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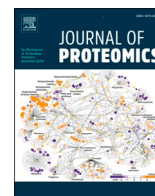
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Ontogeny, not prey availability, underlies allopatric venom variability in insular and mainland populations of *Vipera ammodytes*

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ABSTRACT

Allopatric populations living under distinct ecological conditions are excellent systems to infer factors underlying intraspecific venom variation. The venom composition of two populations of *Vipera ammodytes*, insular with a diet based on ectotherms and mainland with a diet based on ectotherms and endotherms, was compared considering the sex and age of individuals. Ten toxin families, dominated by PLA₂, svMP, svSP, and DI, were identified through a bottom-up approach. The venom profiles of adult females and males were similar. Results from 58 individual SDS-PAGE profiles and venom pool analysis revealed significant differences between juveniles compared to subadults and adults. Two venom phenotypes were identified: a juvenile svMP-dominated and KUN-lacking phenotype and an adult PLA₂/svMP-balanced and KUN-containing phenotype. Despite differences in prey availability (and, therefore, diet) between populations, no significant differences in venom composition were found. As the populations are geographically isolated, the lack of venom diversification could be explained by insufficient time for natural selection and/or genetic drift to act on the venom composition of island vipers. However, substantial differences in proteomes were observed when compared to venoms from geographically distant populations inhabiting different conditions. These findings highlight the need to consider ecological and evolutionary processes when studying venom variability.

Significance: This study provides the first comprehensive analysis of the venom composition of two allopatric populations of *Vipera ammodytes*, living under similar abiotic (climate) but distinct biotic (prey availability) conditions. The ontogenetic changes in venom composition, coupled with the lack of differences between sex and between populations, shed light on the main determinants of venom evolution in this medically important snake. Seven new proteomes may facilitate future comparative studies of snake venom evolution. This study highlights the importance of considering ecological and evolutionary factors to understand snake venom variation.

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1. Introduction

Venomous animals comprise more than 200,000 species and play important ecological roles [1,2], as illustrated by the great diversity of venomous snakes, which use venom to subdue their prey but also to deter potential predators [3]. Snake venoms are complex mixtures of proteins, peptides, and small organic and inorganic compounds [3–5]. Once delivered to the target organism, venoms can cause fatal disturbances of homeostasis by inducing neurotoxic, cytotoxic, hemotoxic, or a combination of these effects [6,7]. Envenomation facilitates overpowering prey before it retaliates or escapes; pain-inducing components play a key role in defence [8,9]. However, adaptations such as venom resistance in snake prey and predators hinder the effectiveness of venom, suggesting that both trophic and defensive adaptations are important aspects of this coevolutionary arms race [10–12]. These processes contribute to the high variability of venoms within and between taxonomic levels, and the emerging field of ecological proteomics provides a framework for understanding venom variation under variable environmental conditions [13].

Changing biotic conditions are assumed to drive snake venom diversity, e.g., variation in prey composition can promote the expression of specific toxins [14]. Diet-dependent feeding success can be a strong selective force in venom composition [8,15,16], particularly in relation to ontogenetic changes in diet [17,18]. Variation in snake venom has also been observed between individuals from the same population (e.g., sex differences [19,20]) and between populations occurring in distinct ecological conditions [21–26]. Geographic isolation, such as in island populations, may contribute to variation in snake venom phenotypes [27], but see [28] as a consequence of different availability of resources compared to mainland populations [29,30].

The Nose-horned viper, *Vipera ammodytes*, Linnaeus, 1758, is a medium-sized venomous snake currently considered a species complex with still unsolved systematics [31–33]. It is widely distributed from north-eastern Italy across the Balkans, reaching some Ionian and Aegean Islands, with scattered populations occurring in the Trentino region (Italy) and Asia Minor [34]. This snake is found in various ecoregions [35]. It is one of the most venomous vipers in Europe [36,37], and it is classified as of the highest medical importance (Category 1) by the World Health Organization [38]. Envenomation by this viper can cause local and systemic haemorrhage and necrosis [39,40], mainly induced by enzymatic components found in large abundances, such as phospholipases A₂ (PLA₂), snake venom metalloproteinases (svMP), and snake venom serine proteases (svSP) [6,41,42]. Occasional neurotoxic effects have been reported, mainly caused by PLA₂ (Ammodytoxin A) [43,44]. Differences in plasma clotting times were observed when comparing the venoms of *V. a. ammodytes* from Montenegro, *V. a. meridionalis* from Greece, *V. a. montandoni* from Bulgaria, and *V. a. transcaucasiana* from Türkiye, along with different potencies of two available antivenoms towards different venom origins, emphasising the need to study venom variation in this species [45]. Studies on *V. ammodytes* venom composition have been developed across geographically distant localities and in different subspecies [*sensu* [46]], such as in *V. a. ammodytes* from Croatia [36], *V. a. ammodytes* and *V. a. meridionalis* from Bulgaria [47], *V. a. ammodytes* from Serbia [48] and *V. a. transcaucasiana* and *V. a. montandoni* from Türkiye [49]. However, no studies have been carried out on populations that are geographically close but live under different ecological conditions. This fine scale is essential to identify the determinants likely to lead to the evolutionary divergence of venoms.

Populations of *V. ammodytes* (belonging to *V. a. montandoni*, *sensu* [46]) found on Golem Grad Island in the Prespa Lake, North Macedonia, and the neighbouring mainland population offer the possibility of studying variation in venom composition in allopatric populations. Separated by a 4.5 km water distance, the island and the mainland populations are under distinct biotic conditions. These localities differ in prey availability, resulting in island vipers having a diet based on

ectothermic prey like wall lizards (*Podarcis muralis*) and Mediterranean banded centipedes (*Scolopendra cingulata*), while on the mainland, vipers are consuming ecto- as well as endothermic prey, e.g. long-tailed field mouse (*Apodemus sylvaticus*) or house mouse (*Mus musculus*) [50,51]. The lack of small mammals (they account for 62 % of the mainland population's diet) could explain the dwarfism observed on the island, with nose-horned vipers being 20 % smaller and having a shorter relative jaw length than mainland individuals [50]. Sex differences are absent in adult diet composition on the island, while on the mainland, differences are primarily quantitative: males consume more lizards than mammals, and females consume lizards and small mammals at similar levels [52]. In both populations, there is a shift in diet composition during ontogeny; island vipers increase the consumption of lizards [50], while mainland counterparts increase the consumption of small mammals [52]. For these reasons, Golem Grad Island and the neighbouring mainland population offer the settings to study the potential roles of sex, age, and biotic conditions on the venom composition.

This study aimed to characterise the venom composition of allopatric populations of *V. ammodytes* from neighbouring island and mainland populations living under similar abiotic (climate) but distinct biotic (prey availability) conditions. Combining a bottom-up proteomic approach and profiling individual venoms using SDS-PAGE for different sex, age and population groups, the following questions were addressed: 1) are there sex differences in venom composition? Given the qualitative lack of sex differences in the diet of the studied populations, it is expected that there will be no significant differences; 2) are there age, hence size, related differences in venom composition? Given the ontogenetic changes in the diet of *V. ammodytes* it is expected that there will be differences; and 3) are there differences in venom composition between the island and mainland populations? Given the differences in prey availability between the two isolated locations, it is expected that there will be differences. By answering these questions, this work will contribute to understanding how biotic conditions influence snake venom variation at the intraspecific level.

2. Material and methods

2.1. Study areas

Venom samples of *V. ammodytes* were collected from island (ISL) and mainland (ML) populations in the Prespa region, North Macedonia. Golem Grad Island (ISL, 40°52'08" N, 20°59'23" E; total area of 18 ha) is located in Prespa Lake, 4.5 km from the shore. The mainland population is located in and around Konjsko village (ML; 40°54'42" N, 20°59'26" E, surveyed area of 24 ha). Populations are in the ecoregion of the Pindus Mountains mixed forests at 850 to 890 m. asl., with a sub-Mediterranean climate with continental influences [35,53].

2.2. Collection and preparation of venom samples

A total of 67 individual venom samples were collected from vipers captured in ISL ($n = 50$) and ML ($n = 17$) during the spring and summer of 2020, 2021, and 2022 (Supplementary Table S1). An Eppendorf tube covered with parafilm was used for venom extraction. The venom samples were stored in a portable fridge (4 °C) during fieldwork and later lyophilised. Date, locality, snout-vent length (SVL, in mm), total length (TL, in mm), and sex were recorded for each sampled individual. Snakes were sexed based on the shape of the tail or hemipenis eversion if necessary. Snakes were aged as 1) juveniles when SVL was smaller than 190 mm (ISL) and 270 mm (ML); 2) adults when SVL was larger than 330 mm (ISL) and 430 mm (ML); and 3) subadults with in-between SVL [52].

2.3. Venom pool analysis

Individual venom samples were assigned to seven venom pools

according to sex, age, and population to obtain representative venom profiles of distinct groups (Table 1). The resulting pools were made based on the successful venom extraction from snakes found during the fieldwork. In-depth qualitative and semi-quantitative profiles of the venoms were generated using the snake venomomics bottom-up approach [54,55].

2.3.1. Snake venom profiles by RP-HPLC

The lyophilised crude venom pools (1 mg) were dissolved in 100 µL aqueous 5 % (v/v) acetonitrile (ACN) with 1 % (v/v) formic acid (HFO) to a final concentration of 10 mg/mL and centrifuged at 10,000 ×g for 5 min to spin down insoluble content. The supernatant was injected into an Agilent 1200 semi-preparative RP-HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a Supelco Discovery BIO Wide Pore C18-3 Column (3 µm particle size, 4.6 × 150 mm column size). The flow rate of 1 mL/min was implemented using ultrapure water with 0.1 % (v/v) HFO (buffer A) and ACN with 0.1 % (v/v) HFO (buffer B). The elution gradient was as follows: 0–5 min, 5 % B; 5–100 min, 5–40 % B; 100–120 min 40–70 % B; 120–130 min 70 % B; 130–130.10 min 70–5 % B; 130.10–132.10 min 5 % B. Absorbance was measured at $\lambda = 214$ nm using a diode array detector (DAD), and 1 mL eluates were automatically collected in a time-based manner (1 fraction/min). When needed, peaks of the chromatograms were manually pooled, and all fractions were vacuum-dried (Thermo Speedvac, Bremen, Germany). The first fractions (peaks that eluted between 0 and 30 min, depending on the venom pool) only containing low molecular peptides were measured without further procession by mass spectrometry. All other fractions were submitted to electrophoretic separation and tryptic in-gel digestion before being submitted to LC-MS.

2.3.2. Second separation by SDS-PAGE

Lyophilised fraction samples were redissolved in 10 µL reducing 2× SDS sample buffer (125 mM Tris HCl pH 6.8, 4 % (w/v) SDS, 17.5 % (w/v) glycerol, 0.02 % (w/v) Bromphenol blue and 200 mM dithiothreitol DTT in ultra-pure (MQ) water) and heated at 95 °C for 10 min. They were separated using 12 % SDS-PAGE (SurePage Bis-Tris, Genscript, Piscataway, NJ, USA) and run with an MES buffer at 200 V for 21 min. A PageRuler Unstained Protein Ladder (Thermo Scientific, Waltham, MA, USA) was used as a standard to determine the apparent mass of the proteins. The gels were washed three times with water, and the proteins were fixed by submerging them for 10 min in a hot fixation buffer (aqueous, 40 % (v/v) methanol, 10 % (v/v) acetic acid), three times under constant mild shaking. After that, they were stained with a hot fast staining buffer (aqueous, 0.3 % (v/v) HCl 37 %, 100 mg/L Coomassie 250G) under mild shaking conditions for 45 min and washed three times shortly in washing buffer (aqueous, 40 % (v/v) methanol, 10 % (v/v) acetic acid). The gels were then kept overnight at 4 °C in a storage buffer (aqueous, 20 % (v/v) methanol, 10 % (v/v) acetic acid) to destain, after which they were scanned for documentation and quantification purposes.

Table 1

Venom pools of *V. ammodytes*. Number of individual venoms of *V. ammodytes* assigned to each pool according to age (A, adult; S, subadult; J, juvenile), sex (F, females; M, males), and population (ISL, island; ML, mainland). The code of each pool is provided.

Venom pool	Code	Total	Females	Males
Adult females Island	A_F_ISL	20	20	–
Adult males Island	A_M_ISL	18	–	18
Subadults Island	S_ISL	3	1	2
Juveniles Island	J_ISL	3	2	1
Adults Mainland	A_ML	3	2	1
Subadults Mainland	S_ML	6	2	4
Juveniles Mainland	J_ML	3	2	1

2.3.3. Tryptic digestion

Protein bands were excised from the gel, dried with 500 µL ACN and stored at –20 °C without ACN until the tryptic digestion. The first step included the reduction of disulfide bridges using 30 µL of 100 mM DTT in 100 mM NH₄HCO₃, pH 8.3, for 30 min at 56 °C, followed by drying with 500 µL ACN. Alkylation of cysteine with 30 µL of 55 mM iodoacetamide in 100 mM NH₄HCO₃, pH 8.3, was performed in the dark, at room temperature, for 20 min. The washing step with 500 µL ACN for 2 min and drying with 500 µL ACN for 15 min preceded the in-gel 30-min incubation with trypsin on ice (13 ng/µL, buffer 10 mM NH₄HCO₃ with 10 % (v/v) ACN, pH 8.3; Thermo, Rockford, IL, USA). If part of the gel was not submerged in trypsin, additional volume was added and left for 90 min on ice. After 2 h of incubation, 20 µL of ABC buffer (10 mM) was added, and samples were incubated overnight at 37 °C. Peptide extraction was done using prewarmed 100 µL elution buffer (aqueous 30 % (v/v) ACN, with 5 % (v/v) HFO) for 15 min at 37 °C. The resulting supernatant was transferred to a separate microtube and vacuum-dried.

2.3.4. Mass spectrometry

The supernatants were re-dissolved in 20 µL aqueous 3 % (v/v) ACN, with 1 % (v/v) HFO. They were submitted to LC-MS/MS analysis using an Orbitrap XL mass spectrometer (Thermo, Bremen, Germany). The chromatographic separation was performed on an Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany) using a reversed-phase Grace Vydac 218MS C18 column (5 µm particle size, 2.1 × 150 mm column size). Ultrapure water with 0.1 % (v/v) HFO (solvent A) and ACN with 0.1 % (v/v) HFO (solvent B) at a flow rate of 0.3 mL/min was used. Peptides were eluted following a linear gradient of 5–40 % B for 10 min, 40–99 % B for 3 min, washed with 99 % B for 3 min, and the system was re-equilibrated to 5 % B for 2 min. In the ESI positive mode, the mass spectrometric parameters were as follows: capillary temperature of 270 °C, sheath gas flow rate of 45 L/min, auxiliary gas flow rate of 10 L/min, source voltage of 4.0 kV, source current of 100 µA, capillary voltage of 20 V, and tube lens voltage of 130 V FTMS measurements were taken with 1 µ scans and a maximal fill time of 1000 ms. AGC targets were set to 106 for full scans and 3 × 105 for MS2 scans. MS2 scans were performed with a mass resolution (R) of 60,000 (at m/z 400) for m/z 250–2000. MS2 spectra were obtained in data-dependent acquisition (DDA) mode as top2 with 35 V normalised CID energy and 500 as the minimal signal required with an isolation width 3.0. The default charge state was set to $z = 2$, and the activation time was 30 ms. Unassigned charge states and charge state 1 were rejected. Directly submitted peptide fractions from earlier retention times of the initial HPLC run were analysed with all charge states accepted.

LC-MS/MS data files (.raw) were converted into .mgf files using MSConvert (Version 3.0.22187) with peak picking (vendor msLevel = 1–) [56] and annotated by pFind Studio with pFind version 3.2.0 [57] and the integrated pBuild. The parameters used: MS Data (format: MGF; MS instrument: CID-FTMS); identification with Database search (enzyme: Trypsin KR_C, full specific up to 3 missed cleavages; precursor tolerance +20 ppm; fragment tolerance +20 ppm); open search setup with fixed carbamidomethyl [C] and Result Filter (show spectra with $FDR \leq 1$ %, peptide mass 500–10,000 Da, peptide length 5–100, and show proteins with number of peptides >1 and $FDR \leq 1$ %). The used databases included UniProt “Serpentes” (ID 8750, reviewed, canonical and isoform, 2674 entries, last accessed on 10 February 2022; available at: <https://www.uniprot.org/>) and the common Repository of Adventitious Proteins (215 entries, last accessed on 10 February 2022; available at: <https://www.thegpm.org/crap/index.html>).

The identified peptides were exported as PSM scores and manually cleared from any decoy entries, contaminations, and artefacts. A list of unique peptide sequences per sample with the best final score was assembled. To confirm identified sequences, all unique ones were analysed using BLAST search [58], with blastp against the non-redundant protein sequences (nr) of the “Serpentes” (taxid: 8570) database. For non-automatically annotated bands, 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. The XTRACT algorithm of Thermo Xcalibur was used for the deconvolution of isotopically resolved spectra.

2.3.5. Relative toxin quantification by snake venomomics

Relative, label-free quantification is based on the snake venomomics method [59] by integrating the values of RP-HPLC peak integral measured at $\lambda = 214$ nm, the SDS-PAGE band intensity and, if necessary, the MS ion intensity of the most abundant peptides identified following the protocol by Damm et al., 2024 [55]. Briefly, when more than one venom protein was present in an HPLC fraction, their proportions (% of total protein bands area) were measured by densitometry of the scanned gels using ImageJ software [60]. The colour depth was set to 8-bit grayscale, and the area and integrated density of each SDS-PAGE band were measured.

2.4. Individual venom analysis

Individual analyses were conducted to assess the diversity of expressed proteins in 58 venom samples and thus complement the in-depth proteomic analysis. Lyophilised samples (20 μ g) were dissolved in 10 μ L reducing 2 \times SDS sample buffer and separated on SDS-PAGE using the same protocol as for venom pools. The gels were scanned, and high-resolution digital images were used to create a matrix indicating the presence or absence of bands. To ensure the accuracy of the matrix, the identification process was repeated three times, and the results were averaged to obtain the final matrix (Supplementary Table S2).

2.5. Statistical analyses

Ordination, cluster, and linear regression analyses were used to compare assessed protein components identified in venom pools and individual venoms concerning three categorical variables: sex, age, and population. Given that two types of numerical data were used in the analyses, i.e., binary presence/absence data of specific SDS-PAGE bands and compositional data of relative abundance of venom components in each proteome, two ordination approaches were used. Three-dimensional Non-metric Multidimensional Scaling (NMDS) was used for binary data of SDS-PAGE bands, and Principal Component Analysis (PCA) was used for compositional data of proteomes.

The NMDS was used to assess sex, age, and population differences in venom composition. The Jaccard similarity index (ISj) was used in these analyses to calculate the distances between groups, fitting a stress value lower than 0.2 [61]. To evaluate whether there is a statistically significant relationship between categorical variables and venom composition, the individual scores of the first NMDS axis (i.e. NMDS1) were used as a response variable in univariate linear regression models considering each categorical variable (sex, age and population) as a predictor.

The PCA was used to visualise potential age and population differences in venom composition across proteomes and to identify components contributing to proteome differentiation. Before analyses, compositional data (relative abundance of venom components expressed as percentages between 1 % and 100 %) were transformed into log-centred ratios [62].

Hierarchical Cluster Analysis (HCA) was further used to assess sex, age, and population differences in venom composition. The ISj was used to analyse the presence/absence data of specific SDS-PAGE bands and Euclidean distances to analyse the relative abundance of venom components in each proteome. The UPGMA clustering method was used to visualise HCA results.

NMDS, PCA and HCA analyses were performed in Past4.03 [63], while univariate linear regression was performed in R version 4.3.2 [64] using packages *vegan* for NMDS analysis and *car* for linear regression models.

3. Results

3.1. Venom composition

Seven venom proteomes of *V. ammodytes* were assessed using the snake venomomics bottom-up approach covering different sex and age groups from island and mainland populations in North Macedonia (Table 2). This allowed the identification and relative quantification of the toxin families in the studied *V. ammodytes* venoms (Supplementary Tables S3-S16, Supplementary Fig. S1-S7).

The most abundant toxin families were phospholipases A₂ (PLA₂), snake venom metalloproteinases (svMP), disintegrins (DI), and snake venom serine proteases (svSP). Snake C-type lectin-like proteins (CTL), cysteine-rich secretory proteins (CRISP), vascular endothelial growth factors (VEGF), and L-amino-acid oxidases (LAAO) belonging to secondary viperine toxin families (classification according to Damm et al. 2021 [65]) were present in all proteomes. Kunitz-type serine protease inhibitors (KUN), a secondary toxin family, were annotated in all proteomes except the juveniles from both populations. Venom nerve growth factors (NGF) and non-annotated components (NA) were of low abundance (<1 %). Distinct peptides were also present in all venoms, such as svMP inhibitors (svMP-i; pEKW), Natriuretic peptide-related (NP-related; DNEPPKKVPPN) and Bradykinin-potentiating peptides (BPP; PSPKVPV).

The PLA₂ was the major toxin family identified per relative abundance in proteomes of adults (23–24 %) and lower in juveniles (14–18 %). Homolog peptides to sequences of neutral ammodytin I2 (A') [Q6A3D9.1] and ammodytin I2 (B) [Q6A3B4.1] were identified, as well as to the enzymatically inactive basic PLA₂ ammodytin L [P17935.1], with a myotoxic role. Tryptic peptides, including the conserved His48 followed by Asp49 (e.g. CCFVHDCCYGR) for active and Ser49 (e.g. CCFVHSCCYAK) for inactive PLA₂ homologs, identified both PLA₂ types in venoms of islands as well as mainland populations. Another ubiquitously found proteoform was ammodytoxin C [Q6A361], identified by the variant-specific C-terminal NYPDILCKEESK. All seven venom pools were rich in svMP, including Vaa-MPII-1 [A0A119KNT0], Vaa-MPII-3 [A0A119KNS3], Vaa-MPIII-1 [A0A119KNR6], Vaa-MPIII-5 [A0A6B7FNN4] known from *V. ammodytes*, and an *Echis ocellatus* svMP [E9KJY2]. DI has been identified in the form of homo and heterodimers, like VA6 [POC6A5.1] and heterodimers VB7A [POC6A6.1], VB7B [POC6A7.1] and CC8B [P83044.1]. The main svSP identified showed high similarity with nikobin [E5AJX2.1], serpentokallikrein-1 [XP_015671564.1], and gussurobin isoform X1 [XP_039181623.1]. Sequence homolog to neutral Vaa-SPH-1 [A0A119KNP0.1], which lacks the typical svSP enzymatic activity but expresses an anticoagulant effect, was found in all studied proteomes.

Found CTL were annotated as Snaclec 1 [Q6X5S3.1], C-type lectin-

Table 2

Snake venom composition of *V. ammodytes* from island and mainland populations. Relative abundances of the toxin families identified in the seven *V. ammodytes* venom pools analysed by bottom-up snake venomomics. Venom codes are based on Table 1.

Toxin family	Island				Mainland		
	A_M_ISL	A_F_ISL	S_ISL	J_ISL	A_ML	S_ML	J_ML
PLA ₂	24.4	23.8	20.3	14.1	23.1	38.6	18.0
svMP	16.8	22.1	22.8	36.9	22.4	14.9	36.0
svSP	10.6	9.2	11.0	3.8	11.9	9.2	4.3
CTL	4.6	7.0	6.1	7.3	6.7	4.6	5.5
DI	15.6	13.6	15.7	14.8	10.0	8.5	13.1
LAAO	1.4	2.2	2.1	2.1	3.1	1.6	1.8
CRISP	6.1	4.2	6.3	11.0	7.8	5.5	8.5
VEGF	3.9	2.8	3.2	1.1	1.6	1.5	0.7
KUN	3.5	2.0	2.1	0.0	3.7	4.2	0.0
NGF	0.9	0.4	0.4	0.5	0.2	0.3	0.1
peptides	11.4	12.1	9.5	7.9	8.8	10.2	11.0
NA	0.7	0.5	0.4	0.6	0.7	0.9	1.0

like protein 3A [AJ070726.1], as well as the factor X activator light chain 2 [Q696W1] described from *Macrovipera lebetina*. CRISP included annotations from *V. berus* [B7FDI1.1] and *Echis coloratus* [PODMT4.1]. The less abundant toxin families (<4 %) were LAAO, VEGF, KUN and NGF. Some of the LAAO were enzymes described from *Sistrurus catenatus edwardsii* [BOVXW0.1] and *Daboia russelii* [G8XQX1.1]. In the current study, KUN was found, with Kunitz/BPTI inhibitor-7 [AMH40739.1] being one of the proteins identified. VEGF was annotated as Vammin-1 [APB93447.1], while NGF, differentially abundant in studies samples, as a homolog of β -nerve growth factor from *D. russelii* [ASU45040.1], among other identified proteins (Supplementary Tables S3-S16).

3.2. Sex-based differences in venom composition

The Non-metric Multidimensional Scaling (NMDS) analysis based on the presence/absence matrix of SDS-PAGE bands of 38 individual adult venoms (20 females and 18 males) from both populations showed an overlap of female and male venoms (Fig. 1A; NMDS scores in Supplementary Table S17). The linear regression analysis based on the NMDS1 scores displayed no significant difference in the venom composition when females and males were tested ($p = 0.056$). The HCA showed no sex-specific groups in band composition (Supplementary Fig. S8).

Individual female and male venom samples from the island population were pooled (Table 2). The chromatograms of both pools were highly similar regarding the peak pattern (Supplementary Fig. S1A and S2A). Prominent differences were in the peak intensities at $R_t = 85$ – 90 min in peaks containing svMP, leading to minor sex differences (female = 22 %, male = 17 % svMP). No sex-specific bands in the SDS-PAGE were detected (Supplementary Fig. S1B and S2B). The qualitative

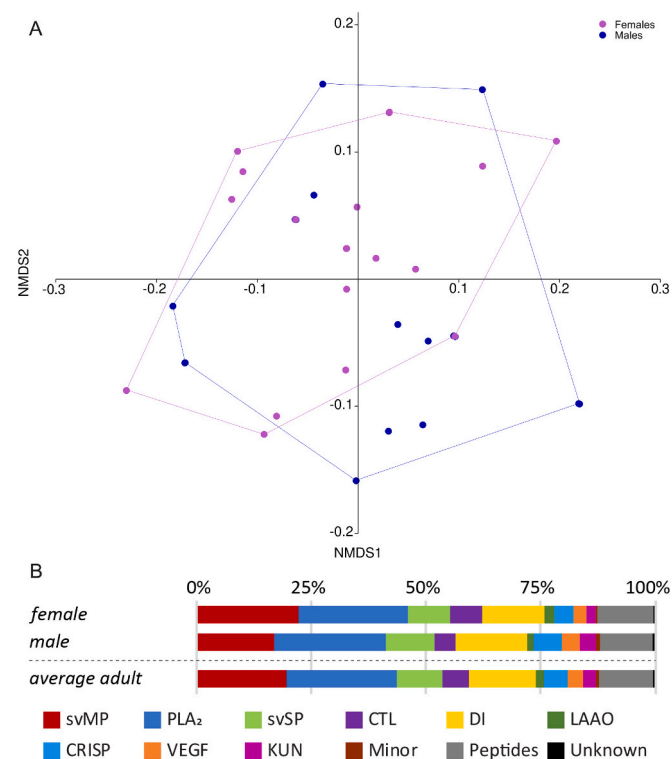


Fig. 1. Sex-based venom comparison of adult *V. ammodytes*. (A) NMDS analysis of a binary matrix of 20 females and 18 males individual SDS-PAGE profiles from the island and mainland populations. An overlap of individual female and male venoms is shown. Linear regression analysis based on the NMDS1 scores revealed no significant difference ($p = 0.056$). (B) Venom composition of females and males from the island population, based on relative abundances of toxin families. A_{ISL} represents the average adult venom composition for the island population.

composition of the female and male proteomes was highly similar, with substantial overlaps across the relative abundances and no protruding proteins identified (Fig. 1B). Due to the absence of significant sex differences in venom composition, female and male venoms were pooled in the following analyses. Their relative toxin abundances were averaged to represent the adult venom proteome from the island population (A_{ISL}, Fig. 1B).

3.3. Ontogenetic shifts in venom composition

The NMDS analysis based on the presence/absence matrix of SDS-PAGE bands of 58 individual venoms (37 adults, 15 subadults and 6 juveniles) from both populations showed a clear separation of juvenile venom in relation to subadult and adult venoms (Fig. 3). Linear regression based on the NMDS1 scores (Supplementary Table S18) revealed a significant difference between juveniles and the other two age groups ($p < 0.001$). The HCA also clustered juveniles in one group and the two other age groups in another cluster (100 % bootstrap support (BS), Supplementary Fig. S9).

In both populations, svMP and CRISP decreased with age, while PLA₂, svSP, VEGF and KUN increased (Fig. 2B, Supplementary Table S19). The other toxin families displayed no clear trend across ontogeny. The biggest changes during ontogeny were observed in svMP, svSP, and KUN abundances. The most prominent HPLC profile differences were observed between juveniles and the other age groups in both populations (Supplementary Fig. S1A-S7A). In the island population, peaks eluting at $R_t = 30$ – 40 min had low abundances in juveniles. Peaks found at this retention time in adults contained KUN, a toxin family lacking in the juvenile venom. In juveniles, DI (peak 14) and svMP (peak 36) were more abundant than in adult venom, and CRISP (peak 29) occurred in juvenile-specific SDS-PAGE bands (29a, b and c). A similar

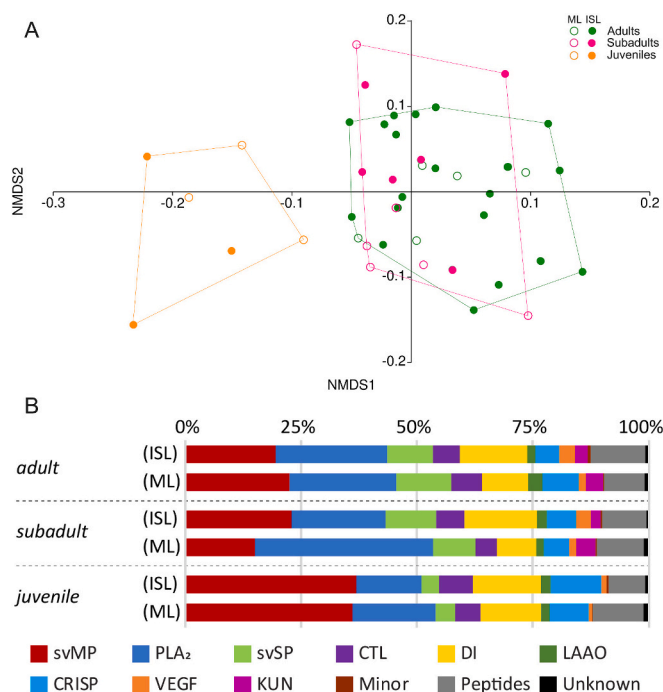


Fig. 2. Age-based venom comparison of *V. ammodytes*. (A) Binary NMDS analysis of a binary matrix of 37 adults, 15 subadults and 6 juveniles individual SDS-PAGE profiles from the island and mainland populations. Differentiation of juvenile venoms in relation to subadult and adult venoms at the individual level is shown. Linear regression based on the NMDS1 scores revealed a significant difference ($p < 0.001$). (B) Venom composition of age groups (A, adult; S, subadult; J, juvenile) from the island (ISL) and mainland (ML) populations, based on relative abundances of toxin families. Pools contain venoms from both sexes.

pattern was observed in the mainland population (Supplementary Fig. S1B-S7B). KUN was absent in juveniles, while in adults, it was found in peaks eluting at $R_t = 30\text{--}40$ min. A more complex peak pattern at $R_t = 50\text{--}60$ min was observed in adult venom compared to juveniles. Juvenile venom was characterised by abundant svMP (peaks 30, 31), while subadults had a high abundance of PLA₂ (peaks 24, 27).

To identify components contributing to the proteome differentiation, logarithmically transformed relative abundances of venom pools were used in a Principal Component Analysis (PCA). In the PCA plot of the toxin families identified in six proteomes, the first two axes (PC1 and PC2) explained 97.1 % of the total variance, with a 92.1 % contribution of PC1 (Fig. 3, Supplementary Table S20). The PC1 depicts the variation in venom composition between age groups, where juveniles are separated from subadults and adults, while the PC2 depicts the variation between island and mainland populations. The HCA grouped juveniles on one branch and adults and subadults on another branch with 100 % BS (Supplementary Fig. S10). Within adults and subadults, additional grouping was based on the island (BS = 93 %) and mainland (BS = 78 %) populations.

3.4. Population differences in venom composition

To further carve out differences between subadult and adult venoms observed in cluster analysis (Supplementary Fig. S10) and PCA (Fig. 3), juvenile venoms were removed from the following analysis. This step was done because juveniles contributed most substantially to explain the variation in the datasets, potentially covering differentiation patterns between island and mainland populations. However, no differences were found between island and mainland populations in analysing the binary matrix of 52 individual SDS-PAGE venom profiles of adults and subadults (40 from island, 12 from mainland; Fig. 4, Supplementary Fig. S11). The linear regression based on the NMDS1 scores (Supplementary Table S21) also confirmed the absence of significant differences between the island and the mainland populations ($p = 0.713$).

4. Discussion

This work provides the first comprehensive study of *V. ammodytes* venom variation from two closely located allopatric populations under comparable abiotic (climate) but distinct biotic (prey availability) conditions. The major findings were that venom composition changes throughout ontogeny, with no evidence for significant sex or population differences. Age differences in venom composition and the lack of sex differences align with the ecology of *V. ammodytes*, while the observed reduced geographic variation in venom composition is contrary to

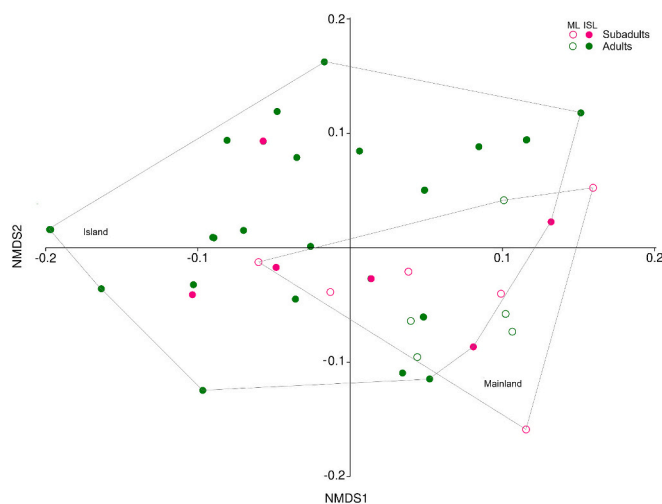


Fig. 4. Population-based venom comparison of *V. ammodytes*. NMDS analysis of a binary matrix of 40 island and 12 mainland individual SDS-PAGE profiles from the island and mainland populations. An overlap of individual venoms from the island and mainland is shown. Linear regression based on the NMDS1 scores revealed no significant differences ($p = 0.713$).

expected relationships.

4.1. Venom composition

The snake venomics bottom-up approach led to the identification of 10 toxin families along with various peptides. The most abundant toxin families were PLA₂, svMP, svSP, and DI (approximately 70 % of the proteome), which likely explains the hemotoxic and neurotoxic effects reported in the clinical picture of *V. ammodytes* envenomation. The PLA₂ was found in diverse isoforms that can induce different physiological symptoms [66,67]. The ubiquitously expressed proteoform in this study was the ammodytoxin C variant, a basic PLA₂ that acts on the neuromuscular junction by inhibiting neurotransmission [68]. Most of the local and systemic haemorrhages are caused by svMP, especially svMP P-III, by disrupting the basal membrane of blood vessels [69]. DI, the third most abundant toxin family, modulates platelet aggregation, cell migration and angiogenesis [70,71]. Compared to the venoms from other species in the genus *Vipera*, the abundance of DI in the current study is among the highest reported [55]. In other strike-and-release predators, e.g., *Crotalus atrox*, it has been shown that DI play an

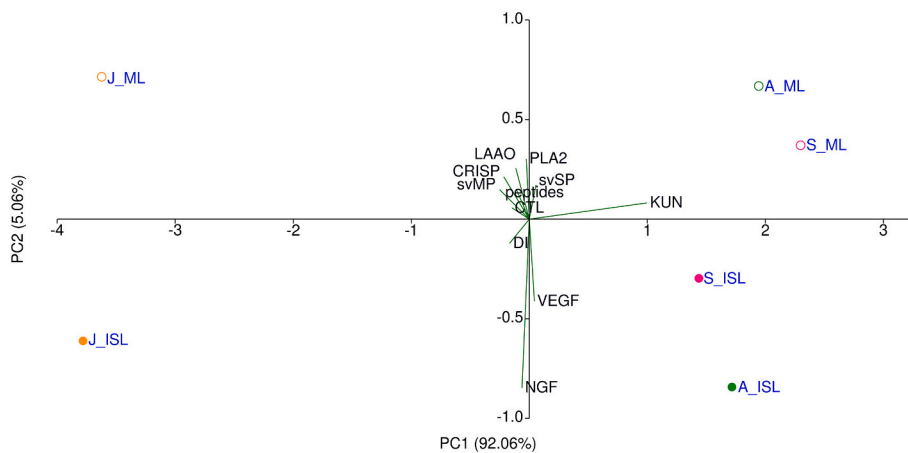


Fig. 3. PCA biplot of the toxin families in *V. ammodytes*. PCA biplot of log-transformed relative abundances of the toxin families identified in six proteomes of *V. ammodytes* (A, adult; S, subadult; J, juvenile; ISL, island; ML, mainland). The direction and length of the vectors (green lines) indicate which components contribute to proteome differentiation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

important role in chemically tagging and tracing envenomated prey [72]. DI could have a similar role in *V. ammodytes*, which employs a similar foraging strategy [73], but this requires further investigation. The last major protein family is svSP, which contributes to coagulation disorders by cleaving or inhibiting specific proteins in the blood clotting cascade [74,75].

The other less abundant toxin families included CTL, CRISP, VEGF, LAAO, KUN and NGF. The CTLs also contribute to prompt disturbance in coagulation by inducing inhibition or activation of platelet aggregation [76,77]. Their activity could be one of the reasons for thrombocytopenia in *V. ammodytes* envenomation [39]. A widespread protein family in snake venoms are CRISP, but the biological functions of many of them are still to be determined. VEGF targets growth factor receptors, leading to increased vascular permeability and may promote the spread of venom [36,78]. Other toxin families contributing to the envenomation are LAAO and KUN, the former producing hydrogen peroxide and leading to myotoxicity, haemorrhage, and haemolysis [79], and the latter contributing significantly to the venom-induced pathophysiology through synergistic action with other venom components as in the case of protease inhibitors [80]. Further work should explore the diversity within each protein family, including peptide identification and detecting post-translational modifications. The in-depth exploration will allow additional assessment of venom composition, which is essential for understanding the evolutionary pathways of venom divergence and the clinical consequences of envenomation.

4.2. Venom composition is not determined by sex

Within the two populations, no significant differences were found between the proteomes of adult females and males from the island nor in the individual adult venoms from both populations. The absence of significant venom differences according to sex in this study aligns with the previously reported lack of variation in venom composition in other vipers, like in some studies in the genus *Bothrops* [81,82]. Still, certain sex differences were reported in other Viperinae species, e.g., *Vipera latastei* [83], *Vipera seoanei* [84] and *Vipera kaznakovi* [85]. Most likely, the similar venom composition across sexes is related to the diet of adult *V. ammodytes*, which is characterised by a different abundance of prey consumed between females and males but essentially identical prey types [50,52]. Overall, the selective pressures acting on the envenomation mechanisms of common types of prey taken by both sexes seem to result in similar venom composition.

4.3. Ontogenetic shift in venom composition

Two venom phenotypes were identified in populations from North Macedonia: a juvenile svMP-dominated and KUN-lacking versus an adult PLA₂/svMP-balanced and KUN-containing venom. Significant differences between the venoms of juveniles and the other age groups (subadults and adults) were also confirmed at the individual level. The svMP decrease and PLA₂ increase throughout ontogeny have been reported in several Viperidae species, e.g., in *V. latastei* [83], *V. kaznakovi* [85], and *Bothrops jararacussu* [86,87]. The ontogenetic shifts in diet composition are a common trend for European vipers [88,89]. In the case of mainland *V. ammodytes*, juveniles tend to consume primarily lizards, while adults increase the consumption of small mammals [50,52,90]. Therefore, the ontogenetic shift in venom composition found in the mainland population is likely linked to the shift in diet, as reported in several other venomous snakes [86,91]. In contrast, juveniles on the island tend to consume lizards and centipedes, while adults consume primarily lizards without a striking qualitative change in diet due to the lack of prey diversity in Golem Grad [50]. Still, changes in venom composition throughout the ontogeny were found in the island population, suggesting additional underlying factors of venom composition shifts (further discussed in the following chapter). Additional studies on the biological activity of specific venom components, such as

PLA₂ and svMP, could give more insights into their role in subduing specific prey types throughout the ontogeny.

4.4. Venom composition and prey availability

Despite the distinct biotic conditions observed, no major differences in the venom composition between proteomes and individual venoms from the island and mainland populations were found. The statistical tests may be limited based on the asymmetrical venom sample represented in the two populations (ISL = 40, ML = 12). Still, the patterns observed at the individual level were consistent with those found at the proteome level, suggesting that sample size did not affect the results. The absence of major differences in venom composition from islands has already been reported in populations of *Crotalus helleri caliginis*, even when the diet composition is distinct [28]. Potential explanations for these findings include gene flow between island and mainland populations and/or insufficient isolation time [92]. Molecular studies estimating the separation time of the island population and testing for gene flow between *V. ammodytes* populations in the Prespa region are currently unavailable. However, the 4.5 km water distance between the island and the mainland should constitute a significant geographic barrier for a medium-sized terrestrial viper, such as *V. ammodytes* [51]. If gene flow is indeed absent, then geographic isolation can cause a decrease in the diversity of venom components in the island population if enough time passes [27]. The time of island colonisation by vipers is unknown, but potentially, the time spent in isolation has not been sufficient for natural selection or genetic drift to act; hence, minor differences were observed in the venom composition of these populations. On the contrary, the venom of adult vipers from the island retains components found in the venom of vipers from the mainland that feed on small mammals despite the absence of such prey on the island. This suggests that venom composition in *V. ammodytes* occurring in geographically separated populations is relatively well conserved in the same age groups, even in the presence of distinct prey availability.

4.5. Geographic variation

Six *V. ammodytes* venom proteomes from Bulgaria, Türkiye, Croatia and Serbia have been described, along with proteomes from North Macedonia from the current work [36,47–49]. Given the different protocols used for protein identification and quantification, a strict quantitative comparison with the ones from this study is only possible with two published proteomes [49]. The comparable proteomes originate from venoms collected in European Türkiye (published as *V. a. montandoni*) and Asia Minor (as *V. a. transcaucasiana*). The most prominent differences between venoms collected in vipers from Türkiye and this study (corresponding to *V. a. montandoni*) were observed in a higher abundance of svMP and CTL and a lower abundance of PLA₂ in populations from North Macedonia, and the absence of DI in populations from Türkiye (Fig. 5). Potential reasons for these substantial differences

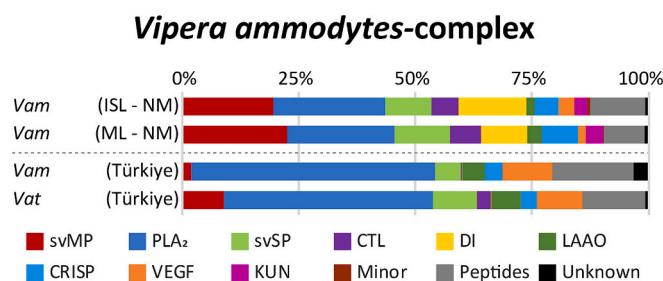


Fig. 5. Comparative venom profiles of *V. ammodytes*. Relative abundances based on snake venomomics across *V. ammodytes* taxa (*V. a. montandoni*, Vam; *V. a. transcaucasiana*, Vat) from the island (ISL) and mainland (ML) populations in North Macedonia (NM) and from Türkiye [48].

might be related to ecological conditions where these populations occur and/or genetic distances between analysed populations. The three proteomes originated from distinct ecoregions: *V. a. montandoni* from Prespa Lake in the Pindus Mountains mixed forests, *V. a. montandoni* from European Türkiye in the Balkan mixed forests, and *V. a. transcaucasiana* in Northern Anatolian conifer and deciduous forests [35]. These ecoregions are characterised by distinct abiotic (i.e. temperature and rainfall) and biotic (i.e. distinct flora and fauna communities), likely influencing potential prey available to *V. ammodytes* and, consequently, venom phenotypes [26]. Another complementary reason for distinct venom compositions could be phylogenetic divergence, as observed in *Vipera monticola* [93]. Indeed, the venom samples originated from individuals belonging to distinct phylogenetic subclades: *V. a. montandoni* (Prespa Lake) to the South 1 + 2 subclade, *V. a. montandoni* (European Türkiye) to the East subclade, and *V. a. transcaucasiana* to the Türkiye subclade [32]. The observed geographic variation in venom composition, potentially related to ecological variation and/or phylogenetic relationships, calls for further compositional and functional studies investigating the underpinning factors related to venom diversity across the broad distribution of this species.

5. Conclusions

This study provides the first comprehensive analysis of *Vipera ammodytes* venom composition from two allopatric populations inhabiting localities characterised by similar abiotic (climate) but distinct biotic (prey availability) conditions. Venom proteomics facilitated the identification of 10 toxin families dominated by PLA₂, svMP, svSP, and DI. The ontogenetic shift in venom composition was characterised by juvenile venom dominated by svMP but lacking KUN and an adult venom with similar abundances of PLA₂ and svMP and an increase in KUN. No evidence was found for significant sex-based venom differences and no venom specialisation towards the distinct prey availability. However, prominent differences in venom proteomes were observed when comparing venoms from geographically distant populations under distinct ecological conditions. This study underscores the importance of considering ecological and evolutionary processes in studies addressing snake venom variability.

Ethics approval

The research was done with the permission of the Ministry of Environment and Physical Planning of North Macedonia (permit not. UP-11/1-418/2021). All applicable international, national, and/or institutional guidelines for the use of animals were followed. The snakes were caught in the field, milked and returned to the capture site. No snakes were injured during processing, and no snakes showed signs of distress at release.

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CRedit authorship contribution statement

Margareta Lakušić: Writing – original draft, Visualization, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Maik Damm:** Writing – review & editing, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Vukašin Bjelica:** Writing – review & editing, Resources, Funding acquisition. **Marko Anđelković:** Writing – review & editing, Resources, Funding acquisition. **Ljiljana Tomović:** Resources, Funding acquisition, Conceptualization. **Xavier Bonnet:** Writing – review & editing, Resources, Methodology, Conceptualization. **Dragan Arsovski:** Writing – review & editing, Resources, Funding acquisition. **Roderich D. Süßmuth:** Writing – review & editing, Resources, Funding acquisition. **Juan J. Calvete:** Writing – review & editing, Methodology. **Fernando Martínez-Freiría:** Writing – review & editing, Visualization, Supervision, Software, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Mass spectrometry proteomics data have been deposited under the project name "DATASET - Mass Spectrometry - Snake venom proteomics of island and mainland *V. ammodytes* populations from North Macedonia" to the MassIVE partner repository (<https://massive.ucsd.edu/>) with the dataset identifier "MSV000095060" and in the Zenodo repository (<https://zenodo.org>) with the dataset identifier "11482457" [doi: <https://doi.org/10.5281/zenodo.11482457>].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2024.105320>.

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