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Venom variation among the three subspecies of the North African mountain viper *Vipera monticola* Saint Girons 1953

Maik Damm [a, b, c, d, 1, 2, *], Ignazio Avella [a, b, d, 1], Reema Merzara [c], Nahla Lucchini [e, f, g], Jon Buldain [e, g], Frederico Corga [h], Abdellah Bouazza [i], Soumia Fahd [j], Roderich D. Süssmuth [c, 2, **], Fernando Martínez-Freiría [e, f, 2, ***]

[a] *Institute for Insect Biotechnology, Justus-Liebig University Giessen, Heinrich-Buff-Ring 26-32, 35392, Gießen, Germany*
[b] *LOEWE-Centre for Translational Biodiversity Genomics, Senckenberganlage 25, 60325, Frankfurt Am Main, Germany*
[c] *Institut für Chemie, Technische Universität Berlin, Straße des 17. Juni 135, 10623, Berlin, Germany*
[d] *Animal Venomics Lab, Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Ohlebergsweg 12, 35392 Gießen, Germany*
[e] *CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Campus de Vairão, Universidade do Porto, 4485-661, Vairão, Portugal*
[f] *BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661, Vairão, Portugal*
[g] *Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Porto, Portugal*
[h] *Departamento de Biologia, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal*
[i] *Equipe de recherche Exploration, Gestion des Ressources naturelles et Environnementales, Polydisciplinary Faculty of Taroudant, Ibn Zohr University, Morocco*
[j] *Laboratoire Ecologie, Systématique, Conservation de la Biodiversité, LESCB URL-CNRST N°18, FS, Abdelmalek Essaadi University, Tétouan, Morocco*

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ABSTRACT

The North African mountain viper (*Vipera monticola*) is a medically relevant venomous snake distributed in Morocco, Algeria, and Tunisia. Three subspecies of *V. monticola*, exhibiting differences in morphotypes and dietary regimes, are currently recognised: *V. m. monticola*, *V. m. atlantica*, and *V. m. saintgironsi*. Through the application of snake venomics, we analysed the venoms of specimens of Moroccan origin belonging to each of the three subspecies. Snake venom metalloproteinases (svMP), snake venom serine proteases (svSP), C-type lectin and C-type lectin-related proteins (CTL), and phospholipases A_2 (PLA_2) were predominant, with PLA_2 being the most abundant toxin family overall. Disintegrins (DI) and cysteine-rich secretory proteins (CRISP) were exclusive to *V. m. monticola* and *V. m. atlantica*, while L-amino-acid oxidases (LAAO) were only found in *V. m. saintgironsi*. The differences detected in the venom profiles, as well as in presence/absence and relative abundances of toxin families, indicate the occurrence of intraspecific venom variation within *V. monticola*. The identified patterns of venom similarity between subspecies seem to align more with their phylogenetic relationships than with the reported differences in their feeding habits.

1. Introduction

Snake venom comprises a complex mixture of proteins, peptides, salts, and organic molecules such as carbohydrates and lipids, and presents a variety of characteristic physical and chemical properties [1–3]. Extensive evidence suggests that the primary

* Corresponding author. Institute for Insect Biotechnology, Justus-Liebig University Giessen, Heinrich-Buff-Ring 26-32, 35392, Gießen, Germany.
** Corresponding author. Institut für Chemie, Technische Universität Berlin, Straße des 17. Juni 135, 10623, Berlin, Germany.
*** Corresponding author. BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661, Vairão, Portugal.
E-mail addresses: maik.damm@agrar.uni-giessen.de (M. Damm), roderich.suessmuth@tu-berlin.de (R.D. Süssmuth), fmartinez-freiria@cibio.up.pt (F. Martínez-Freiría).
[1] Shared first author.
[2] Shared corresponding author; for questions strictly concerning the venomics analysis performed, please contact Maik Damm (maik.damm@agrar.uni-giessen.de) or Roderich D. Süssmuth (roderich.suessmuth@tu-berlin.de); for questions strictly concerning the fieldwork or biology of the model species contact Fernando Martínez-Freiría (fmartinez-freiria@cibio.up.pt).

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function of snake venom is to aid predation, although selection on venom composition driven by defensive purposes has been demonstrated in some species (i.e., spitting elapids) [4–7]. With over 50 protein families identified among their components, snake venoms exhibit extensive variation in terms of composition and activities, occurring at all taxonomic levels [8,9]. For instance, although viperid venoms tend to mainly comprise haemotoxic and cytotoxic compounds [10–13], several viper species have been found to possess components in their venoms able to cause neurotoxic effects (e.g., *Vipera ammodytes, Daboia russelii*, *Hypnale hypnale*) [14–16].

Over the past two decades, the tremendous advancement of snake venom research has allowed to analyse the venoms of hundreds of different taxa, particularly through the application of proteomics [12,17,18]. This wealth of information has highlighted the occurrence of venom variation not only between different species but also at an intraspecific level, occurring for instance between populations and even between individuals of the same population [19–22]. This variability has been often associated with natural selection shaping venom compositions in response to ecological pressures influencing the snakes' feeding ecology, such as differences in prey availability among different populations [4,9]. Changes in venom composition can result in significant differences in the clinical symptoms and impact the efficacy of antivenoms. Therefore, the characterisation of snake venom and its variability is of crucial importance not only for primary venom research but also for snakebite treatment [9,11,23,24].

Human envenomation by snakes is an important cause of death and morbidity worldwide, particularly in developing countries across Africa, Asia, and Latin America [25–28]. Situated in northwestern Africa, Morocco is home to six snake species officially listed as medically relevant by the World Health Organization [29]. With the exception of the elapid *Naja haje*, five of these species belong to different genera of the subfamily Viperinae, and are responsible for the vast majority of snakebite accidents in the country: *Bitis arietans*, *Cerastes cerastes*, *Daboia mauritanica*, *Echis leucogaster*, and *Vipera monticola* [30]. While the venoms of most of these species have been investigated at different levels [12,31], there has been no exploration of *V. monticola* venom to date.

The North African mountain viper, *V. monticola* Saint Girons 1953, is a medically relevant Old World viper (subfamily Viperinae). It is distributed across several mountain ranges in the Maghreb, including the High Atlas, Middle Atlas and Rif in Morocco, as well as the Aurès Atlas, and the Tell Atlas in Algeria and Tunisia [29,32–36] (Fig. 1). It is a rare, highly elusive species, phylogenetically close to Lataste's viper *Vipera latastei* [33,37–39] and with an intricate evolutionary history that has long posed a systematic and taxonomic conundrum [34,40–42]. This was partially solved through the application of an integrative taxonomic approach based on mitochondrial DNA (mtDNA) inferences, which led to the recognition of all North African *Vipera* snakes as belonging to the species *V. monticola*, thus establishing it as the only African representative of the *Vipera* genus, as recently supported by phylogenomic studies [32,39].

The current taxonomic scenario recognises three subspecies within the North African mountain viper, each corresponding to a major mtDNA lineage (Fig. 1): *V. monticola monticola* in the Central High Atlas, *V. monticola atlantica* in the Western High Atlas, and *V. monticola saintgironsi* in the Eastern High Atlas, Middle Atlas, the Rif, and the Tell Atlas [32]. Generally considered a "dwarf" viper, *V. monticola* typically exhibits a small body size, rarely reaching 350 mm in snout-vent length (SVL). However, variation occurs between the three subspecies, notably with *V. m. saintgironsi* being able to attain considerably larger sizes (SVL >500 mm) in certain areas across its distribution (e.g., Middle Atlas, Rif and Tell Atlas)

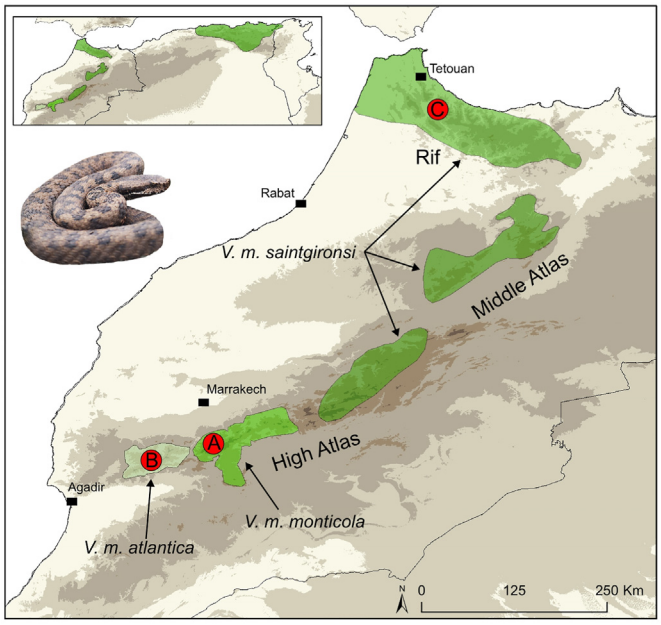

Fig. 1. Distribution of *Vipera monticola* subspecies in Morocco. The red marks represent the locations of origin in Morocco of the samples considered in this study, with: A) Oukaimeden (*V. m. monticola*); B) Tichka Plateau (*V. m. atlantica*); C) Talassemtane N. P. (*V. m. saintgironsi*). Inset shows the major distribution areas of the species across North Africa (adapted from Martínez-Freiría et al. [32,33]). Photo credits: Ignazio Avella (*V. m. monticola*).

[32,34]. Previous studies have suggested the occurrence of the dwarf *V. monticola* morphotype in the High Atlas to be the result of adaptation to the harsh environmental conditions and the scarce resources available in this mountain area [32,34]. Indeed, the three subspecies seem to present markedly different dietary regimes: while smaller North African mountain vipers appear to primarily feed on small ectotherms (e.g., geckos of the genus *Quedenfeldtia*) [43], larger *V. m. saintgironsi* adults likely include bigger, endothermic prey (e.g., rodents) in their diet, as typically observed in several other "non-dwarf" *Vipera* species (e.g., *Vipera aspis, V. latastei*, *Vipera seoanei*) [32,44–47]. In light of the strong link between diet and venom evolution in snakes, and the suggested adaptation to different diets between the three subspecies of *V. monticola*, this species could likely represent an intriguing yet still overlooked model system for the study of intraspecific venom variation in vipers.

Herein we present the first characterisation of the venom of the North African mountain viper, *Vipera monticola*. Through the application of snake venomics, we investigate the presence of intraspecific venom variation within this species, providing a thorough comparison of the venom proteomes for each of the three subspecies currently recognised. We hypothesise that there will be greater venom similarity between the two "dwarf" subspecies, *V. m. monticola* and *V. m. atlantica*, than with the larger *V. m. saintgironsi*, due to their suggested dietary differences. Furthermore, we anticipate that the venom of larger *V. m. saintgironsi* will resemble the venoms of other *Vipera* species that commonly consume endotherms, particularly the phylogenetically close *V. latastei*, more than the venoms of the other two *V. monticola* subspecies.

2. Material and Methods

2.1. Snake venoms

Individual venom samples from 28 adult (SVL

>240 mm) *V. monticola* specimens were used in the present study. Of these, 15 belonged to the subspecies *V. m. monticola*, 12 to the subspecies *V. m. atlantica*, and one to the subspecies *V. m. saintgironsi*. Individuals were collected in May 2019 (n = 10), September 2021 (n = 13) and May 2022 (n = 5) from three distinct locations in Morocco (Fig. 1): Oukaimeden (*V. m. monticola*), the Tichka Plateau (*V. m. atlantica*), and Talassemtane National Park (*V. m. saintgironsi*). All *V. m. monticola* and *V. m. atlantica* specimens presented "dwarf" morphotype, characterised by reduced body size (maximum SVLs were 300 mm and 310 mm, respectively). The single *V. m. saintgironsi* specimen collected was the largest of all sampled animals (SVL: 480 mm, female), and the only viper from a non-dwarf population. Information on the sampled vipers is reported in SI-Table 1. Venoms were collected as described by Avella et al. (2022), by letting each viper bite a parafilm-covered 1.5 ml tube (Eppendorf, Germany) [22]. After venom extraction, vipers were released exactly where they had been captured. Venoms were lyophilised and stored at −20 °C until being transported to the Süssmuth Laboratory of the Institut für Chemie, Technische Universität Berlin (Germany) for analysis. Viper sampling and venom collection were performed with permission of the Haut Commissariat aux Eaux et Forêts et à la Lutte Contre la Désertification of Morocco (HCEFLCD/DLCDPN/DPRN/CFF N°35/2018, N°26/2021 and N°12/2022).

2.2. Snake venomics

Because of the minimal venom quantity obtained from each sampled "dwarf" viper, *V. m. monticola* and *V. m. atlantica* venoms were pooled by subspecies, while the single *V. m. saintgironsi* specimen provided enough venom for individual analysis. Lyophilised venom (1 mg) from each pool and the individual *V. m. saintigironsi* sample was analysed by bottom-up snake venomics, following our previously outlined protocol [48]. Briefly, we fractionated the venoms by a C-18 reversed-phase HPLC system, using a diode array detector (DAD) at λ = 214 nm detection wavelength. The peaks were time-based fractionated (1 fraction/min) and dried in a centrifugal vacuum evaporator. Peaks eluting later than 25 min were further processed by the snake venomics steps of SDS-PAGE profiling and Coomassie-stained bands were cut. The bands were DTT reduced, cysteines IAC alkylated and tryptic digested. Furthermore, tryptic peptides were submitted to an HR-LC LTQ Orbitrap XL mass spectrometer system. Peaks with earlier retention times (Rt) are known for their low molecular mass peptide content, and were directly sent to the LC-MS. The tripeptide pEKW (with pE for pyroglutamate) signal at around 25 min, frequently seen in viperine venoms, was set as a benchmark.

2.3. Bottom-up data analysis

The BU LC-MS/MS data RAW files were converted into the MASCOT generic file (MGF) format using MSConvert (version 3.0.10,577 64-bit) with peak picking (vendor msLevel = 1−) [49]. For an automated database comparison, files were analysed using pFind Studio [50], with pFind (version 3.2.0) and the integrated pBuild, with the previously described parameters in Damm et al. (2024) [48]. The used databases included UniProt 'Serpentes' (ID 8750, reviewed, canonical and isoform, 2747 entries, last accessed on March 8, 2023 via https://www.uniprot.org/). The results were batch-exported as PSM score of all peptides identified with pBuild and manually cleared from decoy entries, contaminations, and artefacts to generate the final list of unique peptide sequences per sample with the best final score. For a second confirmation of identified sequences, unique entries were analysed using BLAST search with blastp against the non-redundant protein sequences (nr) of the "Serpentes" (taxid: 8570) database [51,52]. In case of non-automatically annotated band identity, files were manually checked using Thermo Xcalibur Qual Browser (version 2.2 SP1.4), *de novo* annotated, and/or compared on MS1 and MS2 levels with other bands to confirm band and peptide identities. Deconvolution of isotopically resolved spectra was carried out by using the XTRACT algorithm of Thermo Xcalibur.

2.4. Proteome quantification

The relative quantification protocol based on the common three-step 'snake venomics' approach with the parameters previously described by Damm et al. (2024) [48]. Briefly, the approach involves: step 1) quantification of each HPLC peak area in ratio to the total protein peak area of the chromatogram, regarding each toxin family; step 2) relative abundances of identified proteins co-eluting within the HPLC fractions, by densitometry of the corresponding SDS-PAGE gel bands; step 3) relative abundances of proteins co-migrating in the same SDS-PAGE band by relative ion intensities of the three most abundant peptide ions assigned to the corresponding co-migrating proteins via MS.

2.5. Data accessibility

Mass spectrometry proteomics data have been deposited under the project name "DATASET - Mass Spectrometry - Snake venom proteomics of three subspecies of the North African mountain viper (*Vipera monticola*, Saint Girons 1953) from Morocco" to the MassIVE partner repository (https://massive.ucsd.edu/) with the dataset identifier "MSV000094724" [53] as well as in the Zenodo repository (https://zenodo.org) with the dataset identifier "11103867" [54].

3. Results

The venom compositions of three different subspecies of *V. monticola* determined by snake venomics are presented in Fig. 2. All three venom samples showed complex patterns of HPLC peaks as well as SDS gel bands, as typically seen in viperine venoms (SI Figure S1-3).

3.1. Composition of V. monticola venom

Overall, the venom of *V. monticola* comprises the major, secondary and minor toxin families typically found in the venoms of Old World vipers [12]. The most abundant toxin family in all three subspecies are phospholipases A_2 (PLA_2, 21–49 %), while the other major toxin families identified are snake venom serine proteases (svSP, 11–18 %), C-type lectins and C-type lectin-related proteins (CTL, 8–13 %), and snake venom metalloproteinases (svMP, 6–13 %). For the secondary families, only Kunitz-type inhibitors (KUN, 4–5%) and venom endothelial growth factors (VEGF, 4–5%) were detected in similar amounts within each venom, while disintegrins (DI, 4–6%) and cysteine-rich secretory proteins (CRISP, 1–4%) were exclusively identified in the dwarf vipers *V. m. monticola* and *V. m. atlantica*. The lesser abundant L-amino-acid oxidases (LAAO) were detected in the venom of *V. m. saintgironsi* (1 %), and only traces of this family were found in *V. m. atlantica* venom. Nerve growth factors (NGF, 1–4%) were the only minor toxin family detected in the venoms. The portions of low-molecular-weight peptides range from 6 % in *V. m. saintgironsi* to 15–21 % in the other two subspecies. The amount of non-annotated compounds in each venom was less than 1 %.

The early HPLC fractions of the analysed *V. monticola* venoms include a few dominant low-molecular masses, with the most abundant small peptide being an inhibitor of snake venom

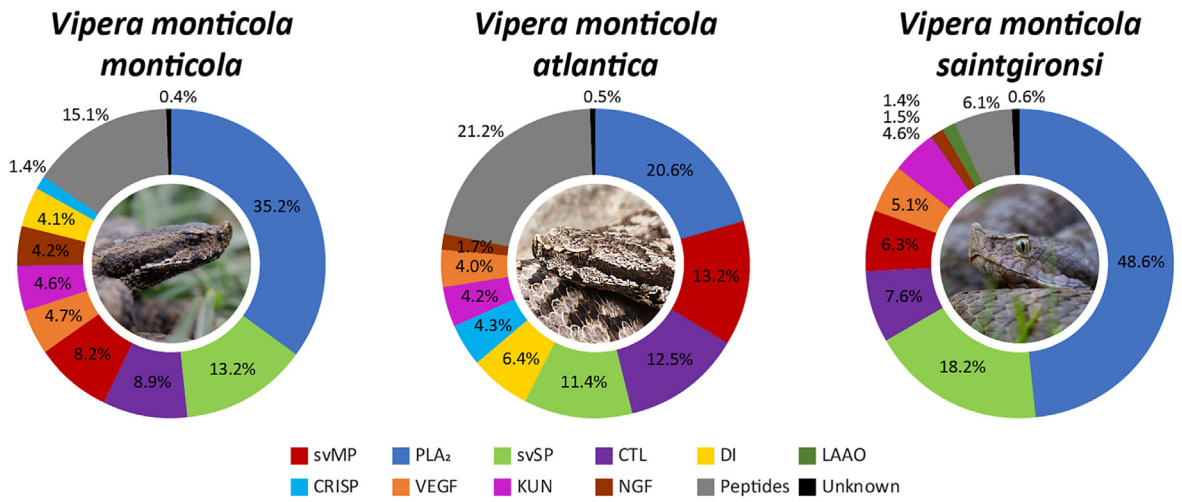

Fig. 2. Venom compositions of the three *Vipera monticola* subspecies from Morocco. The venom proteomes of *V. m. monticola*, *V. m. atlantica* and *V. m. saintgironsi* have been quantified by snake venomics. Toxin families are arranged clockwise by abundances, followed by peptides (grey) and non-annotated parts of the venom (unknown, black). Abbreviations: svMP, snake venom metalloproteinases; PLA_2, phospholipases A_2; svSP, snake venom serine proteases; CTL, C-type lectins and C-type lectin-related proteins; DI, disintegrins; LAAO, L-amino-acid oxidases; CRISP, cysteine-rich secretory proteins; VEGF, venom endothelial growth factors; KUN, Kunitz-type inhibitors; NGF, nerve growth factors. Photo credits: Ignazio Avella (*V. m. monticola*, *V. m. atlantica*), Fernando Martínez-Freiría (*V. m. saintgironsi*).

metalloproteinases (svMP-i) pEKW (*m/z* 444.22) [12,48]. The relative abundance of this small peptide ranged from 4 % (*V. m. saintgironsi*) to 10 % and 15 % (*V. m. monticola* and *V. m. atlantica*, respectively). Another abundant mass in all three venoms is *m/z* 1234.66 (DNEPPKKVPPN), with a sequence similar to the short lebetin 1β (DNKPPKKGPPNG), a potent platelet aggregation inhibitor from *Macrovipera lebetina* [55]. Based on the chromatographic profiles, the peptidic compositions between the two smaller subspecies *V. m. monticola* and *V. m. atlantica* are more similar to each other than to the larger *V. m. saintgironsi*. Indeed, some "dwarf"-specific masses, present in *V. m. monticola* and *V. m. atlantica* venoms but absent in *V. m. saintgironsi*, have been identified (e.g., *m/z* 496.24 (PEGPP) and *m/z* 547.22 (GGGGGGW)).

3.2. Differences in venom profiles between subspecies

A detailed comparison of the HPLC venom profiles reveals seven main sections (I-VII) after the dominant, small-peptide-containing peaks at early Rt of the chromatograms (Fig. 3). These sections are primarily responsible for the compositional percentage differences of the toxin families between the three subspecies.

Section I shows a similar peak pattern for *V. m. monticola* and *V. m. atlantica* from Rt = 39–50 min, which includes dimeric DI (Fig. 3A). In *V. m. saintgironsi*, the reduced signal intensity corresponding to this section completely lacks DI bands, as no members of this toxin family have been detected in the larger *V. monticola* subspecies. Section II comprises a unique peak only found in *V. m. monticola*, formed by two abundant gel bands corresponding to DC-fragments (DC) and VEGF (Fig. 3A and B). In the venoms of the other two subspecies, those toxins eluted at later retention times.

The most evident differences between the three HPLC profiles were observed in sections III, IV and V. In section III, at approximately Rt = 65 min, *V. m. monticola* and *V. m. saintgironsi* venoms show a dominant peak with a small shoulder, congruent in size and shape between the two profiles (Fig. 3B). This section is mainly composed of a dominant PLA_2, with weaker coeluting NGF and VEGF bands in the SDS-PAGE. Conversely, the *V. m. atlantica* venom profile presents in this section a smaller, later-eluting peak, which almost completely overlaps with the shoulder present in the other two profiles (Fig. 3A–C). Nevertheless, the SDS-PAGE band patterns are similar across the venoms of all three taxa, with the only evident difference being *V. m. atlantica* exhibiting a smaller portion of NGF.

Concerning section IV, the venom profile of *V. m. atlantica* differs considerably from those of the other two subspecies, which are in turn very similar between them (Fig. 3). Here, *V. m. monticola* and *V. m. saintgironsi* venoms present a reduced peak at Rt = 74 min (F20 in both), accounting for <2 % of their whole venom compositions, while the corresponding peak in the *V. m. atlantica* profile (F19) accounts for more than 5 % of the whole venom. These peaks in section IV include the major CRISP toxins, not detected in *V. m. saintgironsi*, thus leading to the strong differences between the CRISP content across the subspecies. While at Rt = 77 min *V. m. monticola* and *V. m. saintgironsi* have two congruent abundant peaks (F21 and F22 in both) formed by PLA_2, *V. m. atlantica* has only a single peak at Rt = 79 min (F21). In *V. m. atlantica*, the SDS-PAGE line corresponding to this venom fraction contains not only PLA_2, but also svSP and svMP.

In section V, *V. m. saintgironsi* venom exhibits a unique peak (F24/25) at approximately Rt = 83 min, consisting of a dominant svSP, some svMP, as well as a LAAO band. The latter toxin family is unique to this subspecies and has been detected exclusively in these peaks and F30 in section VI (Fig. 3B and C). The later-eluting peaks in section V are more comparable between the two dwarf subspecies (Fig. 3A), with *V. m. monticola* sharing several characteristics with *V. m. atlantica* as well as *V. m. saintgironsi*, while the profiles of the latter two subspecies are considerably different in this region (Fig. 3B).

Section VI shows a few small signals in all three venom profiles, with no evident consensus across subspecies. These peaks include a variety of svMP, svSP and CTL, with CRISP only in the venom of *V. m. atlantica*, and LAAO exclusively in the venom of *V. m. saintgironsi*. Such a diverse peak pattern was also seen in section VII (Fig. 3). The venom fractions included in this section present similar toxin families across the three venoms analysed, with the dominant *V. m. atlantica* signal (F30), including svMP and CTL in higher amounts than *V. m. monticola* and *V. m. saintgironsi* venoms.

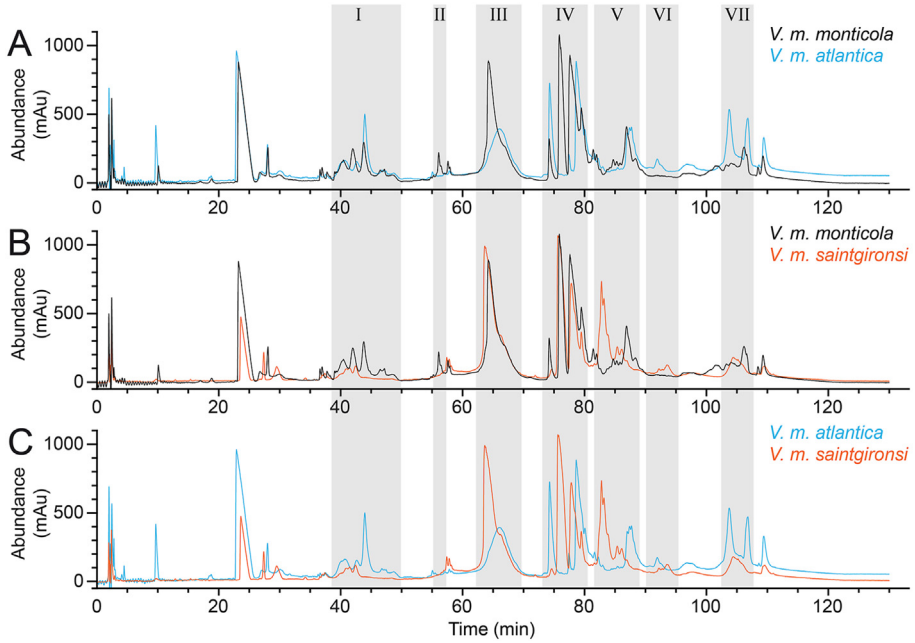

Fig. 3. HPLC venom profile comparisons of the three *Vipera monticola* subspecies. Pairwise chromatogram overlays of *V. m. monticola* (black), *V. m. atlantica* (blue), and *V. m. saintgironsi* (orange) at λ = 214 nm. Main sections (I to VII) of differences in the peak pattern are highlighted in grey areas.

4. Discussion

In this study, we present the first proteomic characterisation of *Vipera monticola* venoms, and describe the venom compositions of the three subspecies *V. m. monticola*, *V. m. atlantica* and *V. m. saintgironsi*. Utilising the snake venomics approach, we show that *V. monticola* venom broadly adheres to the typical compositional profile of Viperinae venoms, and exhibits both qualitative and quantitative intraspecific variation.

The predominant toxin families identified include CTL, PLA_2, svMP, and svSP (Figs. 2 and 4), which are mostly associated with the coagulopathic, haemorrhagic, and cytotoxic effects commonly observed following viperid envenomation [10,11,56]. Specifically, CTL and svSP are recognised for their impact on haemostasis, affecting platelet aggregation and exerting thrombin-like fibrinogenolytic activities [57,58]. Additionally, although PLA_2 can exhibit a range of effects, from neurotoxicity to myotoxicity, they are also known to affect the blood coagulation cascade [59,60]. Finally, svMP are infamous for their ability to induce tissue damage and bleeding disturbances, with those of class P-III possessing particularly strong haemorrhage-inducing activity [56,61].

The high abundance of these main toxin families in the analysed venoms, constituting over half of the produced proteomes (*V. m. monticola*: 66 %; *V. m. atlantica*: 58 %; *V. m. saintgironsi*: 81 %; Fig. 2), suggests that North African mountain viper bites may primarily cause cytotoxic and haemotoxic symptoms.

This is further supported by the presence of other components, such as DI and KUN, which are likely involved in inducing haemotoxicity by inhibiting platelet aggregation and acting as antifibrinolytic agents [62,63]. However, in the absence of any formal analysis of the functional properties of *V. monticola* venom, these conclusions should be approached with caution.

4.1. Intraspecific venom variation

While most identified toxin families and subfamilies are qualitatively similar among the *V. monticola* subspecies, the three venoms show strong variation within the compositions for a subspecies classification. The biggest differences lie in the relative abundances of the main toxin families svMP, svSP and PLA_2, which are typically responsible for haemostatic system disturbance and surrounding tissue damage leading to lifelong disabilities [11,64]. The svMP varies from being a less abundant toxin family in the *V. m. saintgironsi* venom proteome to accounting for more than 13 % in *V. m. atlantica* venom. Conversely, while the lowest relative abundance of PLA_2 was detected in *V. m. atlantica* venom (21 %), this family accounts for approximately half (49 %) of the *V. m. saintgironsi* venom proteome. Notably, all three toxin families in *V. m. monticola* venom are intermediate between those calculated for the other two, as earlier shown in the results (Fig. 2). These differences in svMP and PLA_2 abundances, together with the exclusive detection of secondary toxin families within some taxa like DI and CRISP only present in the “dwarf” vipers, LAAO unique to *V. m. saintgironsi*, may suggest that envenomation by the three *V. monticola* subspecies could result in different clinical symptoms.

The presence of DI and CRISP exclusively in *V. m. monticola* and *V. m. atlantica* supports our hypothesis of greater venom similarity between these two taxa, possibly due to their ectotherm-based diets, in contrast to the supposedly endotherm-including diet of *V. m. saintgironsi* [32,43]. This could be further supported by the fact that LAAO were only found in the *V. m. saintgironsi* venom. Nonetheless, DI, CRISP and LAAO have been reported in the venom proteomes of a considerable number of viperids [12], and their presence or absence do not appear to strictly correlate with any dietary preferences. For example, DIs have been found in the venoms of several species known to primarily feed on endotherms (e.g., *B. arietans*, *D. mauritanica*, *Bothrops asper*) [65–67]. Conversely, these toxins were not detected in the venom of *Vipera ursinii*, a small viper known to almost exclusively feed on ectotherms [68–70]. Similarly, LAAOs were not found in the venoms of two other ectotherm-specialist viper species, *Vipera anatolica* and *Vipera renardi* [71–73]. In light of these considerations, the

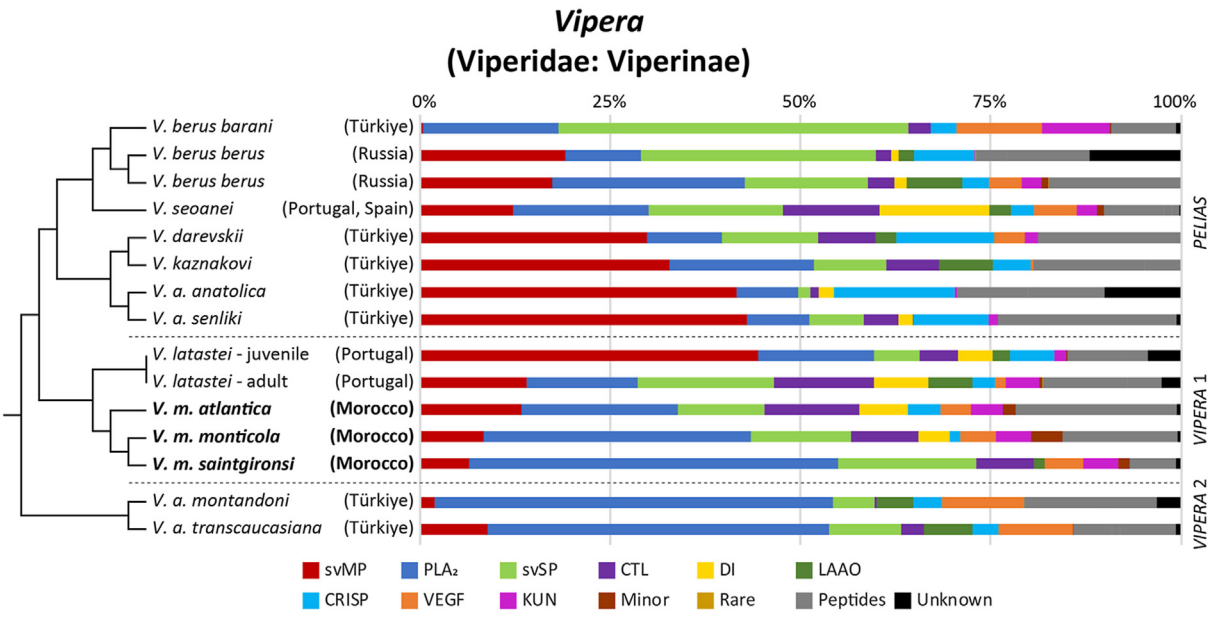

Fig. 4. Snake venomics of *Vipera* species and subspecies. The 15 comparative venom proteomics data of 8 different *Vipera* species including subspecies are lined up following the phylogenetic relationships reported by Martínez-Freiría et al. (2021) [32] and Freitas et al. (2020) [38], including their division into the three clades *Vipera* 1, *Vipera* 2 and *Pelias*. Origins of the investigated venoms are reported in brackets. Proteomes from this study are in bold. Literature references: *Vipera berus barani* [48], *Vipera berus berus* [74,75], *Vipera seoanei* [76], *Vipera darevskii* [48], *Vipera kaznakovi* [77], *Vipera anatolica anatolica* [71], *Vipera anatolica senliki* [72], *Vipera latastei* (averaged by age) [22], *Vipera ammodytes montandoni* and *Vipera ammodytes transcaucasiana* [78]. Exact values are provided in SI-Table S8.

compositional similarities seen between the venoms of the two dwarf *V. monticola* subspecies appear unlikely to be related to comparable dietary regimes.

The qualitative comparison based on the presence or absence of toxin families among the three venom proteomes indicates that *V. m. monticola* and *V. m. atlantica* are more similar to each other than to *V. m. saintgironsi*. However, the RP-HPLC profiles and relative abundances suggest a higher similarity between *V. m. monticola* and *V. m. saintgironsi* venoms. Indeed, their chromatographic profiles overlap considerably, particularly at the central peaks (sections III and IV; Fig. 3). Furthermore, the relative abundances of the major toxin families svMP, PLA_2, svSP, and CTL are more comparable between *V. m. monticola* and *V. m. saintgironsi* than with *V. m. atlantica*. The observed quantitative similarities between the venoms of *V. m. monticola* and *V. m. saintgironsi* appear to align with their phylogenetic relationship as described by Martínez-Freiría et al. [32]. However, considering that evidence suggests that other factors (e.g., diet, geography) can strongly influence snake venom composition, future studies should address their possible roles in determining the similarities detected between the venoms of these two taxa.

4.2. Comparison with Vipera latastei and other Vipera venoms

Considering the close phylogenetic relationship between *V. monticola* and *V. latastei* [32,41] and the ecological similarities between these two taxa, both adapted to primarily inhabit mountain ranges in regions with Mediterranean climate [34,79], we hypothesised the existence of similarities also between their venom compositions. As *V. m. saintgironsi* is presumably feeding on endotherms more frequently than the other two, smaller *V. monticola* subspecies, we anticipated its venom to closely resemble the venom of *V. latastei*, reported to mainly feed on endotherms [45,80].

The comparison between *V. m. saintgironsi* and adult *V. latastei* venoms [22] reveals the presence of qualitative similarities (Fig. 4). *V. monticola* and *V. latastei* share all major toxin families typically found in Viperinae venoms, but not all of the secondary ones. For instance, while *V. m. saintgironsi* venom completely lacks DI and CRISP, these toxin families were found in the venom of *V. latastei*. Additionally, PLA_2 constitutes half of the *V. m. saintgironsi* venom proteome, compared to the 12 %–16 % in adult *V. latastei*. Conversely, svMP accounted for 6 % of *V. m. saintgironsi* venom, but its relative abundance ranged between 6 % and 28 % in adult *V. latastei* venom. Quantitative differences between *V. m. saintgironsi* and *V. latastei* venoms are even more evident when considering the proteomes of the two pools made of juvenile *V. latastei* venoms. Their amount of PLA_2 (15 %) was one third of the PLA_2 found in the venom of *V. m. saintgironsi*, while svMP composed approximately half of the proteomes of the two juvenile *V. latastei* venom pools (44 %; Fig. 4). In light of these considerations, the venoms of *V. m. saintgironsi* and *V. latastei* seem to be similar only qualitatively, while they are considerably different in terms of relative abundances of toxin families.

Intriguingly, the venoms of the dwarf subspecies *V. m. monticola* and *V. m. atlantica* show a higher level of qualitative and quantitative similarity to adult *V. latastei* venom (Fig. 4). Although the venoms of both subspecies lack LAAOs, detected in *V. latastei* venom, they possess the toxin families DI and CRISP, which are absent in *V. m. saintgironsi* but present in *V. latastei*. While *V. m. monticola* venom presents a slightly lower amount of svMP but higher abundance of PLA_2 than *V. latastei* venom, svMP and CTL abundances are almost identical between *V. m. atlantica* and *V. latastei*.

Notably, high abundances of svMP have often been found in the venoms of vipers primarily feeding on ectotherms, such as juvenile *V. latastei* specimens (Fig. 4). Indeed, the venoms of ectotherm-specialists of the *Pelias* clade, such as *Vipera anatolica anatolica*, *Vipera anatolica senliki*, and *Vipera darevskii*, known to mainly feed on orthopterans, seem to follow this compositional pattern [70]. In light of the supposedly ectotherm-based diet of *V. m. monticola* and *V. m. atlantica*, we anticipated the venoms of these subspecies to

also present high amounts of svMP. Contrary to our expectations, this toxin family was present in only moderate amounts in the venoms of the two dwarf vipers (8 % and 13 %), while the relative abundances of PLA_2 were higher than in the ectotherm-specialist European vipers already mentioned (Fig. 4).

With higher PLA_2 relative abundances, the venom of *V. monticola* (and particularly *V. m. saintgironsi*) shows an unexpected analogy to the venoms of the geographically and phylogenetically distant *V. ammodytes* complex [78], further highlighted by the lack of DI (Fig. 4). The members of this species complex are well-known for their PLA_2-based strong neurotoxic venom [14]. The predominance of PLA_2 in *V. m. monticola* and *V. m. saintgironsi* raises the possibility of neurotoxic effects, although the broad functional range of PLA_2 toxins also encompasses haemotoxic and cytotoxic effects [81]. Therefore, further studies are needed to obtain a detailed understanding of the biological activity of this toxin family in the venom of *V. monticola*.

5. Conclusions

This study constitutes the first investigation into the protein composition of *V. monticola* venom. It is a complex, variable phenotype comprising a wide range of components, with svMP, svSP, CTL, and PLA_2 being the predominant toxin families, as commonly observed in Old World vipers. Our analysis shows qualitative and quantitative differences among all three *V. monticola* subspecies. Specifically, DI and CRISP are unique to the "dwarf" subspecies *V. m. monticola* and *V. m. atlantica*, while LAAO is exclusive to the larger *V. m. saintgironsi*. Although we support the theory that dietary habits influence the occurrence of venom diversity, the limited available data on the species' feeding ecology and how it varies among subspecies prevent us from definitively linking the observed venom variation to dietary differences. Furthermore, we acknowledge that analysing the venom of a single *V. m. saintgironsi* specimen is unlikely to offer a comprehensive picture of the qualitative and quantitative features of this subspecies' venom. A larger sample size, more detailed knowledge of the species' dietary habits, and the application of genomics, venom gland transcriptomics, and functional assays would yield a more thorough understanding of *V. monticola* venom variability and the factors determining it.

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Data availability

See 2.5 Material and Methods - Data accessibility.

CRediT authorship contribution statement

Maik Damm: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ignazio Avella:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Reema Merzara:** Data curation, Investigation. **Nahla Lucchini:** Resources, Writing – review & editing. **Jon Buldain:** Resources. **Frederico Corga:** Resources. **Abdellah Bouazza:** Resources. **Soumia Fahd:** Resources. **Roderich D. Süssmuth:** Funding acquisition, Resources, Writing – review & editing. **Fernando Martínez-Freiría:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Visualization, Writing – review & editing.

Declaration of competing interest

All authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2024.07.008.

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