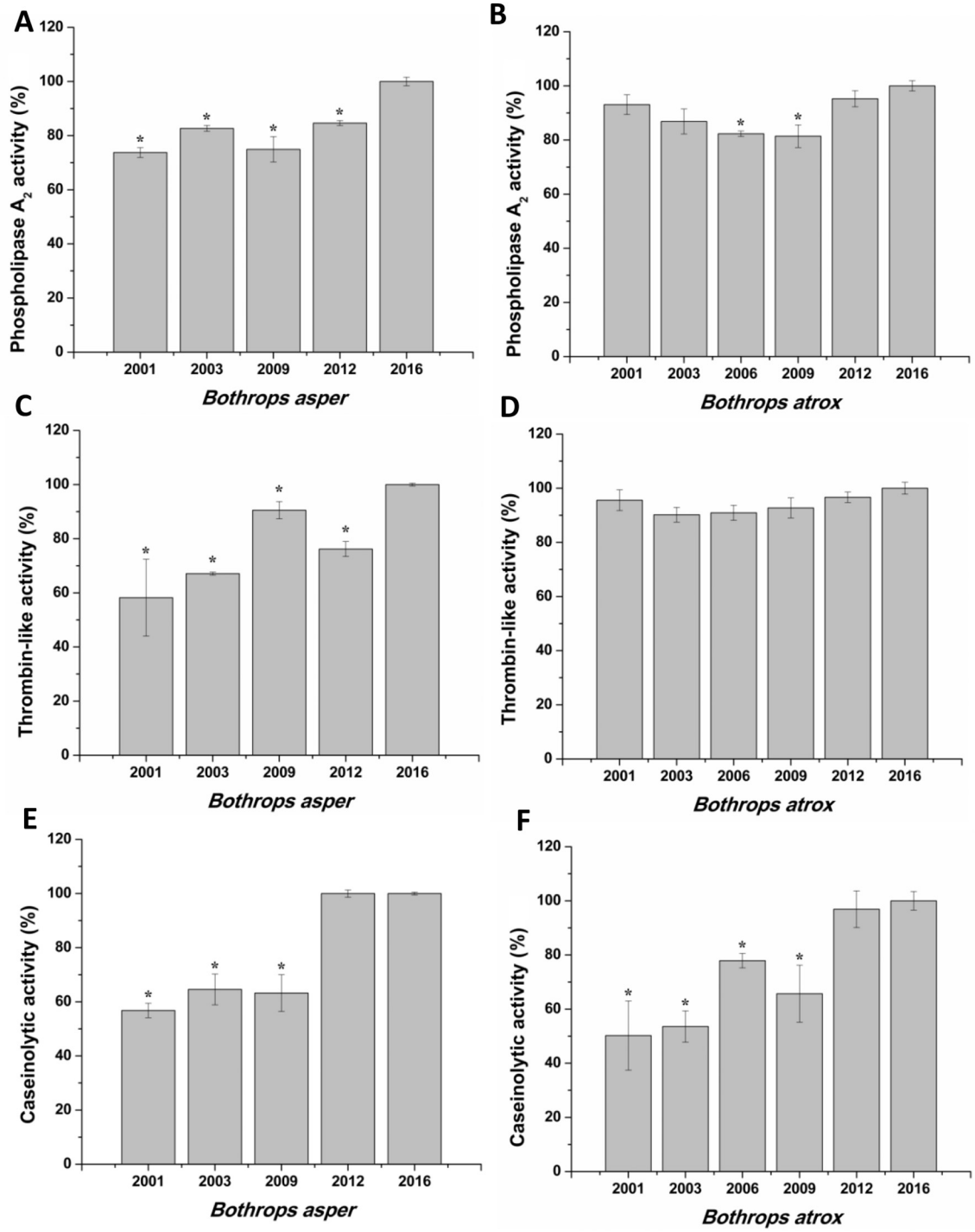

Fig. 5. Enzymatic characterization of desiccated snake venoms: *Bothrops asper* (left) and *Bothrops atrox* (right) from an Ecuadorian historical collection. The phospholipase A_2 (A, B), amydolitic (C, D) and proteolytic (E, F) activities of each desiccated snake sample were determined by colorimetric assays using the chromogenic substrates: NOBA, BapNa and azocasein, respectively. The absorbance readings were carried out using a Versamax 190 multiwell plate reader. *Statistically significant ($p < 0.05$) compared to the venom batch from 2016.

induced greater hemorrhage, as verified in the hemorrhagic halos present in dorsal skin from mice after 3 h of venom injection. Snake venoms that were stored for > 15 years induced the lowest hemorrhagic activity as evidenced by the minimal escape of blood in dorsal skin. Venoms from 2003 and 2006 also showed a significant reduction in this toxicological activity. The induction of this effect is related mainly with SVPMs. Newer venoms were also characterized by inducing more significant muscle damage related mostly with PLA_2 action, with higher plasma creatine kinase levels as determined by colorimetric assay. Venoms from *B. asper* were more myotoxic than *B. atrox* venoms (Fig. 7C and D). No significant differences were found for inflammatory activity, which remained unchanged between the venoms of both species of snakes analyzed (Fig. 7E and F).

4. Discussion

The generation of venom banks is a viable option to conserve and to know the biomolecular arsenal of these rich secretions that can be

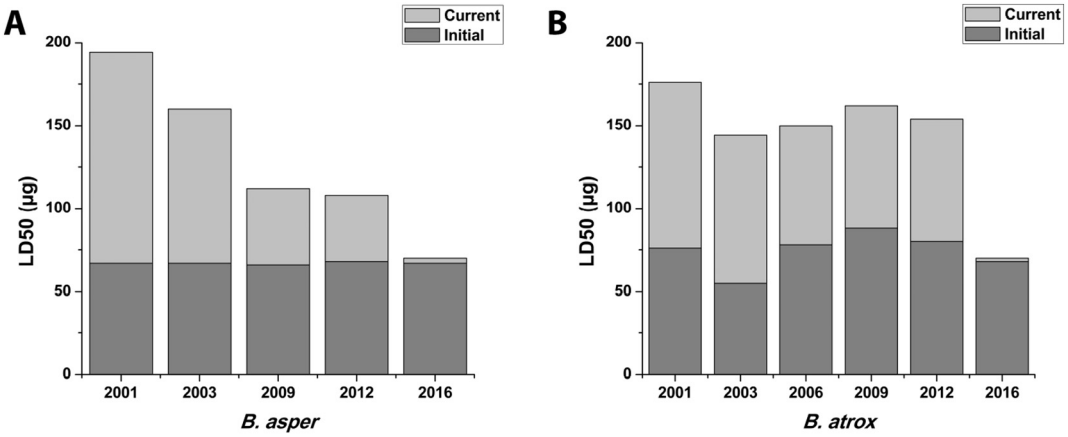

Fig. 6. Lethality of desiccated snake venoms following some years of storage. The LD_{50} of each venom sample was determined at twice (at the milking and in 2017–current) and compared.

explored and investigated by several branches of the science. However, it is extremely important to establish adequate storage conditions to preserve the functional properties of the venoms belonging to the collection (Schöttler, 1951). In the past, INSPI was the main responsible for the production of equine antivenom in Ecuador. Venoms of the two main venomous snakes present in the country have been stored for more than a decade in INSPI laboratory. The venom samples collected were desiccated for storage. According to Willemse and Hattingh (1978) desiccated venoms are less stable than lyophilized ones. This storage type is less favorable for the stability of venom components as showed by the results of a previous study (Schöttler, 1951). Some authors argue the need for certain conditions to maintain the structural and functional integrity of venom pools for several years. It has been described that the storage in a site without light, without exposure to air and with hermetic seal are more efficient and suitable for the preservation of samples. Otherwise, the venom proteins would be affected by degradation (Schöttler, 1951; Jesupret et al., 2014).

Several studies have reported that some of the enzymatic activities, as well as the toxicity of the stored venoms are conserved even in desiccated venoms (Schöttler, 1951; Russell et al., 1960; Jesupret et al., 2014; Hatakeyama et al., 2018). However, the feasibility of its use for research and production of snakebite medication remains a topic under discussion. In one of the first studies that addressed this open question, their results showed that antigenicity is significant affected by time (Schöttler, 1951). On other hand, in a more recent study with an 80 year lyophilized venom collection indicated that the use of these complex biological secretions should be limited to research (Jesupret et al., 2014). Structural changes in the immunogenic components of the samples would compromise their use for the antivenoms production (Willemse and Hattingh, 1978). With this background, we assessed the stability of biochemical composition and enzymatic and toxicological activities of *B. atrox* and *B. asper* venom collection.

The analysis of venom fractions by SDS-PAGE show slight differences between the samples. The band patterns associated with the main families of venom proteins typically characterized in *Bothrops* species are observed in our analyses. This is why our study focuses on the enzymatic and toxicological activity of phospholipases, metalloproteinases and serinoproteases. The elution profile of toxins identified in our study is very similar to proteomic profile of *Bothrops atrox* from Colombia (Nunez et al., 2009) and *Bothrops asper* from Costa Rica (Alape-Giron et al., 2008), with PLA_2s eluting first than serine proteases and metalloproteases.

Gels and chromatograms confirmed that phospholipases A_2 are very abundant in both Ecuadorian venoms according to peak area monitored at 280 nm, similar to biochemical composition demonstrated by other venomic investigations with samples from other Latin American countries (Alape-Giron et al., 2008; Nunez et al., 2009). It was observed that PLA_2 and thrombin-like activities seems not significant altered with storage time. This is in line with the results presented by Hatakeyama et al. (2018) in the study of a venom collection of *Bothrops jararaca.* Phospholipases are toxins very stable, mainly due to presence of disulfide bridges (Almeida et al., 2016). In this case, the differences between activities of some samples would be associated with the variations in the collection areas, especially in *B. asper*, as can be visualized in map of sampling performed. Several studies have shown the phenotypic intraspecific variation in *B. asper* and *B. atrox* populations, which have a wide geographical distribution (Alape-Giron et al., 2008; Nunez et al., 2009; Gutierrez et al., 2010; Sousa et al., 2017). However, this does not apply to all cases, because there are venoms from different years that were collected in the same region. The PLA_2 activity has also been related to the CK activity (Lomonte and Rangel, 2012). In spite of that, it is important to denote the presence of myonecrotic PLA_2s without catalytic activity in snake venoms. Due to this, the muscle damage is not necessarily going to be similar to the enzymatic activity of PLA_2s from the venom pools analyzed.

Edematogenic action of venom batches was also evaluated. The increased venom-injected paw does not show a significant interdependence with the time of storage. Venom enzymes like PLA_2s and SVSPs has been associated with the edema formation (Carneiro et al., 2002). In fact, the snake venom PLA_2 possesses a great similarity with mammalian PLA_2, which participate in the cascades of inflammation (Carvalho et al., 2013). Apparently, by electrophoresis and chromatography from crude venoms there is no significant variation of these proteins in the batches analyzed.

Another characteristic effect of ophidian envenomation is the lethality of the venom. The evaluation of this toxicological feature indicated that the oldest venoms were those with higher LD_{50}, while the newest showed a superior lethality. Similar results were obtained in the evaluation of the collection of *B. jararaca* venoms from Instituto Butantan (Hatakeyama et al., 2018), which demonstrated that the venoms pools most recently collected showed greater lethality than the older samples. Unlike other studies, we had access to initial data taken from the collection. This valuable information allows us to compare the variability in lethal effect with respect to storage time. Our results showed that the most recent pools retained the highest level of lethality observed in the initial assays. The lethality has been correlated with hemorrhagic activity in bothropic species by Ferreira et al. (1992) through statistical analysis. This would be the reason why both activities show similar trend with regard to storage time. The results of hemorrhagic activity shows are similar to those observed in the lethal dose assays. Many studies attribute the variation in the hemorrhagic activity to the geographical variation (Sousa et al., 2017). However, in

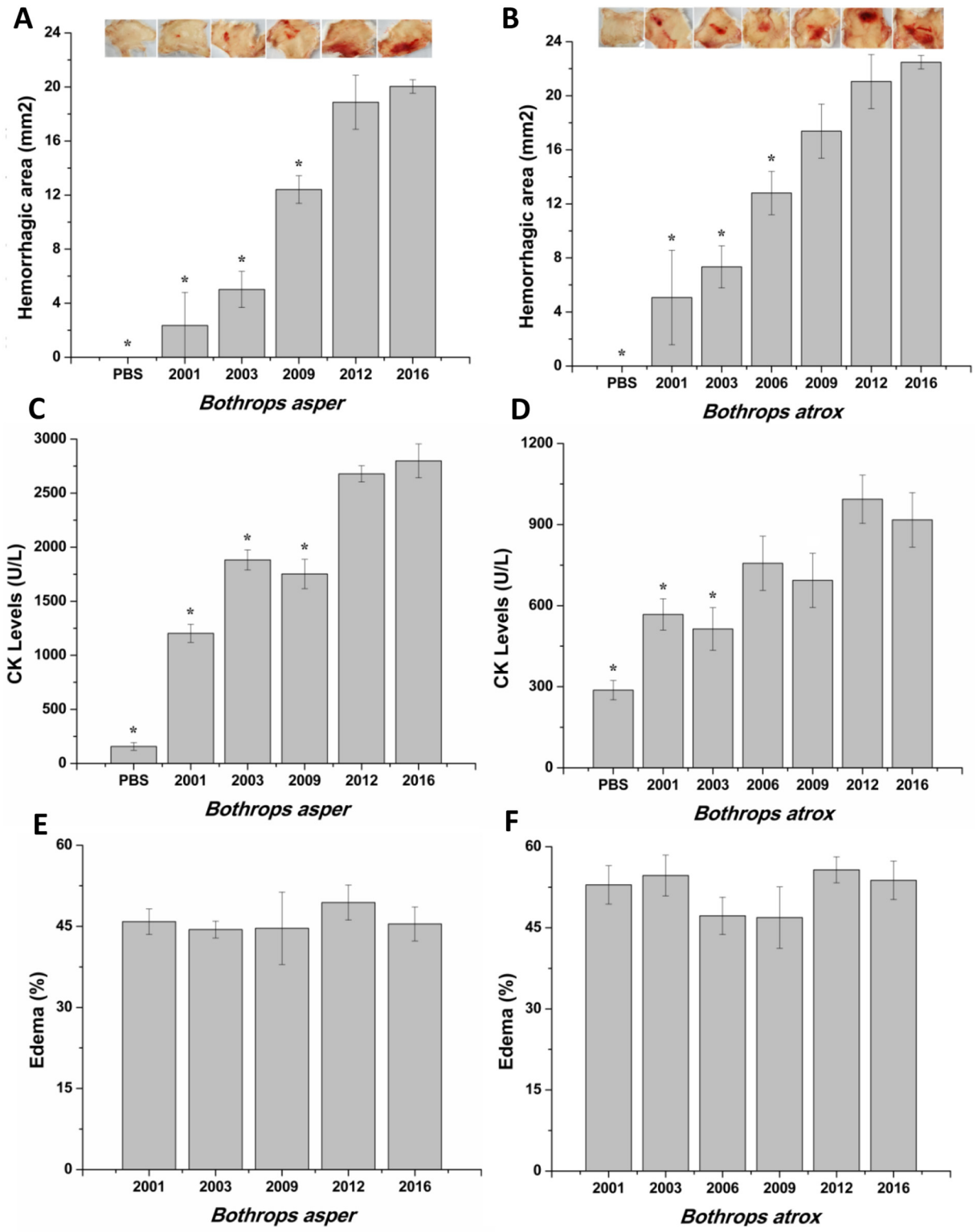

Fig. 7. Toxicological effects induced by desiccated *Bothrops asper* (left) and *Bothrops atrox* (right) snake venoms. The hemorrhagic (A, B), myotoxic (C, D), and edematogenic activities (E, F) from Ecuadorian desiccated samples were assayed using CD-1 male mice (18–20 g; 3 months) according to methodology described in experimental section. *Statistically significant ($p < 0.05$) compared to the venom batch from 2016.

our case the venom samples of *B. atrox* were taken from the same area during the period from 2001 to 2009. This minimizes the probability that geographical factors influence in the variability of the effects observed. For this reason, the effect of storage time may not be ruled out as the driver of the fluctuation.

The diverse components of the venom act in a synergetic way to exert the clinical manifestations of the ophidism (Schezaro-Ramos et al., 2018) but, indubitably, metalloproteinases (PIII-SVMPs) are key biomolecules for microvascular disruption and hemorrhage caused by bothropic envenomations (Gutierrez et al., 2005; Mora-Obando et al., 2014; Gutiérrez et al., 2016; Sousa et al., 2017). Some studies have shown that *Bothrops* venoms with a higher content of these

metalloenzymes have a high lethality and marked hemorrhagic activity (Guércio et al., 2006; Nunez et al., 2009). PIII-SVMPs (40–60 kDa) are considered as the toxins with the highest antigenicity in *Bothrops* venoms (Queiroz et al., 2008; Sousa et al., 2017). Several works have identified that these toxins are found in great abundance in bothropic venoms, and in some cases, they are the major components (Alape-Giron et al., 2008; Tashima et al., 2008; Nunez et al., 2009; Calvete et al., 2011; Mora-Obando et al., 2014). Electrophoresis and chromatogram profiles revealed that both bothropic venoms studied here are rich in these proteins and agreed with the proteomic data of *B. atrox* and *B. asper* previously characterized in other countries. The enzymatic activity of this family of proteins shows similar results as the lethality and the hemorrhagic activity of the venoms. The caseinolytic activity is greater in the samples of 2012 and 2016 compared to the older ones. The RP-HPLC and SDS-PAGE analysis reveals differences between in peak absorbance of samples and the bands located in the PIII-SVMPs mass range. This can be clearly seen in *B. atrox* samples, where the most intense bands are visualized in the fresher venoms. The data are in line with the lack of hemorrhagic activity up to 2.5 mg/mice observed with *B. jararaca* pool from 1963 in a recent study (Hatakeyama et al., 2018). Due to clinical relevance, the degradation or structural destabilization with storage time of metalloproteinase should be investigated, since it would compromise its use for the generation of heterologous immunoglobulins to treat snakebite envenoming. On other hand, the presence of inhibitors of autolytic proteins as components of *Bothrops asper*, *Daboia russelii,* rattlesnakes and other snake species has been reported (Francis and Kaiser, 1993; Munekiyo and Mackessy, 2005; Yee et al., 2017). In the absence of these inhibitors, it has been observed that there is degradation of venom toxins (Munekiyo and Mackessy, 1998). During the storage time, the absence or degradation of these inhibitors may then cause the activity of the proteases. Specifically in this case, the metalloproteinases would be the most affected group.

5. Conclusions

Venom banks represent promising source of valuable samples for research. However, it is very important to control the storage conditions, which can cause changes in the activities of the venom batches. It should be considered that these changes can affect directly the immunogenic characteristics of venoms, so their clinical use for the production of antivenoms should be better investigated. The metalloproteinases are abundant toxins and relevant immunogens in bothropic venoms, therefore, more studies are needed on the endogenous venom inhibitor peptides, and how their integrity is correlated with the stability of these and other toxins in venoms stored for years.

Declaration of competing interest

The authors declare no relevant conflicts of interest concerning the present manuscript.

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