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Interpopulational variation and ontogenetic shift in the venom composition of Lataste's viper (*Vipera latastei*, Boscá 1878) from northern Portugal

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Abstract

Lataste's viper (*Vipera latastei*) is a venomous European viper endemic to the Iberian Peninsula, recognised as medically important by the World Health Organization. To date, no comprehensive characterisation of this species' venom has been reported. Here, we analysed the venoms of juvenile and adult specimens of *V. latastei* from two environmentally different populations from northern Portugal. Using bottom-up venomics, we produced six venom proteomes (three per population) from vipers belonging to both age classes (i.e., two juveniles and four adults), and RP-HPLC profiles of 54 venoms collected from wild specimens. Venoms from juveniles and adults differed in their chromatographic profiles and relative abundances of their toxins, suggesting the occurrence of ontogenetic changes in venom composition. Specifically, snake venom metalloproteinase (SVMP) was the most abundant

toxin family in juvenile venoms, while snake venom serine proteinases (SVSPs), phospholipases A_2 (PLA$_2$s), and C-type lectin-like (CTLs) proteins were the main toxins comprising adult venoms. The RP-HPLC venom profiles were found to vary significantly between the two sampled localities, indicating geographic variability. Furthermore, the presence/absence of certain peaks in the venom chromatographic profiles appeared to be significantly correlated also to factors like body size and sex of the vipers. Our findings show that *V. latastei* venom is a variable phenotype. The intraspecific differences we detected in its composition likely mirror changes in the feeding ecology of this species, taking place during different life stages and under different environmental pressures.

Keywords: snake venom; toxins; bottom-up proteomics; ontogenetic change; geographic variability; venom variation

Significance

Lataste's viper (*Vipera latastei*) is a medically important viper endemic to the Iberian Peninsula, inhabiting different habitats and undergoing a marked ontogenetic dietary shift. In the current study, we report the first proteomic analysis of *V. latastei* venom from two environmentally different localities in northern Portugal. Our bottom-up venomic analyses show that snake venom serine proteinases (SVSPs), phospholipases A2 (PLA2s), and C-type lectin-like (CTLs) proteins are the major components of adult *V. latastei* venom. The comparative analysis of young and adult venoms suggests the occurrence of ontogenetic shift in toxin abundances, with snake venom metalloproteinases (SVMPs) being the predominant toxins in juvenile venoms. Moreover, geographic venom variation between the two studied populations is also detected, with our statistical analyses suggesting that factors like body size and sex of the vipers are possibly at play in its determination.
Our work represents the first assessment of the composition of *V. latastei* venom, and the first step towards a better understanding of the drivers behind its variability.

INTRODUCTION

Snake venoms typically comprise dozens to hundreds of bioactive compounds able to disrupt the homeostasis of the organism in which they are injected, affecting its cardiovascular,

neuromuscular, and/or haemostatic systems [1,2]. Fulfilling both predatory and defensive purposes, and being therefore directly linked to the snake's fitness and survival, snake venom is an ecologically critical functional trait, and under strong natural selection [3-12]. Variation in snake venom composition can be found at multiple taxonomic levels [13,14], with several factors potentially involved in its occurrence (e.g., phylogeny, age, sex, prey preference) [15-19]. Recent reports suggest that high prey diversity, for instance, may play a key role in determining a higher variety of toxic components. The effective subjugation of prey items with different physiologies, in fact, very likely requires different venom compositions [9, 20-22].

Compositional and functional variation between the venoms of juvenile and adult conspecifics (i.e., ontogenetic variation) has been documented for numerous snake species [23-25]. Frequently, the occurrence of this variation has been correlated with differences in diet and/or foraging strategies between individuals belonging to different age classes [26-28]. As an example, in *Bothrops asper* and *Crotalus viridis*, two species undergoing an ontogenetic dietary shift from a mainly ectotherm based diet to a diet including mostly endotherm prey, juveniles appear to produce more metalloproteinases than the adults [29,30]. Such variation is common also between specimens originating from different areas (i.e., geographic variation) [31-35]. Its occurrence is often associated with differences in the diets of snakes from different geographic regions [3,16]. Indeed, environmental pressures varying across geography likely determine changes in prey communities, ultimately influencing the feeding ecology of snake populations [36,37]. In light of the critically adaptive value and fast evolution rates of snake venoms [38], these changes are thus likely to determine differences in their composition [39,40].

Lataste's viper (*Vipera latastei*, Boscá 1878) is a medium-sized European viper (family Viperidae, subfamily Viperinae) endemic to the Iberian Peninsula (see [41]), included within the clade of the Western Mediterranean vipers (i.e., *Vipera* 1; [42,43]). This species inhabits a wide variety of Mediterranean ecosystems and habitats, ranging from sea level to 2800 m a.s.l. (in Spain), and shows a general pattern of population isolation in mountain ranges [44]. An ambush predator, *V. latastei* feeds on a wide variety of prey items, including mainly reptiles and small mammals and, more occasionally, arthropods, amphibians, and birds [45-47]. Interestingly, Santos et al. [48] found a certain degree of variation in diet composition across different environments, with small mammals being the vipers' main prey in colder, more humid areas, whereas the proportion of reptiles was higher in warmer, drier

environments. Ontogenetic shift in diet has also been reported for *V. latastei*: juveniles feed mainly on ectothermic prey (i.e., invertebrates, reptiles, amphibians) and, to a lesser extent, on endotherms (e.g., shrews), whereas the diet of adult vipers is mainly composed of rodents ([44] and references within).

Lataste's viper is recognised as a medically important snake species by the World Health Organization [49], and is one of the few native animal species of the Iberian Peninsula with the potential to cause severe illnesses to humans [50,51]. Although the venom produced by *V. latastei* is less toxic (intravenous $LD_{50 =} 15.1$ μg/mouse, 18-20 g mice [52]) than the venoms of other congeneric European vipers (e.g., *Vipera ammodytes ammodytes*, intravenous LD50 = 8.4 μg/mouse, 18-20 g mice [52]), this species is able to inject considerable amounts of it (Detrait et al. [53] estimated that the subspecies *V. l. gaditana* can produce about 20 mg of dry venom per 100 g of body weight). Studies aiming at characterising the effects of *V. latastei* venom found it to possess coagulopathic and haemorrhagic activity [53-55]. Furthermore, comparative analyses of SDS-PAGE protein patterns of venoms collected from several species of the genus *Vipera* suggested the presence of a certain degree of geographic variation in the composition of the venom of Lataste's viper [56]. Nevertheless, detailed venomic studies for this species are missing.

In the present work, we (a) provide the first proteomic characterisation of the venom of *V. latastei*, (b) investigate the presence of ontogenetic change in the composition of the species' venom; (c) assess the level of geographic venom variation between two environmentally different localities within the species' north-western distributional range in Portugal. Considering the existence of an ontogenetic dietary shift in this species, we expect to detect a corresponding ontogenetic shift in venom composition. Furthermore, we hypothesise that the environmental differences between the two sampled localities (one located on the coast and under considerable anthropic disturbance, the other located in the mountains and less impacted by human activities) might influence factors affecting the vipers' feeding ecology (e.g., prey community), thus driving interpopulational venom variability.

MATERIALS AND METHODS

Study areas

Vipers were collected in two environmentally different localities about 75 km from each other, situated in the north-western distributional range limit of the species (see Fig. 1).

The locality hereafter named "Vila Chã" is located on the northern Atlantic coast of Portugal (41.28 N, 8.72W), close to the village of Vila Chã in the municipality of Vila do Conde, and within the Regional Protected Landscape of the Vila do Conde Coast. The climate in this area is temperate, with dry or mild summers (Csb climate type [57]). The average annual temperature is around 14°C, with total annual rainfall ranging from 1600 to 2000 mm/year [58]. The landscape is characterised by sandy beaches with granitic rock outcrops, surrounded by agricultural fields (mostly dedicated to maize farming) with stonewalls (Fig. 1B). The natural vegetation is mainly herbaceous or scrubby (e.g., *Erica* sp., *Ulex* sp.), with the invasive ice plant (*Carpobrotus* sp.) being very abundant, especially in the areas closest to the coast. While an exhaustive description of the feeding ecology of Lataste's vipers from Vila Chã is currently missing, an estimate based on the analysis of prey remains obtained from 23 adult specimens collected there suggests that adult vipers from this population mainly prey on small mammals (e.g., genus *Microtus*; authors. unpublished data).

The locality hereafter named "Gerês" is located in the Gerês/Xurés Mountains, within the Gerês-Xurés Transboundary Biosphere Reserve (Northern Portugal - North Western Spain), at an elevation ranging from 700 to 900 m a.s.l. (41.80 N, 8.13W). The area is characterised by temperate climate, with a dry season and temperate summer (Cfb climate type[57]), with average annual temperature around 9°C. Precipitation levels are generally high, with total annual rainfall ranging from 2800 to 3200 mm/year [58]. The landscape consists of granite mountains, with abundant rock piles (Fig. 1C), and vegetation generally composed of woodlands (dominated by *Quercus robur*) and scrublands (with *Erica* sp., *Genista* sp., *Rubus* sp., *Ulex* sp.). From the analysis of prey remains collected from 101 *V. latastei* specimens from the Gerês Mountains, Brito [45] found evidence of ontogenetic dietary shift, with adults feeding almost exclusively on small mammals (i.e., mainly genus *Apodemus*, and, less abundantly, genera *Crocidura*, *Sorex* and *Microtus*), composing >90% of their diet, and juveniles mainly feeding on ectotherms (e.g., genus *Podarcis*), composing >60% of their diet.

Vipers from Vila Chã and Gerês share a very recent common ancestor, as demonstrated by the fact that they belong to the same mitochondrial lineage, and even the same haplotype (i.e., the West CNW lineage, haplotype CNW1 [43,59]. Differences in venom composition may thus be due to different local selective regimes rather than neutral evolutionary divergence.

Viper sampling and venom collection

A total of 63 specimens of *V. latastei* were used in this study. Specifically, 38 specimens were collected in Vila Chã, and 25 specimens were collected in Gerês (Table S1). Variation in head scale fragmentation (analysed through the software APHIS v.1.0.0 [60]), body scale counts, and dorsal colouration, were used as criteria to identify each specimen, and thus recognise potential recaptures.

Sampling was performed between 2018 and 2021, from spring to late summer/early autumn. Venom was obtained by letting each viper bite a parafilm-covered 1.5 ml tube (Eppendorf, Hamburg, Germany). After venom extraction, sex was determined, and the snout-vent length (SVL) of each sampled snake was measured. Vipers were then released exactly where they had been captured. All vipers and venom samples were collected with permission of Instituto da Conservação da Natureza e das Florestas (ICNF), Portugal (ref. 537/2018, 362/2019, and 295/2020), and Xunta de Galicia, Spain (ref. EB 017 2019).

We followed Pleguezuelos et al. [61] to assign each animal to an age class, i.e., all males with SVL > 240 mm and all females with SVL > 263 mm were considered adults, while vipers with SVLs smaller than these values were considered juveniles. In the light of this classification, our dataset ultimately consisted of 32 adult and 6 juvenile venom samples from Vila Chã, and 20 adult and 5 juvenile venom samples from Gerês (Table S1). Crude venoms were stored at -20°C, and lyophilised in a Scanvac (Coolsafe, Lynge, Denmark) freeze dryer. After lyophilisation, the venoms were transported to the Evolutionary and Translational Venomics Laboratory of the Institute of Biomedicine of Valencia (Spain) for proteomic analyses.

Preliminary screening of the venoms

For a first assessment of similarities and differences among the collected venoms, all venom samples were screened through reverse-phase high-performance liquid chromatography (RP-HPLC). Specifically, 0.2 mg of each lyophilised venom were dissolved in 80 μL of 0.1% trifluoroacetic acid (TFA) and 5% acetonitrile (ACN). Insoluble material was spun down in an Eppendorf centrifuge at 13,000 g for 10 min at room temperature, and the proteins contained in 40 μL were fractionated by RP-HPLC using a Discovery Bio Wide C18 (150 ×

2.1 mm, 3 μm particle size, 300 Å pore size) Supelco column and Agilent LC 1200 chromatograph equipped with DAD detector. The column was developed at 0.4 mL/min flow rate applying the following gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B): the following gradient was used for elution: 5% isocratically (5%B) solution B for 1 min, followed by 5–25% B for 5 min, 25–45% B for 30 min, and 45–70% for 5 min. Venom samples were analysed individually. However, due to the very low amount (<5 μL) of venom we were able to collect from some of the juveniles, we pooled them. Two pools were generated: one for the population of Vila Chã, comprising samples from five juveniles (i.e., 20VL001, 20VL003, 20VL038, 21VL011,21VL019), and one for the population of Gerês, made of samples from four juveniles (i.e., 18VL260, 19VL034, 19VL456, 19VL458). These pools were analysed using the same chromatographic protocol mentioned above. Details about the pooled venom samples are reported in Table S1.

All the chromatographic profiles produced were then overlapped and compared visually, in order to identify the most different ones in number and relative intensities of their toxin peaks, to be submitted to bottom-up venomics analysis [62,63].

Venom fractionation by RP-HPLC

Pre-MS decomplexation of individual venoms was performed by reverse-phase chromatography (RP-HPLC), following the protocol described by Pla et al. [34]. As a first step, 1 mg of each lyophilised individual venom was dissolved in 200 μL of 0.1 % trifluoroacetic acid (TFA) and 5% acetonitrile (ACN). In the case of 19VL013, we could use only 0.3 mg of lyophilised venom, because of the low amount of venom we were able to collect from this specimen. Insoluble material was spun down in an Eppendorff centrifuge at 13,000 g for 10 min at room temperature, and the proteins contained in 40 μL were fractionated by RP-HPLC using a Teknokroma Europa C18 (250 × 4 mm, 5 μm particle size, 300 Å pore size) column and Agilent LC 1100 High Pressure Gradient chromatography system equipped with DAD detector. The following gradient was used for elution: 5% isocratically solution B (0.1% TFA in ACN) for 5 min, followed by 5–25% B for 10 min, 25–45% B for 60 min, and 45–70% for 10 min, at 1 mL/min flow rate. Peaks were collected manually and dried in a centrifugal vacuum evaporator (SpeedVac®, ThermoSavant).

Molecular mass determination

Molecular masses of the RP-HPLC-purified proteins were estimated by SDS-PAGE analysis or determined by electrospray ionization (ESI) mass spectrometry (MS). For SDS-PAGE analysis, dried aliquots of the RP-HPLC fractions were redissolved in sample buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 30% glycerol, 0.02% bromophenol blue, with or without 1% 2-mercaptoethanol) and heated at 100 °C for 15 min. SDS-PAGE analysis was run under non-reducing and reducing conditions and the gels were stained with Coomassie Brilliant Blue G-250. For ESI-MS mass profiling, the proteins eluted in the different RP-HPLC fractions of individual venoms were separated by nano-Acquity UltraPerformance LC® (UPLC®) using BEH130 C_{18} (100μm x 100mm, 1.7 μm particle size) column in line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. The flow rate was set to 0.6 μL/min and the column was developed with a linear gradient of 0.1% formic acid in water (solution A) and 0.1% formic acid in ACN (solution B), isocratically 1% B for 1 min, followed by 1-12% B for 1 min, 12-40% B for 15 min, 40-85% B for 2 min. Monoisotopic and isotope-averaged molecular masses were calculated by manual deconvolution of the isotope-resolved multiply-charged MS1 mass spectra.

Characterisation of the toxin families present in the venoms

Protein bands were excised from Coomassie Brilliant Blue stained SDS-PAGE gels, and subjected to automated in-gel reduction (10 mM dithiothreitol, 30 min at 65 °C) and alkylation (50 mM iodacetamide, 2 h in the dark at room temperature), followed by overnight digestion with sequencing grade trypsin (66 ng/μL in 25 mM ammonium bicarbonate, 10% ACN; 0.25 μg/sample). The procedure was performed on a ProGest Protein Digestion Workstation (Genomics Solution). Tryptic digests were dried in a vacuum centrifuge (SpeedVac®, ThermoSavant), redissolved in 15 μL of water containing 0.1% formic acid, and submitted to LC-MS/MS. Tryptic peptides were separated by nano-Acquity Ultra Performance LC® (UPLC®) using a BEH130 C_{18} (100μm x 100mm, 1.7 μm particle size) column in-line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. The flow rate was set to 0.6 μL/min and the column developed a linear gradient of 0.1% formic acid in MilliQ® water (solution A) and ACN (solution B) with the following conditions: isocratically 1% B for 1 min, followed by 1–12% B for 1 min, 12–40% B for 15 min, 40–85% B for 2 min. For peptide ion fragmentation by collision-induced dissociation tandem mass

spectrometry (CID-MS/MS), the electrospray ionization (ESI) source was operated in positive ion mode, and both singly and multiply charged ions were selected for CID-MS/MS at sample cone voltage of 28 V and source temperature of 100 °C. The UPLC eluate was continuously scanned from 300 to 1990 m/z in 1 s and peptide ion MS/MS analysis was performed over the range m/z 50–2000 with scan time of 0.6 s. The parent proteins were identified by interpretation of fragmentation spectra: a) manually (*de novo* sequencing), b) searched against the NCBIprot/SwissProt non-redundant databases using the online form of the MASCOT Server (version 2.6) at http://www.matrixscience.com or in an automated way, and c) processed in Waters Corporation's ProteinLynx Global SERVER 2013 version 2.5.2. (with Expression version 2.0) The search parameters were: taxonomy: bony vertebrates; enzyme: trypsin (two-missed cleavage allowed); MS/MS mass tolerance was set to ±0.6 Da; carbamidomethyl cysteine and oxidation of methionine were selected as fixed and variable modifications, respectively. All matched MS/MS data were manually checked. For missing/incomplete identifications, the MS/MS spectra were interpreted manually (*de novo* sequencing), and amino acid sequence similarity searches were performed at https://blast.ncbi.nlm.nih.gov/Blast.cgi against the non-redundant protein sequences database, using the default parameters of the BLASTP program [64]. Fragmentation spectra of peptides that yielded daughter ions diagnostic of the endogenous peptides BPP (m/z 116.1, y1=P, and m/z 213.1, y2=PP) or tripeptide inhibitors of metalloproteinases (SVMPi) at m/z 205.1 (y1=W), m/z 112.1 (b1=pyroglutamate, Z) and m/z 240.1 (b2=Z(K/Q)) or m/z 226.1 (b2= ZN) [65,66] were also sequenced manually.

Relative quantification of the venom proteomes

To compile the relative composition of toxin families in the venom proteomes, we applied the 3-step hierarchical venom proteome quantification protocol developed in the Evolutionary and Translational Venomics Laboratory of the Institute of Biomedicine of Valencia [62,63]. In the first step, the relative abundances of the reverse-phase chromatographic peaks were calculated by dividing peak areas by the total area of the chromatogram, using ChemStation B.01.01.069 (Agilent Technologies). Recording the eluate at the absorbance wavelength of the peptide bond [190–230 nm], and applying the Lambert-Beer law ($A = \varepsilon c l$, where A = absorbance; ε is the molar absorption [extinction] coefficient, $[M^{-1} cm^{-1}]$; c = concentration $[M]$; and l = light path length $[cm]$, these percentages correspond to the "% of total peptide

bond concentration in the peak". For chromatographic peaks containing single components (as judged by SDS-PAGE and/or MS), this figure is a good estimate of the % by weight (g/100 g) of the pure venom component [67]. When more than one venom protein was present in a reverse-phase fraction, their proportions (% of total protein bands area) were estimated by densitometry of Coomassie-stained SDS polyacrylamide gels using Image Studio Lite®, version 5.2 (LI-COR Biosciences) software. Conversely, the relative abundances of different proteins contained in the same SDS-PAGE band were estimated based on the relative ion intensities of the three most abundant peptide ions associated with each protein by MS/MS analysis. The relative abundances of the protein families present in the venom were calculated as the ratio of the sum of the percentages of the individual proteins belonging to the same family to the total area of venom protein peaks in the reverse-phase chromatogram.

Assessment of interpopulational venom diversity

Considering the environmental differences present between the montane site of Gerês and the coastal site of Vila Chã, we sought to investigate the potential presence of variation among the *V. latastei* venoms collected in these two localities. To assess the levels of such variation, we followed the individual-based approach reported by Zancolli et al. [68]. Chromatograms recorded for the two juvenile venom pools, not providing individual-level information, were not taken into account. Briefly, we generated a presence-absence matrix of the peaks present in each individual RP-HPLC chromatogram we produced. Peaks with frequency equal to 1 (i.e., present in every chromatogram, and thus not informative) or difficult to identify were excluded from the dataset before performing the analyses. The final binary matrix was used to calculate individual venom richness (i.e., total number of peaks detected in each chromatogram), and to analyse patterns of venom variation using non-metric multidimensional scaling (NMDS) based on pairwise Bray-Curtis similarity distances among the chromatograms [69]. In NMDS analyses, the stress value provides an estimate of the goodness of fit of the analysis performed. An overall stress value above 0.2 generally indicates poor fit [70]. In order to keep the stress value below the 0.2 threshold, we included an additional dimension to our NMDS analysis, thus opting for a three-dimensional NMDS analysis. The individual NMDS scores on the first two axes (i.e., NMDS1 and NMDS2) were then used to produce an ordination plot representing the associations between the RP-HPLC venom profiles.

We used simple linear regressions to investigate the relationship between the individual scores on the first NMDS axis (i.e., NMDS1, used as response variable) and snout-vent length (SVL), sex, date of collection of the venom samples (i.e., the ordinal day of the year), and population of origin (Gerês or Vila Chã). We scaled all continuous variables (i.e., mean = 0; SD = 1) for easier comparison of their effect size. Collinearity between explanatory variables was low [Variance Inflation Factors (VIF) always < 2], thus we included all of them in the models. We built models using all possible combinations of independent variables, and ranked them on the basis of the Akaike's Information Criterion corrected for small sample sizes (AICc) [71]. The final set of candidate models was obtained by removing all models which had a simpler nested version with lower AICc [72].

We also tested whether the presence or absence of specific peaks identified in the venom chromatograms was significantly correlated with snout-vent length, sex, date of collection of the venom samples, and population of origin. Specifically, we used binomial Generalised Linear Models (GLMs), considering the presence/absence of each peak as binomial dependent variable, and the four above-mentioned variables as predictors. Also in this analysis, VIF values lower than 2 confirmed low collinearity between the explanatory variables, which were thus all included in the models. These were again built using all possible combinations of independent variables, and ranked on the basis of their AICc scores.

We used the packages *vegan* [73] to perform NMDS, *MuMIn* to build the full set of models [74], and *ggeffects* [75] to plot model predictions. All analyses were performed in R environment (version 4.1.1 [76]).

RESULTS AND DISCUSSION

Analysis of the chromatograms

A total of 56 chromatograms were recorded, namely 54 for individual venoms and two for the juvenile venom pools (see Supplementary File 1). By visually comparing the chromatographic profiles obtained through this approach, we detected differences in chromatographic peaks present within each of the two sampled populations. The most striking difference concerned the intensity of a peak appearing around minute 17, evident in some venoms (although with different intensity/height) and completely absent in others (Fig. 2). This peak was absent in the profiles produced from the individual juvenile venoms (i.e., 19VL013 and 19VL026) and

in the pool of juvenile venoms from Gerês, but it was evident in the pool of juvenile venoms from Vila Chã (Fig. S1). We also noted other differences among the analysed chromatograms, mainly involving different intensities in the initial reverse-phase chromatographic peaks (eluting between 7-10 min) and in late-eluting peaks (39-44 min; see Fig. 2). Based on these differences, we selected the following six representative venoms to be used for the proteomic analyses: 19VL013 (juvenile), 18VL253 (adult), and 18VL011(adult) from the Vila Chã population; 19VL026 (juvenile), 18VL258 (adult), and 19VL029 (adult) from the Gerês population.

Protein composition of *V. latastei* venom

The number of chromatographic peaks recovered from the six venoms selected for proteomic analyses varied between 43 (in the juvenile 19VL013 from Vila Chã) and 69 (in the adult 18VL253, from Vila Chã; see Supplementary File 2). The number of toxin classes identified across the analysed venoms varied between 11 (19VL013) and 18 (18VL253, from Vila Chã). Table 1 and Figure 3 display the relative abundances of the components of the six venom proteomes produced. The most abundant protein families retrieved across the six venom proteomes, although unequally distributed, were: snake venom metalloproteinase (SVMP), particularly of class PIII (PIII-SVMP) snake venom serine proteinase (SVSP); D49-phospholipase A$_2$ (PLA$_2$); and C-type lectin-like (CTL; see Table 1 and Fig. 3). These four toxin families are the predominant components of all the Old World viper (i.e., subfamily Viperinae) venoms characterised to date, accounting for 60-90% of their total venom proteomes [77]. Whether acting alone or synergistically, they likely contribute to the coagulopathic and haemorrhagic effects induced by *V. latastei* venom [53-55]. Specifically, CTLs and SVSPs affect blood coagulation, fibrinolysis, and platelet aggregation [78-80], PLA$_2$s can cause haemolysis and inhibition of the coagulation cascade [81], and PIII-SVMPs are notorious for their mainly haemorrhagic effects [82-84]. The potent action of PIII-SVMPs, resulting in severe haemorrhage and tissue lesions [85], has typically been correlated to fast prey subjugation/killing [2,3,86]. These toxins were particularly abundant in the venoms of juvenile Lataste's vipers (Table 1; Fig. 3). Given the inverse relationship existing in venomous snakes between venom yield and SVL [87], it is possible that high levels of PIII-SVMPs might aid young *V. latastei* specimens in subduing their prey, despite the low amount of venom they can deploy. PIII-SVMPs might also be at play in aiding prey digestion, but

evidence supporting this role is controversial (see [88]). In addition to these toxins, we found other haemotoxic components in all analysed *V. latastei* venoms, namely BPPs and KTSPis, reported to induce vasodilation and to potentially act as antifibrinolytic agents, respectively [89,90]. The coagulopathic effects of *V. latastei* venom could also be amplified by VNGFs and VEGFs, which we only detected in the venoms of adult specimens. The former are known to cause inflammatory reactions and increase vascular permeability [91], while the latter are angiogenic and lymphangiogenic regulators [92].

A new RTS-disintegrin from the venom of *V. latastei*

A short RTS-disintegrin (monoisotopic ESI-MS of m/z 4432.9, structurally similar to KTS-disintegrins obtustatin [P83469, 93,94], lebestatin [Q3BU14, 95], russelistatin [96], and viperistain [P0C6E2, 97] was identified in five of the six venom proteomes produced (i.e., 18VL253, 18VL011, 19VL026, 18VL258, and 19VL029, see Tables S2-S7). This subfamily of non-canonical disintegrins, which selectively vit the collagen (I and IV)-binding α1β1 integrin [98], form a distinct clade of short disintegrins that emerged by neofunctionalization of a copy of a *RPTLN* gene [99]. (K/R)TS-disintegrins had so far been found only in the venoms of a few Eurasian vipers of the genera *Macrovipera* and *Daboia* (i.e., *D. mauritanica, D. palaestinae, D. russelii, M. lebetina*) [96,100]. Our finding of another member of this subfamily of short disintegrins, for which we have coined the name "latastin", in the genus *Vipera*, indicates that the evolutionary origin of (K/R)TS-disintegrins predates the split of the genera *Daboia* and *Vipera* about 25 Mya, between Oligocene and Miocene [42]. This predicts the occurrence of this subfamily of toxins in the venoms of other species of *Vipera*.

Ontogenetic shift in the venom of *V. latastei*

The venomic data gathered from the six produced proteomes highlighted the presence of distinct compositional differences between juvenile and adult viper venoms, suggesting the occurrence of ontogenetic changes. Specifically, regardless of geographic origin, the venom proteomes exhibited similar patterns of relative toxin family abundances within the same age class, but distinct ones between different age classes (Table 1; Fig. 3). For example, the two juvenile venom proteomes 19VL013 (Vila Chã) and 19VL026 (Gerês) were characterised by very high abundances of PIII-SVMPs (> 40% of the total venom proteomes), followed by

D49-PLA$_2$s (13-17%), CRISPs (5.5-6%), SVSPs (6%), and CTLs (3-7%) (Table 1; Fig. 3). Adult *V. latastei* venom proteomes 18VL253 (Vila Chã) and 18VL258 (Gerês) also shared some compositional features: similar abundances of CTLs (8.5-19%), SVSPs (16-26%), and D49-PLA$_2$s (12-16%), and low levels (5-12%) of SVMPs (Table 1; Fig. 3). The peak eluting in adult venoms at 17 min (see Supplementary File 1), not detected in the two juvenile venoms, contained VEGF, VNGF, D49-PLA$_2$, and CRISP. The content of the peak in question for each of the four adult *V. latastei* venoms produced is reported in Tables S3 (Spot ID 31), S4 (Spot ID 25), S6 (Spot ID 21), and S7 (Spot ID 20).

Interestingly, the proteome produced for venom 19VL029 from Gerês shares features with both juvenile (i.e., predominance of PIII-SVMPs, 23% of the venom proteome) and adult (i.e., high abundances of PLA$_2$s (14.9%), SVSPs (12%), and CTLs (13%)) venom proteomes (Table 1; Fig. 3). The venom sample in question was obtained from an adult female with 280 mm of SVL (see Table S1). While according to Pleguezuelos et al. [61] this value is enough to consider the viper as sexually mature/adult, Brito and Rebelo [101] reported that female Lataste's vipers from Gerês to reach sexual maturity at 350-400 mm of SVL. Geographic variation in growth rate has already reported for some European vipers (e.g., *Vipera berus* [102]), and also suggested for *V. latastei* [44]. Furthermore, evidence suggests that ontogenetic changes in snake venom composition do not occur sharply during the snake's development [103]. In light of the above considerations, it is reasonable to hypothesise that the intermediate venom phenotype observed for viper 19VL029 may indicate that the specimen was collected at a transitional stage between immaturity and maturity.

Snakes are typically considered gape-limited predators, with a positive correlation between the size of a snake and the size of the prey it can ingest [104]. The limited mouth gape of juvenile vipers likely is a morphological constraint, forcing them to essentially feed on ectotherms, with a generally smaller diameter than endotherm prey, until they reach a larger size [45,105]. Juvenile Lataste's vipers from the two studied populations likely mainly feed on small-sized prey with ectotherm physiology (e.g., lizards of the genus *Podarcis*), very abundant in both Vila Chã (authors' personal observation) and Gerês [45]. Conversely, the diet of adult *V. latastei* mainly comprises endotherms (i.e. small mammals), almost absent in the diet of juvenile Lataste's vipers [44,45]. Further highlighting the influence that trophic ecology has on the evolution of snake venom, ontogenetic shifts in snake diet are often mirrored by changes in venom composition [11,15,26,27,30]. In light of this, we suspect the

different venom formulations secreted by juvenile and adult Lataste's vipers may represent adaptive solutions for the effective disruption of their prey's different physiologies.

Assessment of interpopulational venom diversity

The final binary matrix used to assess the interpopulational diversity in venom composition included 34 polymorphic peaks (i.e., not appearing in every chromatogram). Their number varied between 11 (19VL013) and 23 (19VL029 and 19VL454) across the analysed 54 individual chromatograms (see Table S8), and was on average significantly lower in venoms from Vila Chã (17.3) than in those from Gerês (19.3) (Student's t-test: t = 3.37; df = 48.49; p = 0.001). The difference in number of peaks between the two populations was statistically significant ($B \pm SE$ = -2.06 $\pm$ 0.64; t = -3.23; p <0.001). The three-dimensional NMDS analysis achieved an overall stress value of 0.16, indicating that the method could represent the differences between the venom profiles considered. The statistical analyses performed on the individual NMDS1 scores, representing each venom phenotype, found significant differences between Gerês and Vila Chã. This can be seen in the NMDS ordination plot we generated using the individual NMDS scores on the first two axes (i.e. NMDS1 and NMDS2), in which the two populations form two quite distinct groups, although with some level of overlap (Fig. 4).

The model that best described the variation among venom phenotypes (i.e., individual NMDS1 scores) included population and date of collection of the venom samples as independent variables (Table 2). Although venom phenotypes were found to vary significantly between the two studied populations ($B \pm SE$ = -0.31 $\pm$ 0.04; t = -7.07; p <0.001), the effect of the date of collection of the venom sample was marginally significant ($B \pm SE$ = 0.28 $\pm$ 0.14; t = 1.92; p = 0.08). Furthermore, the binomial Generalised Linear Models (GLMs) performed on the 34 peaks taken into account showed that the presence/absence of eleven peaks (i.e., 1, 6, 7, 16, 19, 20, 22, 23, 26, 31, 33) was significantly correlated to at least one of the four independent variables considered, indicating variation of peaks' presence in relation to body size, date of collection, sex, and population (Table S9). More precisely, peaks 16, 20, 22, and 26 were significantly more frequent in vipers from Vila Chã, while the opposite was observed in peaks 1, 6, 7, 23, 31, and 33 (Fig. 5C; Table S10). Peaks 1 and 33 were significantly less detected in individuals collected between October and the end of March (Fig. 5A; Table S10). The probability of detecting peak 6 increased with the

size of the individual (Fig. 5B), while the opposite trend was observed in peak 19 (Fig. 5C; Table S10). Lastly, peak 23 was observed more frequently in females (Fig. 5D).

A fast-evolving phenotype, snake venom has been found to vary in composition depending on factors of different nature, like phylogeny and prey availability [17-19], and high levels of individual toxin variability have been observed in several species (e.g., [3,106,107]). In the light of such a highly dynamic scenario, and with the limited information gathered in this study, trying to understand what could determine the significant differences we detected in venom phenotypes between the two studied populations is difficult. The habitat of the Gerês site is quite pristine, and human activity in its surroundings is limited. On the contrary, human disturbance on the habitat of the Vila Chã sampling site, surrounded by cultivated fields and with people living in its immediate proximity, is very conspicuous. High levels of disturbance are expected to negatively affect species diversity, thus depleting communities [108]. In this context, the better-preserved habitat of Gerês likely hosts a higher number of species and more complex communities than Vila Chã. This could imply the presence in Gerês of more diverse prey items for the vipers to feed on, which might explain the significantly higher average number of peaks detected in their venom profiles. The lack of phylogeographic distinctness between the two populations indeed suggests that differences in venom composition are likely to be due to differences in local selection. In this perspective, it would be interesting for future studies to test whether differences between the *V. latastei* venom phenotypes of Gerês and Vila Chã might be due to differences in prey type or availability between these two populations and thus to rapid venom adaptation.

CONCLUSIONS

This study represents the first assessment of the protein composition of the venom of *Vipera latastei*. Our results show that it is a complex mixture of several components, with four major toxin families (SVMP, SVSP, CTL, and PLA$_2$) likely being the main culprits of the coagulopathic and haemorrhagic effects Lataste's viper envenomation can cause. We found evidence of an ontogenetic shift in the composition of the venom of this species, and showed the presence of a certain degree of variation among adult venoms, with differences between the two populations of Gerês and Vila Chã strongly supported by statistical analyses. While we support the idea of diet being one of the main drivers behind the insurgence of venom variability, with the data currently at our disposal we cannot rule out or confirm the role other

forces might play in determining variation in the venom of *V. latastei*. Our results indeed suggest that other factors, like sex and body size of the viper, might play a role in shaping this species' venom composition. A larger sample size, more detailed information about the species' feeding ecology, and the application of genomics and venom gland transcriptomics shall provide a more comprehensive insight into the drivers behind *V. latastei* venom variability.

The following are the supplementary data related to this article.

Supplementary Figure S1. Comparison between the chromatographic profiles of venoms from single juvenile individuals and juvenile venom pools.

In panel A, the overlapped chromatograms correspond to the venom of specimens from the Vila Chã population, namely 19VL013 (red profile) and the pooled venoms of specimens 20VL001, 20VL003, 20VL038, 21VL011, and 21VL019 (blue profile). In panel B, the overlapped chromatograms correspond to the venom of specimens from the Gerês population, namely 19VL026 (red profile) and the pooled venoms of specimens 18VL260, 19VL034, 19VL456, and 19VL458 (blue profile).

Supplementary File 1. All 56 RP-HPLC profiles of *V. latastei* venoms produced.

Above each chromatogram, code, population of origin (i.e., Vila Chã or Gerês), and age class (i.e., A = adult, J = juvenile) of the corresponding viper are reported. For the chromatograms relative to the two pools of juvenile venoms, the codes of the venoms used to make them are in brackets.

Supplementary File 2. RP-HPLC profiles of the six *V. latastei* venoms used to produce the six venom proteomes by bottom-up venomics.

Above each chromatogram, code, age class, and population of origin (i.e., Vila Chã or Gerês) of the corresponding viper are reported. The numbers linked to the peaks correspond to the numbers of the Spot IDs in Tables S2-S7. Differences in number and elution times of the peaks with the chromatograms reported in Supplementary File 1 are due to the different fractionation conditions.

Supplementary material

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Declaration of Competing Interest

The authors declare no competing interests.

Author contributions

IA, JJC, LS, WW, and FM-F conceived the study. IA and FM-F collected the venom samples. IA, LS, SQB, and YR performed the laboratory analyses. IA and FL performed the statistical analyses. All the authors drafted and revised the manuscript.

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References

1.   B.G. Fry, Research methods, in: B.G. Fry (Ed.), Venomous reptiles and their
        toxins: evolution, pathophysiology and biodiscovery, University Press, Oxford,

2015, pp. 153-214.B.G. Fry, Research methods, in: B.G. Fry (Ed.), Venomous reptiles and their toxins: evolution, pathophysiology and biodiscovery, University Press, Oxford, 2015, pp. 153-214.

2. S.P. Mackessy, Handbook of venoms and toxins of reptiles, 2nd ed, CRC Press, Boca Ratón, 2021.

3. J. Daltry, W. Wüster, R.S. Thorpe, Diet and snake venom evolution, Nature 379 (1996) 537-540. doi:10.1038/379537a0

4. J.J. Calvete, Snake venomics: from the inventory of toxins to biology, Toxicon 75 (2013) 44-62. doi:10.1016/j.toxicon.2013.03.020

5. N.R. Casewell, W. Wüster, F.J. Vonk, R.A. Harrison, B.G. Fry, Complex cocktails: the evolutionary novelty of venoms, Trends Ecol. Evol. 28 (2013) 219-229. doi:10.1016/j.tree.2012.10.020

6. S.A. Smiley-Walters, T.M. Farrell, H.L. Gibbs, Evaluating local adaptation of a complex phenotype: reciprocal tests of pigmy rattlesnake venoms on treefrog prey. Oecologia 184 (2017) 739-748. doi:10.1007/s00442-017-3882-8

7. T.N.W. Jackson, H. Jouanne, N. Vidal, 2019. Snake venom in context: neglected clades and concepts. Front. Ecol. Evol. 7, 332. doi:10.3389/fevo.2019.00332

8. T.N.W. Jackson, I. Koludarov, 2020. How the toxin got its toxicity. Front. Pharmacol. 11, 574925. doi:10.3389/fphar.2020.574925.

9. K. Lyons, M.M. Dugon, K. Healy, 2020. Diet breadth mediates the prey specificity of venom potency in snakes. Toxins. 12, 74. doi:10.3390/toxins12020074xins12020074

10. G. Zancolli, N.R. Casewell, Venom systems as models for studying the origin and regulation of evolutionary novelties, Mol. Biol. Evol. 37 (2020) 2777-2790. doi:10.1093/molbev/msaa133

11. J.J. Calvete, B. Lomonte, A.J. Saviola, F. Bonilla, M. Sasa, D.J. Williams, E.A.B. Undheim, K. Sunagar, T.N.W. Jackson, 2021. Mutual enlightenment: a toolbox of concepts and methods for integrating evolutionary and clinical toxinology via

snake venomics and the contextual stance. Toxicon X. 9-10, 100070. doi:10.1016/j.toxcx.2021.100070

12. T.D. Kazandjian, D. Petras, S.D. Robinson, J. van Thiel, H.W. Greene, K. Arbuckle, A. Barlow, D.A. Carter, R.M. Wouters, G. Whiteley, S.G. Wagstaff, A.S. Arias, L.O. Albulescu, A. Plettenberg Laing, C. Hall, A. Heap, S. Penrhyn-Lowe, C.V. McCabe, S. Ainsworth, R.R. da Silva, P.C. Dorrestein, M.K. Richardson, J.M. Gutiérrez, J.J. Calvete, R.A. Harrison, I. Vetter, E.A.B. Undheim, W. Wüster, N.R. Casewell, Convergent evolution of pain-inducing defensive venom components in spitting cobras, Science 371 (2021) 386-390. https://doi.otg/10.1126/science.abb9303

13. S. Ainsworth, J. Slagboom, N. Alomran, D. Pla, Y. Alhamdi, S.I. King, F.M.S. Bolton, J.M. Gutiérrez, F.J. Vonk, C.H. Toh, J.J. Calvete, J. Krool, R.A. Harrison, N.R. Casewell, The paraspecific neutralisation of snake venom induced coagulopathy by antivenoms. Commun. Biol. 1 (2018) 1-14. doi:10.1038/s42003-018-0039-1

14. T. Tasoulis, G.K. Isbister, 2017. A review and database of snake venom proteomes. Toxins. 9, 290. doi:10.3390/toxins9090290

15. N.R. Casewell, T.N.W. Jackson, A.H. Laustsen, K. Sunagar, Causes and consequences of snake venom variation, Trends Pharmacol. Sci. 41 (2020) 570-581. doi:10.1016/j.tips.2020.05.006

16. N.J. Jr. da Silva, S.D. Aird, Prey specificity, comparative lethality and compositional differences of coral snake venoms, Comp. Biochem. Physiol. Part - C: Toxicol. Pharmacol. 128 (2001) 425-456. doi:10.1016/S1532-0456(00)00215-5

17. M.F.D. Furtado, S.R. Travaglia-Cardoso, M.M.T. Rocha, Sexual dimorphism in venom of *Bothrops jararaca* (Serpentes: Viperidae), Toxicon 48 (2006) 401-410. doi:10.1016/j.toxicon.2006.06.005

18. H.L. Gibbs, L. Sanz, M.G. Sovic, J.J. Calvete, 2013. Phylogeny-based comparative analysis of venom proteome variation in a clade of rattlesnakes (*Sistrurus* sp.). PloS One. 8, e67220. doi:10.1371/journal.pone.0067220

19. S.A. Minton, S.A. Weinstein, Geographic and ontogenic variation in venom of the western diamondback rattlesnake (*Crotalus atrox*), Toxicon 24 (1986) 71-80. doi:10.1016/0041-0101(86)90167-4

20. C.M. Modahl, Mrinalini, S. Frietze, S.P. Mackessy, 2018. Adaptive evolution of distinct prey-specific toxin genes in rear-fanged snake venom. Proc. Royal Soc. B. 285, 20181003. doi:10.1098/rspb.2018.1003

21. E.L. Davies, K. Arbuckle, 2019. Coevolution of snake venom toxic activities and diet: evidence that ecological generalism favours toxicological diversity. Toxins. 11, 711. doi:10.3390/toxins11120711

22. M.L. Holding, J.L. Strickland, R.M. Rautsaw, E.P. Hofmann, A.J. Mason, M.P. Hogan, G.S. Nystrom, S.A. Ellsworth, T.J. Colston, M. Borja, G. Castañeda-Gaytán, C.I. Grünwald, J.M. Jones, L.A. Freitas-de-Sousa, V.L. Viala, M.J. Margres, E. Hingst-Zaher, I.L.M. Junqueira-de-Azevedo, A.M. Moura-da-Silva, F.G. Grazziotin, H.L. Gibbs, D.R. Rokyta, C.L. Parkinson, 2021. Phylogenetically diverse diets favor more complex venoms in North American pitvipers. PNAS. 118, e2015579118. doi:10.1073/pnas.2015579118

23. S.P. Mackessy, J. Leroy, E. Mociño-Deloya, K. Setser, R.W. Bryson, A.J. Saviola, 2018. Venom ontogeny in the Mexican lance-headed rattlesnake (*Crotalus polystictus*). Toxins. 10, 271. doi:10.3390/toxins10070271

24. C.M. Modahl, A.K. Mukherjee, S.P. Mackessy, An analysis of venom ontogeny and prey-specific toxicity in the monocled cobra (*Naja kaouthia*), Toxicon 119 (2016) 8-20. doi:10.1016/j.toxicon.2016.04.049

25. D. Pla, L. Sanz, M. Sasa, M.E. Acevedo, Q. Dwyer, J. Durban, A. Pérez, Y. Rodriguez, B. Lomonte, J.J. Calvete, Proteomic analysis of venom variability and ontogeny across the arboreal palm-pitvipers (genus *Bothriechis*), J. Proteomics 152 (2017) 1-12. doi:10.1016/j.jprot.2016.10.006

26. A. Alape-Girón, L. Sanz, J. Escolano, M. Flores-Diaz, M. Madrigal, M. Sasa, J.J. Calvete, Snake venomics of the lancehead pitviper *Bothrops asper*: geographic, individual, and ontogenetic variations, J. Proteome Res. 7 (2008) 3556-3571. doi:10.1021/pr800332p

27. D.V. Andrade, A.S. Abe, Relationship of venom ontogeny and diet in *Bothrops*, Herpetologica 55 (1999) 200-204.

28. H.L. Gibbs, L. Sanz, J.E. Chiucchi, T.M. Farrell, J.J. Calvete, Proteomic analysis of ontogenetic and diet-related changes in venom composition of juvenile and adult dusky pigmy rattlesnakes (*Sistrurus miliarius barbouri*), J. Proteomics 74 (2011) 2169-2179. doi:10.1016/j.jprot.2011.06.013

29. M.M. Saldarriaga, R. Otero, V. Núñez, M.F. Toro, A. Díaz, J.M. Gutiérrez, Ontogenetic variability of *Bothrops atrox* and *Bothrops asper* snake venoms from Colombia, Toxicon 42 (2003) 405-411. doi:10.1016/0041-0101(03)00171-5

30. A.J. Saviola, D. Pla, L. Sanz, T.A. Castoe, J.J. Calvete, S.P. Mackessy, Comparative venomics of the prairie rattlesnake (*Crotalus viridis viridis*) from Colorado: identification of a novel pattern of ontogenetic changes in venom composition and assessment of the immunoreactivity of the commercial antivenom CroFab®, J. Proteomics 121 (2015) 28-43. doi:10.1016/j.jprot.2015.03.015

31. S. Bhatia, K. Vasudevan, 2020. Comparative proteomics of geographically distinct saw-scaled viper (*Echis carinatus*) venoms from India. Toxicon X. 7, 100048. doi:10.1016/j.toxcx.2020.100048

32. J.J. Calvete, L. Sanz, A. Pérez, A. Borges, A.M. Vargas, B. Lomonte, Y. Angulo, J.M. Gutiérrez, H.M. Chalkidis, R.H.V. Mourao, M.F.D. Furtado, A.M. Moura-Da-Silva, Snake population venomics and antivenomics of *Bothrops atrox*: paedomorphism along its transamazonian dispersal and implications of geographic venom variability on snakebite management, J. Proteomics 74 (2011) 510-527. doi:10.1016/j.jprot.2011.01.003

33. A. Chanda, B. Kalita, A. Patra, W.D.S.T. Senevirathne, A.K. Mukherjee, Proteomic analysis and antivenomics study of Western India *Naja naja* venom: correlation between venom composition and clinical manifestations of cobra bite in this region, Expert Rev. Proteom. 16 (2019) 171-184. doi:10.1080/14789450.2019.1559735

34. D. Pla, L. Sanz, S. Quesada-Bernat, M. Villalta, J. Baal, M.A.W. Chowdhury, G. León, J.M. Gutiérrez, U. Kuch, J.J. Calvete, 2019. Phylovenomics of *Daboia*

russelii across the Indian subcontinent. Bioactivities and comparative in vivo neutralization and in vitro third-generation antivenomics of antivenoms against venoms from India, Bangladesh and Sri Lanka. J. Proteomics. 207, 103443. doi:10.1016/j.jprot.2019.103443

35. R.R. Senji Laxme, S. Attarde, S. Khochare, V. Suranse, G. Martin, N.R. Casewell, R. Whitaker, K. Sunagar, 2021. Biogeographical venom variation in the Indian spectacled cobra (*Naja naja*) underscores the pressing need for pan-India efficacious snakebite therapy. PLOS Negl. Trop. Dis.. 15, e0009150. doi:10.1371/journal.pntd.0009150

36. S. Creer, W.H. Chou, A. Malhotra, R.S. Thorpe, Offshore insular variation in the diet of the Taiwanese bamboo viper *Trimeresurus stejnegeri* (Schmidt), Zool. 19 (2002) 907-913. doi:10.2108/zsj.19.907

37. J.A. Wilkinson, J.L. Glenn, R.C. Straight, J.W. Jr. Sites, Distribution and genetic variation in venom A and B populations of the Mojave rattlesnake (*Crotalus scutulatus scutulatus*) in Arizona, Herpetologica 47 (1991) 54-68. https://www.jstor.org/stable/3892815

38. A. Barua, A.S. Mikheyev, 2020. Toxin expression in snake venom evolves rapidly with constant shifts in evolutionary rates. Proc. Royal Soc. B. 287, 20200613. doi:10.1098/rspb.2020.0613

39. M.L. Holding, M. Margres, D.R. Rokyta, H.L. Gibbs, Local prey community composition and genetic distance predict venom divergence among populations of the northern Pacific rattlesnake (*Crotalus oreganus*), J. Evol. Biol. 31 (2018) 1513-1528. doi:10.5061/dryad.rg098gq

40. K.E. Robinson, M.L. Holding, M.D. Whitford, A.J. Saviola, J.R. Yates III, R.W. Clark, Phenotypic and functional variation in venom and venom resistance of two sympatric rattlesnakes and their prey, J. Evol. Biol. 34 (2021) 1447-1465. doi:10.1111/jeb.13907

41. F. Martínez-Freiría, I. Freitas, G. Velo-Antón, N. Lucchini, S. Fahd, S. Larbes, J.M. Pleguezuelos, X. Santos, J.C. Brito, Integrative taxonomy reveals two species and intraspecific differentiation in the *Vipera latastei-monticola* complex, J. Zoolog. Syst. Evol. Res. 59 (2021) 2278-2306. doi:10.1111/jzs.12534

42. I. Freitas, S. Ursenbacher, K. Mebert, O. Zinenko, S. Schweiger, W. Wüster, J.C. Brito, J. Crnobrnja-Isailovic, B. Halpern, S. Fahd, X. Santos, J.M. Pleguezuelos, U. Joger, N. Orlov, E. Mizsei, O. Lourdais, M.A.L. Zuffi, A. Strugariu, S.R. Zamfirescu, I. Martínez-Solano, G. Velo-Antón, A. Kaliontzopoulou, F. Martínez-Freiría, Evaluating taxonomic inflation: towards evidence-based species delimitation in Eurasian vipers (Serpentes: Viperinae), Amphib-reptil. 41 (2020) 285-311. doi:10.1163/15685381-bja10007

43. F. Martínez-Freiría, I. Freitas, M.A.L. Zuffi, P. Golay, S. Ursenbacher, G. Velo-Antón, Climatic refugia boosted allopatric diversification in western Mediterranean vipers, J. Biogeogr. 47 (2020) 1698-1713. doi:10.1111/jbi.13861

44. F. Martínez-Freiría, J.C. Brito, J.M. Pleguezuelos, X. Santos, *Vipera latastei* Boscá, 1878, in: M. A. Ramos et al. (Eds.). Fauna Ibérica, vol. 10 - Reptiles, 2ª edición revisada y aumentada, Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas, Madrid, 2014, pp. 920-941.

45. J.C. Brito, Feeding ecology of *vipera latastei* in northern Portugal: ontogenetic shifts, prey size and seasonal variations, Herpetol. J. 14 (2004) 13-19.

46. F. Martínez-Freiría, M. Lizana, J.P. do Amaral, J.C. Brito, Spatial and temporal segregation allows coexistence in a hybrid zone among two Mediterranean vipers (*Vipera aspis* and *V. latastei*), Amphib-reptil. 31 (2010) 195-212. doi:10.1163/156853810791069001

47. J.M. Pleguezuelos, J.C. Brito, S. Fahd, X. Santos, G. Llorente, X. Parellada, Variation in the diet of the Lataste's viper *Vipera latastei* in the Iberian Peninsula: seasonal, sexual and size-related effects, Anim. Biol. 57 (2007a) 49-61. doi:10.1163/157075607780001998

48. X. Santos, J.M. Pleguezuelos, J.C. Brito, G.A. Llorente, X. Parellada, S. Fahd, Prey availability drives geographic dietary differences of a Mediterranean predator, the Lataste's viper (*Vipera latastei*). Herpetol. J. 18 (2008) 16-22.

49. World Health Organization, Snakebite Information and Data Platform. https://www.who.int/teams/control-of-neglected-tropical-diseases/snakebite-envenoming/snakebite-information-and-data-platform/overview#tab=tab_1, 2020 (accessed 01 March 2022).

50. M.R. Di Nicola, A. Pontara, G.E. Kass, N.I. Kramer, I. Avella, R. Pampena, S.R. Mercuri, J.L.C.M. Dorne, G. Paolino, 2021. Vipers of major clinical relevance in Europe: taxonomy, venom composition, toxicology and clinical management of human bites. Toxicology. 453, 152724. doi:10.1016/j.tox.2021.152724

51. S.R. Valdoleiros, I. Gonçalves, C. Silva, D. Guerra, L.A. Silva, F. Martínez-Freiría, F. Rato, S. Xará, Venomous animals in the Portuguese territory: clinical management of bites and stings, Acta Med Port. 34 (2021) 784-795. doi:10.20344/amp.15589

52. I.G. Archundia, A.R. de Roodt, B. Ramos-Cerrillo, J.P. Chippaux, L. Olguín-Pérez, A. Alagón, R.P. Stock, Neutralization of *Vipera* and *Macrovipera* venoms by two experimental polyvalent antisera: a study on paraspecificity, Toxicon 57 (2011) 1049-1056. doi:10.1016/j.toxicon.2011.04.009

53. J. Detrait, G. Naulleau, H. Saint Girons, Contribution à l'étude du venin de *Vipera latastei* Boscá, 1878, Munibe 35 (1983) 81-86.

54. A.P. Arez, G.D. Laing, V. Rosário, R.D. Theakston, Preliminary studies on the characterization of venom from *Vipera latastei latastei* (Boscá, 1878) (Reptilia: Viperidae) collected in Nw Portugal. Port. Zool. 2 (1993) 37-42.

55. A. Chowdhury, C.N. Zdenek, M.R. Lewin, R. Carter, T. Jagar, E. Ostanek, H. Harjen, M. Aldridge, R. Soria, G. Haw, B.G. Fry, 2021. Venom-induced blood disturbances by Palearctic viperid snakes, and their relative neutralization by antivenoms and enzyme-inhibitors. Front. immunol. 12, 688802. doi:10.3389/fimmu.2021.688802

56. H. Saint Girons, J. Detrait, Étude électrophorétique des venins de Viperinae (Serpentes) du genre *Vipera*: variations des proteinogrammes et implications phylogénetiques, Bull. de la Soc. Zool. de France 117 (1992) 399-412.

57. Agencia Estatal de Meteorologia, Ministerio de Medio Ambiente y Medio Rural y Marino e Instituto de Meteorologia de Portugal, Atlas Climático Ibérico - Temperatura do ar e Precipitação (1971-2000), Lisbon, 2011.

58. Instituto Português do Mar e da Atmosfera, Área educativa – Clima de Portugal continental. https://www.ipma.pt/pt/educativa/tempo.clima, 2021 (accessed 21 May 2021).

59. G. Velo-Antón, R. Godinho, D.J. Harris, X. Santos, F. Martínez-Freiría, S. Fahd, S. Larbes, J.M. Pleguezuelos, J.C. Brito,Deep evolutionary lineages in a Western Mediterranean snake (*Vipera latastei/monticola* group) and high genetic structuring in Southern Iberian populations, Mol. Phylogenet. Evol. 65 (2012) 965-973. doi:10.1016/j.ympev.2012.08.016

60. O. Moya, P.L. Mansilla, S. Madrazo, J.M. Igual, A. Rotger, A. Romano, G. Tavecchia, G., APHIS: a new software for photo-matching in ecological studies, Ecol Inform 27 (2015) 64-70. doi:10.1016/j.ecoinf.2015.03.003

61. J.M. Pleguezuelos, X. Santos, J.C. Brito, X. Parellada, G.A. Llorente, S. Fahd, Reproductive ecology of *Vipera latastei* in the Iberian Peninsula: implications for the conservation of a Mediterranean viper, Zoology 110 (2007b) 9-19. doi:10.1016/j.zool.2006.11.002

62. J.J. Calvete, Next-generation snake venomics: protein-locus resolution through venom proteome decomplexation, Expert Rev. Proteom. 11 (2014) 315-329. doi:10.1586/14789450.2014.900447

63. S. Eichberg, L. Sanz, J.J. Calvete, D. Pla, Constructing comprehensive venom proteome reference maps for integrative venomics, Expert Rev. Proteom. 12 (2015) 557-573. doi:10.1586/14789450.2015.1073590

64. S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403-410. doi:10.1016/S0022-2836(05)80360-2

65. A. Coutinho-Neto, C.A.S. Caldeira, G.H.M.F. Souza, K.D. Zaqueo, A.M. Kayano, R.S. Silva, J.P. Zuliani, A.M. Soares, R.G. Stábeli, L.A. Calderon, ESI-MS/MS identification of a bradykinin-potentiating peptide from Amazon *Bothrops atrox* snake venom using a hybrid Qq-oaTOF mass spectrometer, Toxins 5 (2013) 327-335. doi:10.3390/toxins5020327

66. L. Menin, A. Perchuć, P. Favreau, F. Perret, S. Michalet, R. Schöni, M. Wilmer, R. Stöcklin, High throughput screening of bradykinin-potentiating peptides in *Bothrops moojeni* snake venom using precursor ion mass spectrometry, Toxicon 51 (2008) 1288-1302. doi:10.1016/j.toxicon.2008.02.019

67. F. Calderón-Celis, L. Cid-Barrio, J.R. Encinar, A. Sanz-Medel, J.J. Calvete, Absolute venomics: absolute quantification of intact venom proteins through elemental mass spectrometry, J. Proteomics 164 (2017) 33-42. doi:10.1016/j.jprot.2017.06.001

68. G. Zancolli, L. Sanz, J.J. Calvete, W. Wüster, 2017. Venom on-a-chip: a fast and efficient method for comparative venomics. Toxins. 9, 179. doi:10.3390/toxins9060179

69. P.R. Minchin, An evaluation of relative robustness of techniques for ecological ordinations, Vegetatio 69 (1987) 89-107. doi:10.1007/BF00038690

70. J.B. Kruskal, Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis, Psychometrika 29 (1964) 1-27. doi:10.1007/BF02289565

71. K.P. Burnham, D.R. Anderson, Model selection and multimodel inference: a practical information-theoretic approach, Springer, New York, 2002.

72. S.A. Richards, M.J. Whittingham, P.A. Stephens, Model selection and model averaging in behavioural ecology: the utility of the IT-AIC framework, Behav. Ecol. Sociobiol. 65 (2011) 77-89. doi:10.1007/s00265-010-1035-8

73. J. Oksanen, Design decisions and implementation details in vegan. Vignette of the package vegan. https://cran.r-project.org/web/packages/vegan/vignettes/decision-vegan.pdf, 2016 (accessed 9 November 2021).

74. K. Bartoń, Package "MuMIn": Multi-Model Inference. R package version 1.43.17. https://CRAN.R-project.org/package=MuMIn, 2020 (accessed 9 November 2021).

75. D. Lüdecke, ggeffects: tidy data frames of marginal effects from regression models, J. Open Source Softw. 3 (2018) 772. doi:10.21105/joss.00772

76. R Core Team, R: A language and environment for statistical computing. https://www.R-project.org, 2021 (accessed 13 October 2021).

77. M. Damm, B.F. Hempel, R.D. Süssmuth, 2021. Old World Vipers - A review about snake venom proteomics of Viperinae and their variations. Toxins. 13, 427. doi:10.3390/toxins13060427

78. T. Matsui, Y. Fujimura, K. Titani, Snake venom proteases affecting hemostasis and thrombosis, Biochim Biophys Acta Proteins Proteom 1477 (2000) 146-156. doi:10.1016/S0167-4838(99)00268-X

79. R.M. Kini, Serine proteases affecting blood coagulation and fibrinolysis from snake venoms, Pathophysiol Haemos Thromb 34 (2005) 200-204. doi:10.1159/000092424

80. T. Ogawa, T. Chijiwa, N. Oda-Ueda, M. Ohno, Molecular diversity and accelerated evolution of C-type lectin-like proteins from snake venom, Toxicon 45 (2005) 1-14. doi:10.1016/j.toxicon.2004.07.028

81. H. Xiao, H. Pan, K. Liao, M. Yang, C. Huang, Snake venom PLA2, a promising target for broad-spectrum antivenom drug development, Biomed Res. Int. 2017 (2017) 1-10. doi:10.1155/2017/6592820

82. O.H.P. Ramos, H.S. Selistre-de-Araujo, Snake venom metalloproteinases - structure and function of catalytic and disintegrin domains, Comp. Biochem. Physiol. Part - C. Toxicol. Pharmacol. 142 (2006) 328-346. doi:10.1016/j.cbpc.2005.11.005

83. J.M. Gutiérrez, A. Rucavado, T. Escalante, Snake venom metalloproteinases: biological roles and participation in the pathophysiology of envenomation, in: S.P. Mackessy (Ed.), Evolution of venomous animals and their toxins, first ed., CRC Press, Boca Ratón, 2010, pp. 115-130.

84. J.W. Fox, J.M. Gutiérrez, 2017. Understanding the snake venom metalloproteinases: an interview with Jay Fox and José María Gutiérrez. Toxins. 9, 33. doi:10.3390/toxins9010033

85. A.M. Moura-da-Silva, D. Butera, I. Tanjoni, Importance of snake venom metalloproteinases in cell biology: effects on platelets, inflammatory and

endothelial cells, Curr. Pharm. Des. 13 (2007) 2893-2905.
doi:10.2174/138161207782023711

86. D. Mebs, Snake venom composition and evolution of Viperidae, Kaupia 8 (1999) 145-148.

87. S.P. Mackessy, Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *Crotalus v. oreganus*, Copeia 1988 (1988) 92–101. doi:10.2307/1445927

88. M.D. McCue, Prey envenomation does not improve digestive performance in western diamondback rattlesnakes (*Crotalus atrox*), J Exp Zool A Ecol Genet Physiol 307 (2007) 568-577. doi:10.1002/jez.411

89. B. Ding, Z. Xu, C. Qian, F. Jiang, X. Ding, Y. Ruan, Z. Ding, Y. Fan, Antiplatelet aggregation and antithrombosis efficiency of peptides in the snake venom of *Deinagkistrodon acutus*: isolation, identification, and evaluation, Evid. Based Complementary Altern. Med. 2015 (2015) 1-6. doi:10.1155/2015/412841

90. M. Morjen, S. Honoré, A. Baza, Z. Abdelkafi-Koubaa, A. Ellafi, K. Mabrouk, H. Kovacic, M. El Ayeb, N. Marrakichi, J. Luis, PIVL, a snake venom Kunitz-type serine protease inhibitor, inhibits in vitro and in vivo angiogenesis, Microvasc. Res. 95 (2014) 149-156. doi:10.1016/j.mvr.2014.08.006

91. K. Sunagar, B.G. Fry, T.N.W. Jackson, N.R. Casewell, E.A.B. Undheim, N. Vidal, S.A. Ali, G.F. King, K. Vasudevan, V. Vasconcelos, A. Antunes, 2013. Molecular evolution of vertebrate neurotrophins: co-option of the highly conserved nerve growth factor gene into the advanced snake venom arsenal. PloS One. 8, e81827. doi:10.1371/journal.pone.0081827

92. I.G. Ferreira, M.B. Pucca, I.S. de Oliveira, F.A. Cerni, B.D.C. da Silva Jacob, E.C. Arantes, Snake venom vascular endothelial growth factors (svVEGFs): unravelling their molecular structure, functions, and research potential, Cytokine Growth Factor Rev. 60 (2021) 133-143. doi:10.1016/j.cytogfr.2021.05.003

93. C. Marcinkiewicz, P.H. Weinreb, J.J. Calvete, D.G. Kisiel, S.A. Mousa, G.P. Tuszynski, R.R. Lobb, Obtustatin: a potent selective inhibitor of α1β1 integrin in vitro and angiogenesis in vivo, Cancer Res. 63 (2003) 2020-2023.

94. D. Monleón, M.P. Moreno-Murciano, H. Kovacs, C. Marcinkiewicz, J.J. Calvete, B. Celda, Concerted motions of the integrin-binding loop and the C-terminal tail of the non-RGD disintegrin obtustatin, J. Biol. Chem. 278 (2003) 45570-45576. doi:10.1074/jbc.M307030200

95. O. Kallech-Ziri, L. José, S. Daoud, A. Bazaa, N.S. Abid, N. Andreotti, M. Lehmann, R. Zouari, K. Mabrouk, J. Marvaldi, J.M. Sabatier, M. El Ayeb, N. Marrakchi, Lebestatin, a disintegrin from *Macrovipera* venom, inhibits integrin-mediated cell adhesion, migration and angiogenesis, Lab. Investig. 85 (2005) 1507-16. doi:10.1038/labinvest.3700350

96. R. Sanz-Soler, C. Lorente, B. Company, L. Sanz, P. Juárez, A. Pérez, Y. Zhang, Y. Jin, R. Chen, J.A. Eble, J.J. Calvete, G. Bolás, Recombinant expression of mutants of the Frankenstein disintegrin, RTS-ocellatusin. Evidence for the independent origin of RGD and KTS/RTS disintegrins, Toxicon 60 (2012) 665-675. doi:10.1016/j.toxicon.2012.05.010

97. D.G. Kisiel, J.J. Calvete, J. Katzhendler, A. Fertala, P. Lazarovici, C. Marcinkiewicz, Structural determinants of the selectivity of KTS-disintegrins for the α1β1 integrin, FEBS letters 577 (2004) 478-482. doi:10.1016/j.febslet.2004.10.050

98. J.J. Calvete, C. Marcinkiewicz, L. Sanz, KTS and RTS-disintegrins: anti-angiogenic viper venom peptides specifically targeting the α1β 1 integrin, Curr. Pharm. Des. 13 (2007) 2853-2859. doi:10.2174/138161207782023766

99. R. Sanz-Soler, L. Sanz, J.J. Calvete, Distribution of RPTLN genes across Reptilia: hypothesized role for RPTLN in the evolution of SVMPs, Integr. Comp. Biol. 56 (2016) 989-1003. doi:10.1093/icb/icw031

100. A.J. Saviola, J.J. Calvete, Reptile venom disintegrins, in: S.P. Mackessy (Ed.), Handbook of venoms and toxins of reptiles, second ed., CRC Press, Boca Ratón, 2021, pp. 211-224.

101. J.C. Brito, R. Rebelo, Differential growth and mortality affect sexual size dimorphism in *Vipera latastei*, Copeia 2003 (2003) 865-871. doi:10.1643/h202-279.1

102. A. Forsman, Variation in sexual size dimorphism and maximum body size among adder populations: effects of prey size, J. Animal Ecol. 60 (1991) 253-267. doi:10.2307/5458

103. J. Durban, A. Pérez, L. Sanz, A. Gómez, F. Bonilla, S. Rodríguez, D. Chacón, M. Sasa, Y. Angulo, J.M. Gutiérrez, J.J. Calvete, Integrated "omics" profiling indicates that miRNAs are modulators of the ontogenetic venom composition shift in the Central American rattlesnake, *Crotalus simus simus*, BMC Genom. 14 (2013) 1-17. doi:10.1186/1471-2164-14-234

104. H.R. Mushinsky, Foraging ecology, in: R.A. Seigel, T. Collins, S.S. Novak (Eds.), Snakes: ecology and evolutionary biology, MacMillan, New York, 1987, pp. 1624-1629.

105. A. Forsman, L.E. Lindell, The advantage of a big head: swallowing performance in adders, *Vipera berus*, Funct. Ecol. 7 (1993) 183-189. doi:2389885

106. D.R. Amazonas, L.A. Freitas-de-Sousa, D.P. Orefice, L.F. de Sousa, M.G. Martinez, R.H.V. Mourão, H.M. Chalkidis, P.B. Camargo, A.M. Moura-da-Silva, 2019. Evidence for snake venom plasticity in a long-term study with individual captive *Bothrops atrox*. Toxins. 11, 294. doi:10.3390/toxins11050294

107. N. da Costa Galizio, C. Serino-Silva, D.R. Stuginski, P.A.E. Abreu, S.S. Sant'Anna, K.F. Grego, A.K. Tashima, A.M. Tanaka-Azevedo, K. de Morais-Zani, Compositional and functional investigation of individual and pooled venoms from long-term captive and recently wild-caught *Bothrops jararaca* snakes, J. Proteomics 186 (2018) 56-70. doi:10.1016/j.jprot.2018.07.007

108. J.H. Connell, Diversity in tropical rain forests and coral reefs, Science 199 (1978) 1302-1310. doi:10.1126/science.199.4335.1302

Table 1. Relative abundances (in percentage of the total venom proteome) of the components identified in the six representative *V. latastei* venoms analysed by bottom-up proteomics. 5'NT = 5'-nucleotidase; AP = aminopeptidase; BIP = bradykinin-inhibitory peptide; BPP = bradykinin-potentiating peptide; CRISP = cysteine-rich secretory protein; CTL = C-type lectin-like; DISI = disintegrin; Hyal = hyaluronidase; KTSPi = Kunitz-type

serine proteinase inhibitor; LAAO = L-amino-acid oxidase; D49/K49-PLA$_2$ = D49/K49-phospholipase A$_2$; PLB = phospholipase B; PDE = phosphodiesterase; PI-/PIII-SVMP = snake venom metalloproteinase of class PI or PIII, respectively; DC = disintegrin-like/cysteine-rich domain released from PIII-SVMPs; QC = glutaminyl cyclase; SVMPi = endogenous tripeptide inhibitors of SVMP; SVSP = snake venom serine proteinase; VEGF = vascular endothelial growth factor; VNGF = venom nerve growth factor; Unidentified = unassigned venom component. Snake venom metalloproteinases which could not be assigned unambiguously to any of the three classes PI, PII, or PIII, were classified as "SVMP".

| Venom components | Vila Chã | | | Gerês | | |
	19VL013 (juvenile)	18VL253 (adult)	18VL011 (adult)	19VL026 (juvenile)	18VL258 (adult)	19VL029 (adult)
5'NT	-	0.2	-	-	0.12	0.12
AP	-	0.09	0.15	0.07	0.05	0.03
BIP	-	-	0.14	-	-	-
BPP	4.85	2.81	8.89	1.4	4.38	1.83
CRISP	6.15	2.29	2.98	5.54	2.88	3.75
CTL	7.08	19.16	8.52	3.01	14.95	9.98
DC	-	1.03	0.62	0.54	-	2.98
DISI	-	-	0.12	1.56	6.94	4.38
Dimeric DISI	0.99	3.09	3.66	2.52	1.18	2.56
(K/R)TS DISI	-	0.65	1.67	3.69	3.14	0.7
Medium-sized DISI	-	-	0.55	0.33	-	-
QC	-	0.001	-	-	-	-
Hyal	0.005	-	-	0.04	-	-
KTSPi	1.51	1.93	5.79	1.6	6.09	3.93
LAAO	1.9	5.13	6.48	2.68	8.26	3.39
PDE	-	0.12	-	0.06	0.06	0.05
D49-PLA$_2$	17.14	11.94	16.23	13.43	15.41	14.89
K49-PLA$_2$	-	-	0.02	-	-	-
PLB	-	0.05	0.003	0.31	-	0.01
PIII-SVMP	21.92	9.39	2.07	21.66	3.14	13.62
PI-SVMP	-	0.24	0.001	1.2	1.28	0.89
SVMP	20.06	3.05	3.23	19.67	2.18	9.6

SVMP fragment	0.15	0.23	0.23	3.55	0.001	0.93
SVMPi	5.87	15.42	7.99	8.94	11.36	9.77
SVSP	5.9	16.21	26.87	6.08	16.73	11.99
VEGF	-	3.29	0.69	-	1.65	-
VNGF	-	0.24	0.42	-	0.01	0.02
Unidentified	6.49	2.53	2.67	2.1	0.2	4.59

Table 2. Final set of candidate simple linear regression models tested. The reported models relate the individual scores on the first NMDS axis (i.e., NMDS1) with the independent variables snout-vent length (SVL), date of collection of the venom samples (Day year), and population of origin (i.e., Gerês or Vila Chã). The best-fitting linear regression model is reported in bold. The table displays the number of parameters in the model (K), the information score of the model (Akaike's Information Criterion corrected for small sample sizes; AICc), the difference in AICc score between the best model and the model being compared (ΔAICc), and the AICc weight (i.e., the proportion of the total amount of predictive power provided by the full set of models containing the model being assessed; wAICc).

Model	K	AICc	ΔAICc	wAICc
Population + Day year	**5**	**-40.92**	**0.00**	**0.50**
Population	3	-40.86	0.05	0.49
SVL	3	-19.42	21.50	1.08e-5
Null	2	-8.53	32.39	4.68e-8

Figure 1. Sampling localities of the two studied *V. latastei* populations. In Panel **A**, the star indicates the Vila Chã population, while the triangle indicates the Gerês population. The grey squares correspond to the current distribution range of *V. latastei* (adapted from [51]). Panels **B** and **C** show the habitats of the localities Vila Chã and Gerês, respectively.

Figure 2. Overlapped chromatograms of the six venom samples analysed through bottom-up proteomics. Panels **A** and **B** display chromatographic traces of the venoms from Vila Chã and Gerês, respectively. Sample codes and corresponding colours are reported in the panel insets. Notice the differences in the intensities of the peaks, particularly the ones in the two time intervals highlighted by the brackets (i.e., minutes 16-18, and minutes 39-44).

Figure 3. Pie chart representations displaying the relative abundances of the toxin families found in the six *V. latastei* venom proteomes sampled. A) 19VL013 (juvenile viper from Vila Chã); **B**) 19VL026 (juvenile viper from Gerês); **C**) 18VL253 (adult viper from Vila Chã); **D**) 18VL258 (adult viper from Gerês); **E**) 18VL011 (adult viper from Vila Chã); **F**) 19VL029 (adult viper from Gerês). For further details, please consult Table 1 and Tables S2-S7.
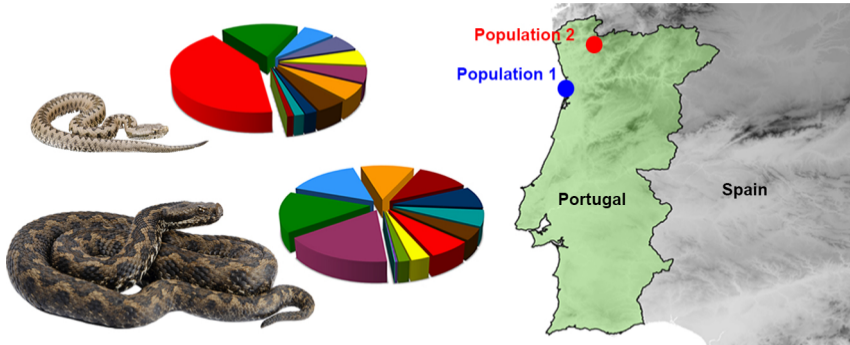
Figure 4. Non-metric multidimensional scaling (NMDS) ordination plot of the 54 *V. latastei* venom chromatographic profiles considered. Venom samples from Vila Chã and Gerês are identified, respectively, by blue and red filled circles (adult) and triangles (juveniles). Blue and red convex hulls delimit the venoms from the Vila Chã and Gerês populations, respectively. Venoms submitted to proteomics analysis are in bold, identified by their sample code (Table S1), and coloured according to their geographic origin (i.e., blue = Vila Chã, red = Gerês). The almost centred position of venom samples 18VL253 and 18VL258 in the two convex hulls implies that these venoms are among the least diverging ones of each population. This suggests that 18VL253 and 18VL258 might be considered "average" adult *V. latastei* venom profiles for Vila Chã and Gerês, respectively.

Figure 5. Best model predictions of occurrence of peaks 1, 6, 19, and 23 in individual chromatograms. The panels display the predicted probability of occurrence of **A**) peak 1 in relation to the day of the year and to population (green = Gerês; red = Vila Chã). The same relation was observed in peak 33; **B**) peak 6 in relation to SVL and to population (green = Gerês; red = Vila Chã); **C**) peak 19 in relation to SVL; and **D**) peak 23 in relation to sex and to population (yellow = females; purple = males).

Graphical abstract

Highlights

- The first proteomic analysis of *Vipera latastei* venom from Portugal is presented.
- A new short RTS-disintegrin is reported for the venom of this species.
- Ontogenetic changes in the composition of *V. latastei* venom are characterised.
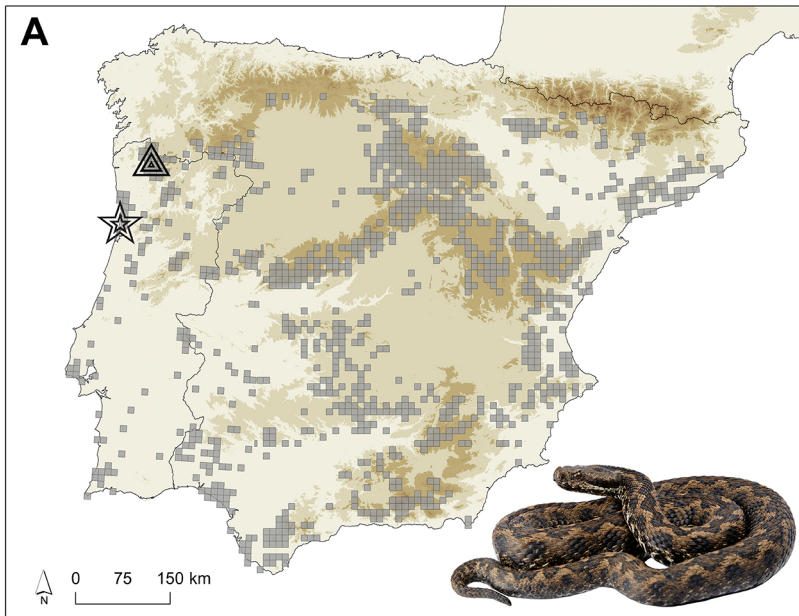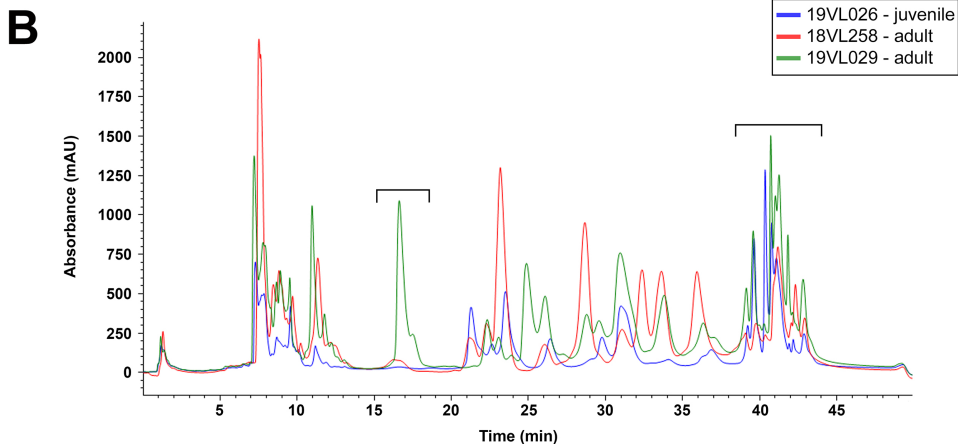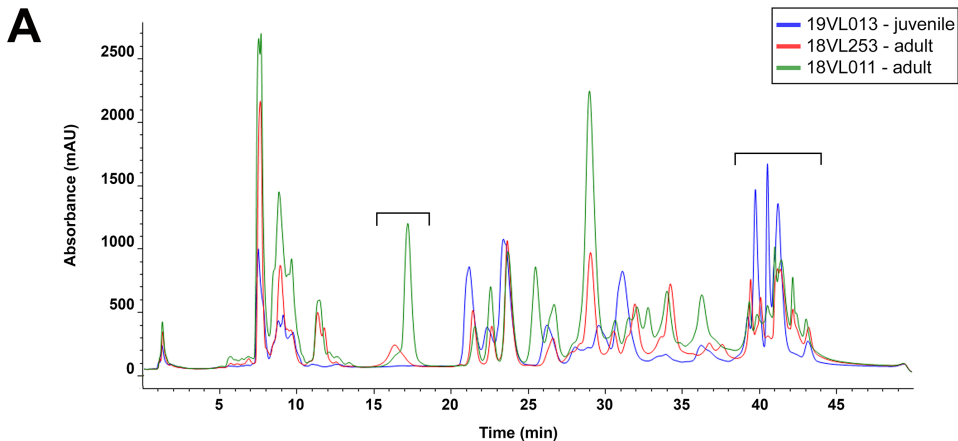- Variability between the venoms of the two viper populations studied is detected.
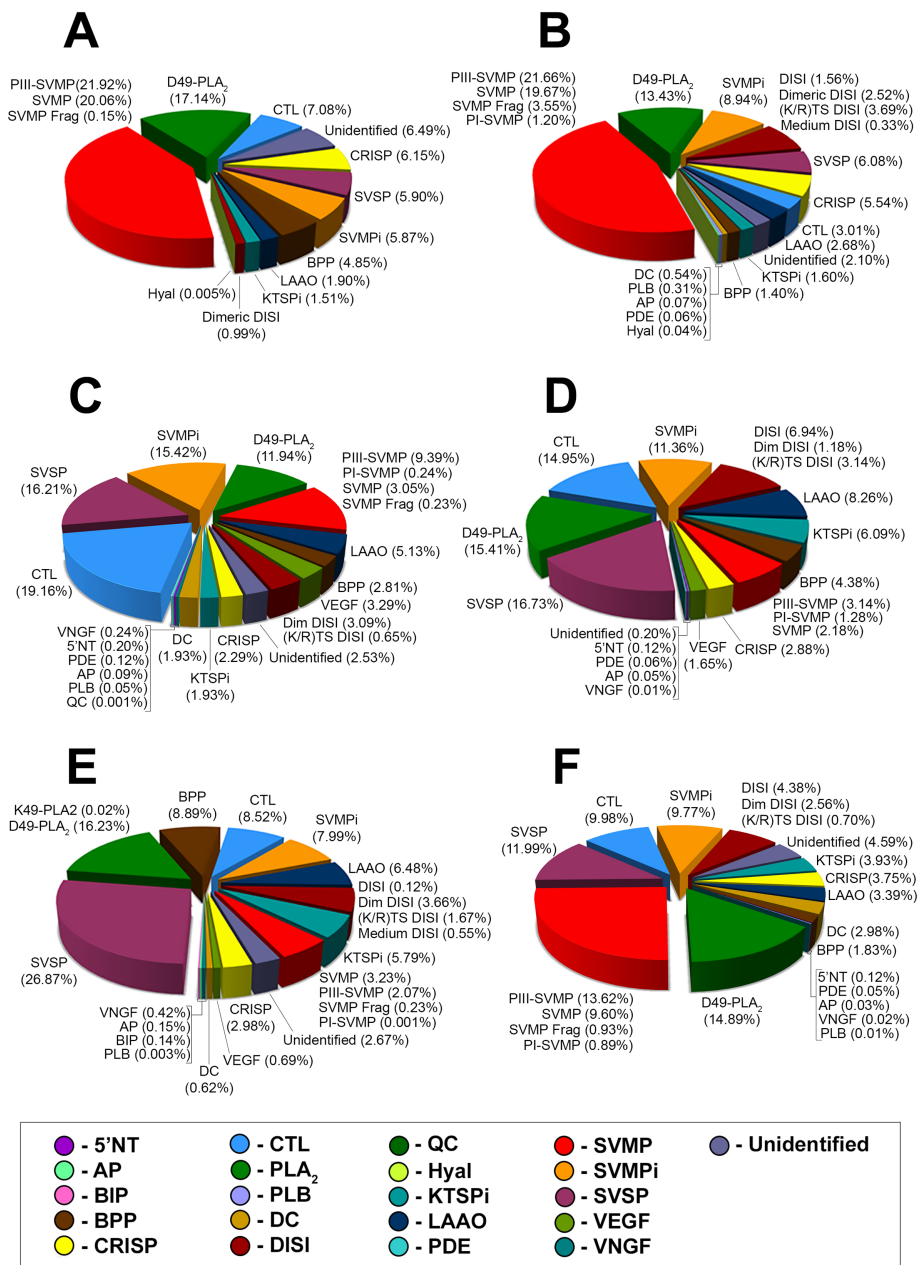
Graphics Abstract

Figure 1

Figure 2

Figure 3

Figure 4

A) Peak 1 — Predicted probability (%) vs Day year

B) Peak 6 — Predicted probability (%) vs SVL (mm)

C) Peak 19 — Predicted probability (%) vs SVL (mm)

D) Peak 23 — Predicted probability (%) vs Population (Gerês, Vila Chã)

Figure 5