Morphological variation, proximity to water, comparative venom proteomics and snakebite epidemiology of the medically important Cypriot blunt-nosed viper *Macrovipera lebetina lebetina*

Dissertation

to attain the degree Doctor of Philosophy (Ph.D.)
of the Faculty of Forest Sciences and Forest Ecology
Georg-August-Universität Göttingen

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Göttingen, September 2023

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Date of oral examination: 11 May 2023

Abstract

Snakes and humans are in a complex relationship. While snakes are of ecological and economic relevance as rodent predators, thus contributing to food security and public health, venomous species are also of medical importance as a cause of serious snakebite envenoming. Among the largest Middle Eastern viper species is the blunt-nosed viper (*Macrovipera lebetina*), whose nominate subspecies *M. lebetina lebetina* is considered endemic for Cyprus, where it is the only dangerously venomous snake species. While their hemotoxic and cytotoxic venom is valuable for drug research, blunt-nosed vipers also cause severe snakebite accidents and are feared and often persecuted by people. The Cypriot blunt-nosed viper is a threatened species listed in Appendix II of the Berne convention.

With published body weight data and sex-specific morphometric data missing for *M. l. lebetina*, the **first objective** of this doctoral thesis was to provide first-time sex-specific morphometric data for *M. l. lebetina* (**Chapters 2 and 3**). The data were collected during four field trips to Cyprus (2014-2022), with wild blunt-nosed vipers being systematically measured regarding their snout-vent length (SVL), tail length (TaL), head length (HL), head width (HW) and body weight, and a body condition index (BCI) calculated. The statistical investigation included a regression and correlation analysis and a sex-specific comparison via the unpaired *t*-test and Mann-Whitney U test. The dataset increased from 34 measured individuals (**Chapter 2, published**) to 46 (**Chapter 3, unpublished**), including 16 adult males, 21 adult females and nine juveniles. Their rounded total lengths (ToL) ranged from 23.5 cm to 133.5 cm, and body weights from 10 g to 1,456 g. Adult females showed a much weaker relationship between SVL and weight (TaL, HW and BCI) than adult males and juveniles, with no significant correlation between SVL and TaL (SVL and BCI). Adult males significantly exceeded adult females in SVL, TaL, ToL and HL, but had a significantly lower mean BCI. The mean BCI and mean ratio TaL/SVL were significantly lower in larger than in smaller adult males. We conclude that the ratios TaL/SVL, TaL/ToL, HL/SVL and HL/HW are unsuitable indicators for sex determination in adult *M. l. lebetina*. Adult males exceed adult females in body size, while the latter tend to have a higher BCI. In large adult males, body condition and tail growth appear to decline with increasing body size.

Although Cypriot blunt-nosed vipers are frequently found near freshwater during summer, little is known about the importance of water for their spatial ecology. Therefore, the **second objective** was to investigate possible seasonal changes in the proximity to water, and thus habitat preferences, of *M. l. lebetina* (**Chapter 2**). Hence, in spring 2014 and late summer 2015, the shortest distances of wild blunt-nosed vipers to the nearest artificial and natural water bodies were measured, before statistical analysis via MWU test and Wilcoxon matched pairs test. In total, 36 blunt-nosed vipers were recorded with corresponding distances to freshwater in 2014 (n = 19) and in 2015 (n = 17). The distances of vipers to the nearest water body in general, as well as to the nearest artificial and natural water body did not differ significantly between both seasons. Also, their nearest distances to natural and artificial water bodies did not differ significantly during each season. As the vipers were mostly observed in richly structured

habitats but without increased water attraction in late summer, the annual drying out of freshwater bodies presumably does not affect their occurrence. Instead, microhabitat structures providing for shelter, thermoregulation and prey are certainly more important for determining their presence or absence. A better understanding of *M. l. lebetina* habitat requirements is needed for minimizing the snakebite risk and improving the conservation of blunt-nosed viper populations and habitats in Cyprus.

The available data on the venom proteome composition of blunt-nosed vipers is insufficient, which hinders the development of more effective antivenoms against *M. lebetina* envenoming. Accordingly, the **third objective** was to conduct the first in-depth venom proteome analysis for the blunt-nosed viper subspecies *M. l. lebetina* and *M. l. cernovi* (**Chapter 4, published**). For the analysis, the venom proteomes of both subspecies were characterized via RP-HPLC separation of crude proteins, SDS-PAGE of fractionated proteins and LC-MS/MS of peptides obtained from in-gel tryptic digestion. Further, high-resolution shot-gun proteomics was used. Both venoms consisted of at least 18 protein families, with snake venom Zn2+-dependent metalloprotease (SVMP), serine protease (SVSP), disintegrin, phospholipase A2 (PLA2), C-type lectin-like (CTL-like), and L-amino acid oxidase (LAAO) together accounting for >80% of the venom protein contents. Also, many low abundance proteins (<1% of the proteome) were discovered. Although sharing mostly similar proteins, both subspecies differed in their venom proteome composition. The P-III class of SVMP toxins was much more abundant in the venom of *M. l. lebetina,* while the P-I class was more common in *M. l. cernovi* venom, implicating that the latter has a more potent myonecrotic effect, with minor systemic hemorrhage. This study extends our understanding of the venom proteome complexity of *M. lebetina* ssp., particularly regarding toxins related to envenoming pathogenesis and hard-detected protein classes.

The **fourth objective** of this thesis was to investigate first-time systematically collected snakebite epidemiology data from the Republic of Cyprus, which is fundamental for better estimating the medical importance of *M. l. lebetina* (**Chapter 5, published**). Hospital data on snakebite envenoming from 2013 to 2019 were analyzed for a retrospective review, while human-viper conflict information was acquired from interviews with 12 representatives of Cypriot institutions. Between 2013 and 2019, 288 snakebite envenoming cases were admitted to public hospitals, averaging 41 people annually. Snakebite envenoming incidence ranged from 6.84 (2015) to 3.36 per 100,000 population (2017). Between 2000 and 2018, the deaths of one man (73 years), and indirectly, one woman (77 years), were related to snakebite envenoming. Of all cases, 92% happened between April and October, culminating in September (31%). Snakebite incidence peaked in the 60–69 years age group (9.19) and was higher in males (6.85) than in females (2.82). Of all admitted patients, 84% were discharged within four days, while the mean hospital stay duration was 2.65 days. Most patients were admitted to the general hospitals in Paphos (51%), Limassol (30%) and Nicosia (11%). The data hint at mostly non-severe clinical courses. Males and middle- to older-aged people are at greatest risk, as well as outdoor workers. Recommended measures for snakebite mitigation include educational workshops, the protection of valuable snake habitats and the deterrence and translocation of blunt-nosed vipers from inhabited areas.

Zusammenfassung

Schlangen und Menschen stehen in einer komplexen Beziehung zueinander. Zum einen sind Schlangen von ökologischer und wirtschaftlicher Relevanz, da sie als Beutegreifer von Nagetieren zur Lebensmittelsicherheit und öffentlichen Gesundheit beitragen. Zum anderen sind Giftschlangen von medizinischer Bedeutung, da sie schwere Bissvergiftungen verursachen. Zu den größten Vipernarten des Nahen Ostens zählt die Levanteotter (*Macrovipera lebetina*), deren Nominatform *M. lebetina lebetina* als endemisch für Zypern gilt und dort die einzige gefährliche Giftschlangenart ist. Einerseits ist ihr hämotoxisch und zytotoxisch wirkendes Gift bedeutsam für die Forschung, andererseits verursachen Levanteottern schwerwiegende Schlangenbissvergiftungen und werden daher von der lokalen Bevölkerung gefürchtet und oft verfolgt. Die zypriotische Levanteotter ist als bedrohte Art im Anhang II der Berner Konvention aufgeführt.

Da bisher noch keine Daten zum Körpergewicht und zu geschlechtsspezifischen morphometrischen Merkmalen von *M. l. lebetina* veröffentlich worden sind, war das **erste Ziel** dieser Dissertation, erstmalig geschlechtsspezifische morphometrische Daten zu *M. l. lebetina* zu sammeln und zu analysieren (**Kapitel 2 und 3**). Für die Datenaufnahme wurde im Rahmen von vier Feldaufenthalten (2014-2022) auf Zypern bei wilden Levanteottern systematisch die Kopfrumpflänge (KRL), Schwanzlänge (SL), Kopflänge (KL), Kopfbreite (KB) und das Körpergewicht gemessen, und ein Body Condition Index (BCI) berechnet. Alle morphometrischen Merkmale wurden anschließend mittels Regressions- und Korrelationsanalyse untersucht und bei adulten Tieren ein morphometrischer Geschlechtervergleich mittels ungepaartem *t*-Test und Mann-Whitney-U-Test (MWU-Test) durchgeführt. Der analysierte Datensatz vergrößerte sich von 34 vermessenen Levanteottern (**Kapitel 2, publiziert**) auf 46 Individuen (**Kapitel 3, unpubliziert**), die sich auf 16 adulte Männchen, 21 adulte Weibchen und neun Jungtiere verteilten. Die gerundeten Gesamtlängen (GL) der Vipern reichten von 23,5 cm bis 133,5 cm und ihre Körpergewichte von 10 g bis 1.456 g. Bei adulten Weibchen korrelierte die KRL schwächer mit den Parametern Gewicht, SL, KL, KB und BCI, und es gab weder einen signifikanten Zusammenhang zwischen KRL und SL, noch zwischen KRL und BCI. Adulte Männchen übertrafen adulte Weibchen signifikant in KRL, SL, GL und KL, hatten aber einen signifikant niedrigeren BCI (Mittelwerte). Große adulte Männchen hatten einen signifikant niedrigeren mittleren BCI und mittlere relative Schwanzlänge als kleinere. Relative Schwanzlänge, relative Kopflänge und relative Kopfbreite können als ungeeignete Indikatoren für die Geschlechtsbestimmung von adulten zypriotischen Levanteottern betrachtet werden. Adulte Männchen von *M. l. lebetina* sind insgesamt größer als adulte Weibchen, letztere sind jedoch tendenziell etwas massiger. Bei großen adulten Männchen scheint sich der Körperzustand mit zunehmender Größe zu verschlechtern, während die relative Schwanzlänge abnimmt.

Obgleich zypriotische Levanteottern während der Sommerzeit häufig an Wasserstellen beobachtet werden, ist wenig über die Bedeutung von Wasser für ihre räumliche Ökologie bekannt.

Somit war das **zweite Ziel** meiner Studie die Untersuchung von möglichen saisonalen Unterschieden in der Wassernähe von *M. l. lebetina* (**Kapitel 2**). Dafür wurden im Frühling 2014 und Spätsommer 2015 die kürzesten Abstände wilder Levanteottern zur nächsten künstlichen und natürlichen Wasserstelle gemessen, bevor im nächsten Schritt die Distanzen mittels MWU-Test und Wilcoxon-Test miteinander verglichen wurden. Insgesamt wurden die Distanzen von 36 Levanteottern zur nächsten Wasserstelle erfasst, davon 19 im Frühling 2014 und 17 im Spätsommer 2015. Die Distanzen der Schlangen zur nächsten Wasserstelle allgemein, sowie zum nächsten künstlichen und natürlichen Wasservorkommen unterschieden sich nicht signifikant zwischen beiden Jahreszeiten. Auch gab es weder im Frühling 2014 noch im Spätsommer 2015 signifikante Unterschiede zwischen den Distanzen der Tiere zu den nächsten künstlichen und natürlichen Wasserstellen. Die Beobachtung von *M. l. lebetina* in meist reich strukturierten Lebensräumen bei gleichzeitig nicht nachweisbarer Angebundenheit der Tiere an Wasservorkommen deutet darauf hin, dass das jährliche periodische Austrocknen von Wasserstellen das Vorkommen der Art wahrscheinlich nicht beeinflusst. Stattdessen sind Mikrohabitatstrukturen, die Schutz- und Thermoregulationsbedürfnisse der Vipern erfüllen und Beutetiere anlocken, wohl wichtiger, um die An- oder Abwesenheit der Art zu bestimmen. Ein besseres Verständnis der Habitatansprüche von Levanteottern ist grundlegend für die Minimierung des Risikos von Bissunfällen durch die Levanteotter, und für einen besseren Schutz der Art und ihrer Lebensräume auf Zypern.

Die Datenlage zur Venomproteomkomposition von Levanteottern ist unzureichend, was ein Hindernis bei der Entwicklung von effektiveren Antiveninen gegen Bissvergiftungen durch diese Art darstellt. Daher war das **dritte Ziel** meiner Dissertation die Durchführung der ersten tiefergehenden Venomproteom-Untersuchung der Levanteotter-Unterarten *M. l. lebetina* und *M. l. cernovi* (**Kapitel 4, publiziert**). Für die Analyse wurden die Venomproteome beider Unterarten durch RP-HPLC-Trennung der Rohgiftproteine sowie mittels SDS-PAGE (Polyacrylamid-Gelelektrophorese) der fraktionierten Proteine charakterisiert, des Weiteren durch LC-MS/MS von durch tryptischen In-Gel-Verdau von Proteinbändern gewonnenen Peptiden. Auch kam hochauflösende Shotgun-Proteomik zum Einsatz.. Beide Gifte bestanden aus mindestens 18 Proteinfamilien. Schlangengift-Metalloproteinase (SVMP), Serinprotease (SVSP), Disintegrine, Phospholipase A2 (PLA2), C-Typ Lectin-artige (CTL-like), and L-Aminosäureoxidase (LAAO) machten zusammen mehr als 80% des Venomproteingehalts aus. Zudem wurden in den Giften auch zahlreiche in geringen Anteilen vorhandene Proteine (<1% des Proteoms) nachgewiesen. Obgleich beide Unterarten überwiegend gleiche Proteinklassen aufwiesen, unterschieden sie sich teilweise in ihrer Venomproteomkomposition. So war die P-III-Klasse der SVMP-Toxine im Gift von *M. l. lebetina* wesentlich stärker ausgeprägt als die P-I-Klasse, während letztere etwas stärker im Gift von *M. l. cernovi* vertreten war. Dies deutet auf darauf hin, dass Bissvergiftungen durch *M. l. cernovi* einen stärkeren myonekrotischen Effekt haben und schwächer hämorrhagisch wirken. Diese Studie erweitert unser Verständnis der Venomproteomkomplexität bei Levanteotterunterarten, vor allem hinsichtlich Toxinfamilien, die bei der Pathogenese von Schlangenbissvergiftungen eine Rolle spielen, und im Hinblick auf schwer zu erfassende Proteinklassen.

Das **vierte Ziel** dieser Arbeit war es, erstmalig systematisch erfasste Daten zur Schlangenbissepidemiologie auf Zypern zu untersuchen, was von großer Bedeutung zur besseren Einschätzung der medizinischen Bedeutung der zypriotischen Levanteotter ist (**Kapitel 5, publiziert**). Für die Studie wurden die Krankenhausdaten zu Schlangenbissvergiftungen in der Republik Zypern von 2013 bis 2019 retrospektiv untersucht und ausgewertet. Zudem wurden auf Grundlage von Interviews mit 12 Vertretern zypriotischer Institutionen gewonnene Informationen zum Konflikt zwischen Menschen und Levanteottern auf Zypern gesammelt. Zwischen 2013 und 2019 wurden insgesamt 288 Schlangenbissunfälle in öffentlichen Krankenhäusern der Republik Zypern erfasst, mit einem Durchschnitt von 41 Schlangenbissopfern jährlich. Die Inzidenz der Schlangenbissvergiftungen bewegte sich zwischen 6,84 (2015) und 3,36 pro 100.000 Einwohner (2017). Zwischen 2000 und 2018 wurden im Zusammenhang mit Schlangenbissvergiftungen der Tod eines Mannes (73 Jahre) und indirekt der Tod einer Frau (77 Jahre) registriert. 92% der Unfälle traten zwischen April und Oktober auf, die meisten davon im September (31%). Die Inzidenz der Schlangenbissvergiftungen war am höchsten in der Altersgruppe der 60-69-jährigen (9,19), und war höher bei Männern (6,85) als bei Frauen (2,82). Von allen eingewiesenen Schlangenbisspatienten wurden 84% innerhalb von vier Tagen entlassen, während die Krankenhausaufenthalte im Mittel 2,65 Tage dauerten. Die meisten Patienten wurden in die Allgemeinkrankenhäuser in Paphos (51%), Limassol (30%) und Nicosia (11%) eingewiesen. Die Daten deuten auf größtenteils recht milde klinische Verläufe hin. Männer sowie Menschen mittleren bis höheren Alters sind am stärksten durch Schlangenbissvergiftungen gefährdet, ebenso wie Arbeiter im Freien. Empfohlene Maßnahmen zur Verringerung der Schlangenbissproblematik umfassen Bildungsworkshops, den Schutz wertvoller Schlangenlebensräume und die Vergrämung und Umsiedlung von Levanteottern aus bewohnten Gebieten.

Combat dancing of male Cypriot blunt-nosed vipers (D. Jestrzemski 2015)

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Chapter 1

Introduction

1.1 Snake venoms, venomous snakes and snakebite

1.1.1 Venom

Venom is defined as a secretion produced in the specialized gland of an animal and delivered to a target organism by the infliction of a wound (e.g., via a bite or sting). Venom starts to act when entering the other organism, with molecules from the secretion disturbing normal physiological processes in the victim to paralyze, kill and often predigest the prey, or to deter, injure and even kill a potential predator (Mebs 2002, Fry et al. 2009). Animal venoms are usually very complex mixtures of bioactive compounds, comprising of proteins and peptides acting as toxins, as well as of salts and organic components including acids and neurotransmitters (Casewell et al. 2013). Among the most important toxin applications are the active use of venom (via injection) as well as the passive application of poison to become inedible or otherwise noxious for natural enemies (Mebs 2002, Murphy 2010). Venoms and their delivery and application techniques have evolved convergently across the animal kingdom, with natural selection for diet as a key driver of venom composition evolution (Mebs 2002, Casewell et al. 2013). Thus, many taxa of specialized predators (e.g., venomous snakes) have developed toxins with a prey-specific lethality, and toxins have been evolving to the biological functions that they fulfil today (Murphy 2010).

1.1.2 Venomous snakes

Fry et al. (2015) date the origin of the reptile venom system at around 170 million years ago, with enlarged glands (containing newly derived regions for protein and mucus production) already being present in the last common ancestor of the toxicoferans, a single clade of scaled reptiles (squamates) with toxin-secreting oral glands, which include Serpentes (snakes), Anguimorpha (gila monsters, alligator lizards and monitor lizards) and Iguania (iguanas, agamas, and chameleons). Venom further proved to be a crucial innovation in the roughly 100-million-year-old advanced snake clade Caenophidia. Today, venomous snakes possessing venom glands and fangs are only found among the modern snakes (superfamily Colubroidea), namely in the families Colubridae, Elapidae, Viperidae and the subfamily Atractaspidinae (e.g., Fry et al. 2009). While the relatively small number of mildly and dangerously venomous colubrid taxa are entirely rear-fanged (opistoglyphous), front-fanged clades include the

Viperidae (solenoglyphous), Elapidae (proteroglyphous) and several species of Atractaspidinae, a cryptic clade with significant variation in fang morphology (Fry et al. 2009, Kerkkamp et al. 2015, Jackson et al. 2017). While Colubridae and Elapidae have fixed upper jaws (maxillae) with attached front fangs, the maxillae of Viperidae and Atractaspidinae are moveable and with mounted front fangs, which permits the fangs to be erected upon biting, and in a parallel position when resting (Kerkkamp et al. 2015).

1.1.3 Snakebite

Snakes (Squamata, Serpentes) are among the medically most important vertebrate animals. At the global level, snakebite envenoming is still a major challenge for tropical medicine in the 21st century. In June 2017, snakebite envenoming was ranked as a Category A Neglected Tropical Disease by the WHO (Chippaux 2017). Some studies estimate that over five million snakebite accidents happen each year (Chippaux 1998) with 150,000 deaths (White 2000) and more than 400,000 permanently disabled people suffering from amputation or internal organ damage (Gutiérrez et al. 2017). However, recent epidemiological studies have demonstrated that these numbers greatly underestimate the global burden of disease due to snakebite (see Longbottom et al. 2018). For example, in Bangladesh alone about 700,000 people are bitten every year (Rahman et al. 2010) and in India there are 46,000 deaths per year from snakebite (Mohapatra et al. 2011). Bites caused by members of the snake family Elapidae commonly cause neuromuscular paralysis leading to respiratory failure, whereas snakes in the family Viperidae are responsible for more complex envenomations including local swelling, severe tissue damage and continuous bleeding from body openings (Murphy et al. 2010, Ghezellou et al. 2018). In addition to physical impairments, snakebite survivors often suffer from severe psychological trauma caused by the incident itself, its economic, and its health consequences, which forces them to retire from work and causes the loss of the main source of income for many families (Rahman et al. 2010).

1.1.4 Snake venoms in medicine and research

For millennia, snake venoms have been used for medical purposes. Since the end of the 19th century, snake venom serves for antivenom production to treat snakebite envenoming. Today, several diseases are treated with drugs discovered in snake venoms (Murphy 2010). From a global perspective, snake venoms are still a highly underexplored resource in pharmacological research, and still much needs to be understood about the molecular mechanisms underlying their diversity. New high-throughput screening systems offer greatly increased speed and efficiency in identifying and extracting therapeutically useful molecules. However, the global biodiversity crisis is threatening the populations of several snake species, and thus the resources on which snake venom research and venom-derived medications depend. A joint approach by biomedical researchers, pharmacologists, clinicians, herpetologists and conservation biologists is now more required than before, if the full potential of snake venom-derived medications is to be realized (Vonk et al. 2011). In this regard, the emergence of ‘omic’ technologies in the field of toxinology (genomics, transcriptomics and proteomics) offers the possibility to expand our understanding of the clinical correlation of the global toxin composition of venoms. The strength of these technologies is their complementarity in venom analysis, enabling to explore the molecular diversity of venoms in depth. Due to the wide dynamic range of proteins in complex proteomes, it is not possible to visualize every component of a proteome using a single proteomic technique. Therefore, multifaceted approaches are needed to maximize proteome coverage (Calvete 2011).

1.1.5 Snake antivenom development

Systemic envenoming is effectively treated by intravenous antivenom administration (Bazaa et al. 2005), which aims at neutralizing toxic effects and undoing pathological symptoms (Ghezellou et al. 2018). Antivenom is monovalent when it contains antibodies against only one snake species, and polyvalent when its antibodies are against multiple snake species (see Murphy 2010). For a better rational design, therapeutic effectiveness and production of antivenoms, an improved understanding of the snake specimens used for venom harvesting and the composition of their venom samples is necessary (Ghezellou et al. 2018).

1.2 Vipers

1.2.1 The family Viperidae

Currently comprising of 387 species (Uetz et al. 2023), the members of the Viperidae family are found on all continents except Australia and Antarctica, from the tropics to the Arctic Circle. Their reproduction techniques include oviparity (egg-laying), ovoviviparity (eggs developing inside the maternal body until hatching) and viviparity (live-bearing). Vipers have triangular heads and their habitus is often stout (Rage 1994, Mebs 2002), stockier than that of elapids and colubrids. As vipers are usually ambush predators, heavy posterior bodies help them to anchor themselves as they strike. The bite-and-release envenoming strategy of vipers greatly reduces the risk of injury by struggling and potentially dangerous prey such as rats (Murphy 2010). The venom injection also enables pre-digestion of the prey by powerful enzymes that break down and destroy blood and body tissue (Baier et al. 2013). All vipers except the African genus *Causus* (night adders) have vertical pupils, and some species possess supraocular horns. Most vipers feed on small vertebrates (i.e., rodents, birds and lizards). Vipers are divided into the three subfamilies Azemiopinae (Fea's vipers), Viperinae (true vipers) and Crotalinae (pit vipers) (Ineich et al. 2006, Phelps 2010). True vipers are only found in the Old World: in Europe, in Africa (except Madagascar) and in Asia, especially in the western part of the Asian continent. Among the medically most important true vipers of Southeastern Europe and Western Asia are the horned viper (*Vipera ammodytes*), the Ottoman viper (*Montivipera xanthina*), the blunt-nosed viper (*Macrovipera lebetina*), the Palestine viper (*Daboia palaestinae*) and the carpet viper (*Echis carinatus*) (Rage 1994, Phelps 2010). Pit vipers are the only Viperidae found in the Americas, where they constitute the medically important genera *Crotalus* (rattlesnakes), *Bothrops* (lanceheads) and *Lachesis* (bushmasters). They can be distinguished by their heat-sensing pit organs (between eyes and nostrils), which allow them to accurately strike even in absolute darkness (Murphy 2010). The taxonomic status of several viper genera (e.g., *Macrovipera*) is in constant change, and sometimes subject of contradicting theories. Consequently, statements about the number of described viper species differ between herpetologists (Mebs 2002, Murphy 2010, Phelps 2010).

1.2.2 The viper venom apparatus

Of all venomous snakes, the venom apparatus of solenoglyph snakes (i.e., vipers) is considered the most advanced, when compared to the evolutionary older proteroglyph snakes (i.e., elapids and sea snakes) and opistoglyph snakes (colubrids) (Mebs 2002, Fry et al. 2009). The viper venom apparatus consists of the main gland, the primary duct, an accessory gland segmented into two parts and a secondary duct. The main venom gland opens into a large lumen, which acts as a venom reservoir (**Figure 1.1**). Venom production stops when the volume is full and continues when venom is discharged. Viper fangs are curved and attached to small mobile maxillary bones containing no other teeth. Both fangs can be erected and independently of each other folded in sheaths against the mouth roof. The longest viper (and snake) fangs are found in *Bitis gabonica* with up to 50 mm in length (Murphy 2010). Replacement fangs are constantly produced in the tissue near the main fangs, which break off from time to time (Mebs 2002).

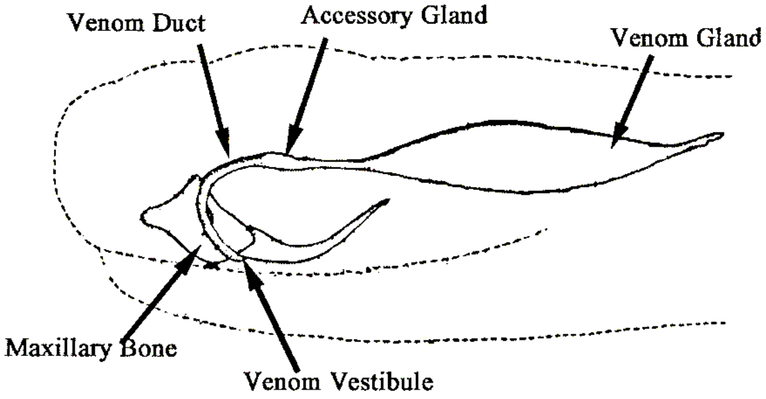

Figure 1.1. The venom apparatus of a viper. Source: Murphy (2010).

1.2.3 Viper venom: effects on the human body

Viper venoms contain toxins and enzymes that cause cell and tissue damage, for example by tissue liquification, which can rapidly spread from the site and continue for days, requiring the amputation of a bitten limb due to severe necrosis. Other effects are swelling, oedema, continuous bleeding from body openings and soft tissues (haemorrhage due to consumption coagulopathy), as well as blood clotting in other body parts. Some species may also induce neurotoxic symptoms. Up to 2-3 liters of fluid can be extravasated, resulting in haemoconcentration followed by hypovolaemic cardiovascular shock. Severe envenoming can lead to haemostatic disturbances, which may even cause consumption coagulopathy (Mebs 2002, Murphy 2010, Phelps 2010).

1.2.4 Viper conservation

Snakes experience a global trend of declining populations (Akani et al. 2013, Reading et al. 2013). Furthermore, widely held negative perceptions of snakes, coupled with insufficient knowledge of their elemental biology, are major challenges to their conservation (Burkhardt et al. 2009, Murphy 2010, Maritz et al. 2016). Possessing highly developed venom delivery mechanisms, potent and complex venoms, a cryptic camouflage coloration and an often strongly defensive behavior, while occupying almost all terrestrial ecosystems, vipers play a critical role in the global public health problem of snakebite envenoming (Chippaux 1998, Warrell 2010, Gutiérrez 2012, Gutiérrez et al. 2017). Consequently, they are among the most maligned snake families (Maritz et al. 2016), with viper persecution in some parts of the world reaching the level of mass eradication campaigns such as the traditional “rattlesnake roundups” in the southern USA (Pisani & Fitch 1993, Burghardt et al. 2009). However, the most important threats to vipers are the loss, transformation, degradation and fragmentation of their habitats (Nilson et al. 1999, Maritz et al. 2016, Console et al. 2020). Viper populations are further decimated by accidental killing due to road traffic, illegal collection and trade (with unknown impacts on the populations), exploitation for food (e.g., in Asia and Africa), collection for venom harvesting, introduced species (e.g., mongooses, pigs and feral cats), and climatic changes (Maritz et al. 2016). The slow growth, late maturation and infrequent, highly seasonal reproduction of vipers (Ineich et al. 2006), as well as their specialization in diet and/or habitats makes them especially vulnerable to habitat loss. As a result of habitat fragmentation, isolated populations with low genetic diversity may face an additionally increased extinction risk (Maritz et al. 2016, Console et al. 2020). Of 429 snake species listed as threatened on the IUCN Red List, 73 species (17%) are vipers (IUCN Viper Specialist Group 2021), although vipers make up only 9.5% of all currently known snake species (Uetz et al. 2023).

1.3 The blunt-nosed viper (*Macrovipera lebetina*)

1.3.1 The genus *Macrovipera* (blunt-nosed vipers)

The monophyletic genus *Macrovipera* (blunt-nosed vipers) was erected by Reuss in 1927 and, after taxonomic controversy, reestablished by Herrmann et al. (1992). Of eight currently described blunt-nosed viper subspecies (Ščerbak & Böhme 2005, Stümpel 2012), four have been verified in a comprehensive molecular investigation by Stümpel (2012): *M. l. lebetina* (Linnaeus, 1758), *M. l. obtusa* (Dwigubsky, 1832), *M. l. turanica* (Chernov, 1940) and *M. l. cernovi* (Chikin & Ščerbak 1992). In contrast, the subspecies *M. l. euphratica* (Martin, 1838) has been synonymized with *M. l. obtusa*, and *M.* (*lebetina*) *schweizeri* (Werner 1935) with *M. l. lebetina*, while the existence of the taxa *M. l. transmediterranea* (Nilson & Andrén, 1988) and *M. l. peilei* (Murray, 1892) could not be confirmed due to an insufficient number of samples (Stümpel 2012). In 2018, *Macrovipera razii* was described as a new blunt-nosed viper species (Oraie et al. 2018). All subspecies of *M. lebetina* are distributed from Northern Africa to Western and Central Asia (Irano-Turanian region), where they inhabit semi-arid habitats (**Table 1).** Their distribution spans over 3,500 km from east to west on the Eurasian mainland (Ščerbak & Böhme 2005, Phelps 2010). The nominate subspecies *M. l. lebetina* is considered endemic for Cyprus, although some authors (e.g., Böhme 1987, Entzeroth 1989) state that it also inhabits the southern Anatolian coast. The border between the *M. l. lebetina* and *obtusa* haplotypes is formed by Iskenderun (Stümpel 2012).

Table 1. *Macrovipera lebetina* ssp. and *Macrovipera razii*.

Species	Subspecies	Taxon author	Common name	Distribution
Macrovipera lebetina	*M. l. transmediterranea*	Nilson & Andrén, 1988	North African blunt-nosed viper	Algeria, Tunisia
	M. l. schweizeri	Werner, 1935	Cycladic blunt-nosed viper	Greece (Milos, Kímolos, Políegos, Sífnos)
	M. l. lebetina	Linnaeus, 1758	Cypriot blunt-nosed viper	Cyprus, possibly also southern Anatolia
	M. l. obtusa	Dwigubsky, 1832	Levant blunt-nosed viper	Turkey, Lebanon, Syria, Jordan, Iraq, Iran, Caucasus, Afghanistan, Pakistan, Kashmir
	M. l. euphratica	Martin, 1838	"Blunt-nosed viper"	Eastern Anatolia, Iraq
	M. l. cernovi	Chikin & Ščerbak 1992	Chernov blunt-nosed viper	Iran, Turkmenistan, Afghanistan and Pakistan
	M. l. turanica	Chernov, 1940	Turan blunt-nosed viper	Turkmenistan, Uzbekistan, Kazakhstan, Tajikistan, Afghanistan, Pakistan
	M. l. peilei	Murray, 1892	"Blunt-nosed viper"	Afghanistan, Pakistan
Macrovipera razii	-	Oraie, Rastegar-Pouyani, Khosrovani, Moradi, Akbari, Sehhatisabet, Shafiei, Stümpel & Joger, 2018	Razi's viper	Iran (Kerman, central Zagros mountains)

1.3.2 Morphology, ecology and IUCN conservation status of *Macrovipera lebetina*

Reaching a length of up to 180 cm on the mainland (Ščerbak & Böhme 2005), and occasionally even 200 cm or more (Mermer et al. 2012), blunt-nosed vipers belong to the largest true viper species of the world. Hence, the scientific name "*Macrovipera*" correctly translates to "large viper". In contrast, insular blunt-nosed vipers reach maximum lengths of 150 cm (*M. l. lebetina*) in Cyprus (Baier et al. 2013), while Cycladic blunt-nosed vipers (*M. l. schweizeri*) reach 98.5 cm on the island of Milos and 107 cm on Sifnos (Nilson 2005). Blunt-nosed vipers inhabit river valleys and slopes with rocky outcrops and shrubs as well as canyons, gorges, gullies, pine forests, orchards, vineyards, caves and ruins. These heavy-bodied vipers are ambush predators of small mammals and birds, and often found in agricultural areas (Baier et al. 2013, Ščerbak & Böhme 2005). After hibernation, mating takes place in spring (April-May) and oviposition in summer (June to August), with a clutch size of up to 35 eggs. New-borns hatch in August and September, with a total length of 18 to 20 cm. Blunt-nosed vipers are diurnal during the cooler time of the year and nocturnal during the hot summer season. Depending on region and climate, hibernation takes place from October/November to March/April. The species may reach an age of 17 years in captivity (Ščerbak & Böhme 2005, Baier et al. 2013). Blunt-nosed vipers show a strongly defensive reaction when cornered (**Figure 1.2**).

Figure 1.2. An adult female Cypriot blunt-nosed viper (*M. l. lebetina*) coiled into a S-shaped defensive posture and hissing constantly, ready to strike and deliver a venomous bite (Polis Chrysochous, 28 April 2014; photo: D. Jestrzemski).

The recent IUCN assessment of *M. lebetina* lists the species as “Least Concern” due to its wide distribution, presumably large population, and because its decline is unlikely fast enough for a classification in a more threatened category. However, *M. lebetina* is probably declining in much of its distribution range, and in some areas may be soon listed as “Near threatened”, or in a threatened category. Thus, its current population trend is regarded as “Decreasing”. In fact, blunt-nosed vipers are considered rare and threatened in several parts of their range. In Central Asia, populations are likely shrinking due to habitat loss and degradation, but also collection from the wild and human persecution pose threats. Thus, *M. lebetina* is included in the Red Data Books of Russia, Turkmenistan, and Tajikistan (Aghasyan et al. 2021).

1.3.3 Medical importance of the blunt-nosed viper

As a large viper species possessing huge amounts of potent venom, *M. lebetina* has been a cause of serious snakebite accidents from the Eastern Mediterranean to Kashmir (e.g., Corkill 1932, Swaroop & Grab 1954, Sharma et al. 2008, Kazemi et al. 2019), with severe snakebite cases also reported from Cypriot specimens (Heidenstam 1881, Fraser 1929, Hopkins 1974, Göçmen 2006). A particularly grave bite accident occurring in southern Turkey and resulting in the amputation of his right arm is described by Schweiger (1983). Thus, as a potentially very dangerous snake species, *M. lebetina* is feared and often persecuted by people (Mermer et al. 2012). Blunt-nosed viper venom consists mainly of hemotoxins (breaking down the blood and body tissue) and cytotoxins (causing necrosis). As the venom is slowly reacting, usually enough time remains for seeking medical treatment in the case of a bite (Baier et al. 2013).

Blunt-nosed viper venoms are of high value for drug research and development (Bazaa et al. 2005, Park et al. 2012), and numerous venomic studies have been conducted on *M. lebetina* ssp., particularly on the widely distributed subspecies *M. l. obtusa*. Of these, many focus on biological venom properties of relevance for health research, drug development and antivenom research (Kurtovic et al. 2014), while some also investigate the function of *M. l. obtusa* venom as environmental pollution indicator (see Abiyev & Topchiyeva 2013). Several articles are on cancer research, especially for *M. l. turanica*, whose toxins show potential of triggering apoptosis and inhibiting cancer growth (e.g., Lee et al. 2015). In contrast, most venomic studies on *M. l. transmediterranea* are not directly related to cancer treatment, although the venom properties examined are potentially beneficial for health-related drug development. No detailed investigations on the venom proteome of *M. l. lebetina* have yet been published, although different characteristics of its venom were analyzed (e.g., Nalbantsoy et al. 2012).

1.3.4 Choice of species and study region

At the core of a viper research project is the selection of a suitable species. The Cypriot blunt-nosed viper was chosen for different reasons. First, *M. l. lebetina* is a large viper species and of high medical importance as a cause of severe snakebite envenoming in Cyprus, while being of economic significance as a key predator of rodents in agricultural areas. Second, there is a lack of knowledge on the conservation biology and snakebite epidemiology of this species in general, and for Cyprus in particular, which hinders a better understanding of its conservation status and its interactions with humans (Ščerbak & Böhme 2005, Baier et al. 2013). Third, relatively little is known about the venomic diversity of *M. lebetina* ssp., since no in-depth studies on the species' venom proteome have yet been published for the subspecies *M. l. schweizeri* and *M. l. turanica*, and before this research project, also not for *M. l. lebetina*.

The island of Cyprus is ideal for studying the blunt-nosed viper, as it harbors the entire currently known population of the nominate subspecies *M. l. lebetina* (sensu stricto). In addition, Cyprus is the only European Union member country with a population of *M. lebetina*, which underlines its national responsibility for preserving the local subspecies and the corresponding genetic diversity within the species complex *M. lebetina*. The ongoing human persecution of *M. l. lebetina* also highlights its role as a snake species of conservation concern both in Cyprus and the EU. Further, *M. l. lebetina* is the largest viper species in Europe and consequently a potential flagship species for reptile conservation. Due to the absence of other front-fanged venomous snake taxa in Cyprus, *M. l. lebetina* is the island's only medically important snake species as well as its only viper species (Baier et al. 2013), which highlights the status of *M. l. lebetina* as a potential source of life-threatening snakebite envenoming in Cyprus, and the relevance of conducting research on its snakebite epidemiology, habitat selection and interactions with humans. It also facilitates the analysis of snakebite envenoming data for Cyprus, since no other Cypriot snake species causes similar envenoming symptoms.

1.4 Cyprus

1.4.1 Cyprus: Geography and climate

Situated between 34°33' and 35°34' N, and 32°16' and 34.37' E, Cyprus is the third largest Mediterranean island, with a maximum length of 224 km, a maximum width of 92 km, an area of 9,251 km² (1,733 km² of forest cover), and a total coastal length of 780 km (Osenegg 1989, Delipetrou et al. 2008). The island is closest to Turkey in the north (75 km), with Syria 150 km in the east and Egypt 380 km in the south (Delipetrou et al. 2008). Geologically, Cyprus is divided into three tectonic units: Troodos Mountains with their ophiolitic tip and basic plutonic rocks, Keryneia Range formed by mostly allochthonous sedimentary formations, and Mamonia Complex in the island's hilly south-west (Osenegg 1989, Delipetrou et al. 2008, Baier et al. 2013). Cyprus is the hottest and driest Mediterranean island, with the highest number of sunshine hours. Its climate is Mediterranean (subtropical), and characterized by dry, hot summers and mild, humid winters, with Köppen climate classifications Csa and BSh (Delipetrou et al. 2008, Baier et al. 2013). The annual precipitation ranges from 300 mm in the dry Mesaoria plain up to 1,100 mm at Troodos peak (Delipetrou et al. 2008). Most rainfall occurs from November to March. The average temperatures range from 3°C on the upper

Troodos and 10°C on the Mesaoria Plain in winter to 22°C on the upper Troodos and 29°C on the Mesaoria Plain in summer (Baier et al. 2013).

1.4.2 Cyprus: Flora and herpetofauna

Although Cyprus was densely forested in the early Holocene (Delipetrou et al. 2008), the forest cover shrank to 18% by the end of the 20th century. Ship construction and copper smelting (in ancient times), land clearing for agriculture, unrestricted goat grazing and forest fires resulted in the conversion of the native Mediterranean forest into agricultural and pastoral landscapes (Osenegg 1989, Delipetrou et al. 2008). By the late 19th century, forests and soils were highly degraded. The subsequent afforestation altered natural landscapes by the introduction of alien tree species (e.g., *Acacia* spp., *Eucalyptus* spp.) (Delipetrou et al. 2008). Today, the natural vegetation of Cyprus is dominated by coniferous forests as the climax vegetation (19% of landcover, mainly *Pinus brutia* and *P. nigra*), and garrigue ("Trachiotis", bushes and shrubs) (Baier et al. 2013). The Cypriot herpetofauna is composed of three amphibian (anurans) and 22 confirmed reptile species, including three venomous snake species: the cat snake (*Telescopus fallax*), the eastern Montpellier snake (*Malpolon insignitus*), which are rear-fanged venomous colubrids (Kuch & Mebs 2002), and the blunt-nosed viper (*M. lebetina*), a medically important, front-fanged viperid (Baier et al. 2013). With its zoogeography of reptile and amphibian species, Cyprus is considered part of Southwestern Asia and is situated at the edge of the East Mediterranean to Irano-Turanian distribution center (Baier et al. 2013).

1.4.3 The study area: Northern Paphos district

In Cyprus, Paphos district holds a high density of *M. lebetina* (Baier et al. 2013), while being delimited by the Mediterranean Sea to the north and west, and by the forested foothills of the Troodos Mountains to the east (Delipetrou et al. 2008) (**Figure 1.3**). The coastal lowland area of northern Paphos district (ca. 35°02' N, 32°26' E) is characterized by agriculture in the Polis Chrysochous valley, with Akamas Peninsula in the west and the foothills of Paphos Forest (the island's largest unfragmented *P. brutia* stand) in the east. While large parts of Paphos Forest are protected as Permanent Forest Reserves, Akamas Peninsula contains three Permanent Forest Reserves and the National Marine Reserve of Lara-Toxeftra (Delipetrou et al. 2008). Known as one of the island's few remaining natural coastal areas, Akamas Peninsula also is a Natura 2000 site, together with the Polis-Gialia beach stretching along Chrysochous Bay. A major threat to Akamas Peninsula are wildfires, of which around 80 were set on purpose only from 2017-2020 (Agapiou 2019). Daily mean temperatures in Paphos district range from 12.1 °C in January to

27.6 °C in July and August and may exceed 40°C during the hot and dry summer. The mean annual precipitation is 394.2 mm, of which 380.9 mm falls between October and April (rainy season), and 13.3 mm during the dry season from May to September (Climatological Data Polis, 1991-2005).

Figure 1.3. Google Earth map of Paphos district (study area). Yellow lines: main traffic routes (A6 from Limassol city to Paphos city, B7 from Paphos city to Polis Chrysochous, E704 from Polis Chrysochous to Pomos). Bright lines: borders between Paphos district, Limassol district and Nicosia district. Red lines: demarcation lines between the Republic of Cyprus, the United Nations Buffer Zone and the Turkish Republic of Northern Cyprus.

1.5 Problem statement and research objectives

1.5.1 Morphometric characteristics of *M. l. lebetina*

Scientific literature on snakes frequently lacks information on their body mass, even though it is considered as an ideal body size measure in life history studies and often a more useful proxy than body length (Feldman & Meiri 2013). Although blunt-nosed vipers are among the largest and heaviest viperids in the Middle East and exceed all other European viper species in length and mass, no body weight data have been published yet for the Cypriot blunt-nosed viper. Furthermore, published data on sex-specific morphological differences in *M. lebetina* ssp. are scarce and sometimes contradictory (e.g., Baier et al. 2013). Therefore, in the second chapter of this thesis (published as Jestrzemski & Kuzyakova 2018), important morphometric characteristics such as snout-vent length (SVL), tail length (TaL) and body condition index (BCI) will be compared between adult males and females of *M. l. lebetina*, and between adult females before and after the mating and oviposition season. For this purpose, the following hypotheses will be tested:

(1) Adult males are larger in mean snout-vent length than adult females of *M. l. lebetina*.

(2) The mean ratio of tail length to snout-vent length (TaL/SVL) is smaller in adult males than in adult females of *M. l. lebetina*.

(3) In late summer (after the mating season), adult females of *M. l. lebetina* are thinner than in spring (during the mating season).

As an update to the data presented in Chapter 2, an extended morphological dataset will be examined in Chapter 3 for testing the first two hypotheses again. Furthermore, since blunt-nosed vipers, and snakes in general, accumulate parasites during their lifespan (see Nasiri et al. 2014), we expect that their body condition will deteriorate with increasing age. Besides body size and body condition, relative tail length is another biologically important trait influencing male mating success. Assuming that smaller adult males of *M. lebetina* face higher sexual selective pressure than larger adults, we predict that they experience faster tail growth than older males, to make up for this disadvantage as quick as possible. Therefore, the following hypotheses will be tested in Chapter 3 as well:

(4) The mean BCI of large adult males (SVL >= 100 cm) is lower compared to smaller adult males (SVL < 100 cm).

(5) The ratio of tail length to snout-vent length (TaL/SVL) of large adult males (SVL >= 100 cm) is lower compared to smaller adult males (SVL < 100 cm).

Thus, the first objective of this thesis (Chapters 2 and 3) is the first-time publication of morphometric data of juvenile and adult blunt-nosed vipers from Cyprus, together with first-time morphometric comparisons between adult males and adult females of *M. l. lebetina*, between adult females during different seasons and between adult males of different size classes. The sex-specific comparison shall also provide hints for possible morphometric traits that could be used to successfully distinguish between sexes in adult blunt-nosed vipers.

1.5.2 Does the proximity to water of the Cypriot blunt-nosed viper change seasonally?

Although *M. l. lebetina* is frequently observed near freshwater in Cyprus during the hot summer period (Baier et al. 2013), and severe bite accidents involving blunt-nosed vipers have occurred at water bodies (see Fraser 1929), there are yet no published studies investigating the importance of water for the spatial ecology of *M. l. lebetina*. Therefore, the second chapter (published as Jestrzemski & Kuzyakova 2018) will also explore the possibility of seasonal changes in the proximity to water of *M. l. lebetina*, via an analysis of the snakes' distances to the nearest artificial and natural freshwater body. For this purpose, the following research questions will be investigated:

(1) Is *M. l. lebetina* found closer to water sources in late summer than in spring?
(2) Is *M. l. lebetina* found closer to natural water sources in spring than in late summer?
(3) Is *M. l. lebetina* found closer to artificial water sources in late summer than in spring?
(4) Is *M. l. lebetina* found closer to natural than to artificial water sources in spring?
(5) Is *M. l. lebetina* found closer to artificial than to natural water sources in late summer?

In this regard, the second objective (Chapter 2) is to examine, for the first time, the belief of local people that Cypriot blunt-nosed vipers are found particularly close to water during the hot summer season, which shall contribute to a better understanding of the habitat preferences of blunt-nosed vipers in Cyprus.

1.5.3 Comparative venom proteomics of Iranian *M l. cernovi,* and *Cypriot M. l. lebetina*

Enormous qualitative and quantitative variation is found in the composition of snake venom proteomes: within and between snake species, but also between sexes, ontogenetic development stages and individuals with different diets and habitats. Even within protein families, toxins depart in their biological targeting (Vonk et al. 2011). Thus, comparing the venom proteomes between snake species and individuals enables us to detect similarities and differences between their venoms, which indicates the phylogenetic relationship between species or environmental influences on their venom proteomes. In this way, the comparison of venom proteomes also has evolutionary and environmental implications (Serrano et al. 2005). Furthermore, obtaining a better understanding of the intraspecific venomic variation of snake species is critical for evaluating the efficacy of currently applied antivenoms and for developing more effective immunization mixtures for future antivenoms (Gutiérrez et al. 2011). The presently known proteomic studies on the venoms of *M. l. transmediterranea* (Bazaa et al. 2005) and *M. l. obtusa* (Sanz et al. 2008, İğci & Demiralp 2012, Pla et al. 2020) have greatly contributed to our current knowledge of the proteomic diversity and relative abundance of toxin protein families in blunt-nosed viper venoms. Yet, the knowledge on the venomic variation in the species complex *M. lebetina* is insufficient, with missing in-depth venomic studies for other subspecies (e.g., *M. l. schweizeri* and *M. l. turanica*). Therefore, the third objective (Chapter 4) is to present, for the first time, a comprehensive venomic investigation for *M. l. lebetina* and *M. l. cernovi*, to unravel and compare their venom proteome compositions with each other, and with those of already investigated subspecies. This chapter has been published as Ghezellou et al. (2022).

1.5.4 The first seven years of systematically recorded hospital admissions due to snakebite envenoming in Cyprus: what do the data tell us?

Despite the occurrence of several medically highly important snake taxa in North Africa and the Middle East, and epidemiological data pointing at up to 80,000 snakebite envenoming cases annually in this region (Kasturiratne et al. 2008), the available data on regional snakebite envenoming are highly fragmentary, and even entirely missing for some countries (e.g., Syria) (Amr et al. 2020). This scarcity of epidemiological data is often due to the absence of reliable reporting systems and a low awareness of health authorities concerning the importance of snakebite envenoming for public health. As snakebites are commonly only reported in local institutions or newspapers, international scientific access to these data is hindered (Sasa & Vazquez 2003). This also affected the Republic of Cyprus until 2013, when the State Health Services Organisation of Cyprus began to first-time systemically provide data on hospital admissions due to snakebite envenoming to the Cypriot Ministry of Health.

Hence, the fourth objective of this thesis (Chapter 5) is to provide for the first time a survey analysis of systematically obtained data on the epidemiology of snakebite envenoming in Cyprus, covering the years from 2013 to 2019, which is a better estimation of the medical importance of Cyprus' only dangerously venomous snake species (*M. l. lebetina*). This is an advantage compared to the snakebite statistics of other Middle Eastern countries, where *M. lebetina* ssp. occur together with several other viper species producing similar envenoming symptoms, which hinders a clearer estimation of the medical importance of individual snake species. Chapter 5 has been published as Jestrzemski et al. (2022).

1.6 References Chapter 1

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Chapter 2

Morphometric characteristics and seasonal proximity to water of the Cypriot blunt-nosed viper *Macrovipera lebetina lebetina* (Linnaeus, 1758)

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Published in: Journal of Venomous Animals and Toxins including Tropical Diseases (JVAT, 2018), 24:42
https://doi.org/10.1186/s40409-018-0175-6
Chapter version: as published in the JVATiTD, with only formal corrections.

Abstract Chapter 2

Background: The blunt-nosed viper *Macrovipera lebetina* (Linnaeus, 1758) is a medically important snake species in the Middle East. Its nominate subspecies *Macrovipera l. lebetina* is confined to Cyprus, where it is the only dangerously venomous snake species and heavily pursued. Despite the viper's large size, data on its body mass and sex-specific morphological differences are scarce. It is commonly believed that *M. l. lebetina* prefers freshwater proximity during summer. Hence, we aimed at investigating *M. l. lebetina* sex-specific morphological differences and its possible attraction to freshwater bodies in late summer.

Methods: Morphometric characteristics, proximity to water and conservation status of *M. l. lebetina* were investigated in Paphos district (Cyprus) in 2014, 2015 and 2017. Vipers were caught in different habitats, examined morphologically for metric and meristic characters, and released back into their habitat. Additionally, local people were interviewed about the conservation situation of the species.

Results: Of 38 recorded blunt-nosed vipers, morphological characteristics were collected from 34 (10 adult males, 16 adult females, eight unsexed juveniles). Rounded total length (ToL) ranged from 23.5 cm to 133.0 cm and weight between 10 g and 1,456 g. Adult males significantly exceeded adult females in tail length (TaL), ToL and head length (HL). No significant sex-specific differences were found in snout-vent length (SVL), head width (HW), weight or body condition index (BCI), nor for the ratios TaL/SVL, TaL/ToL, HL/SVL or HL/HW. Adult females from late summer (2015) had a significantly lower mean BCI than those from spring (2014). Distances of blunt-nosed vipers to the nearest water bodies (natural and artificial, respectively) did not differ significantly between spring (2014) and late summer (2015). There was also no significant difference between the distances of vipers to natural and to artificial water bodies in spring (and late summer).

Conclusions: Adult male blunt-nosed vipers exceed adult females in TaL, ToL and HL. Adult females are likely in a more vulnerable body condition in late summer than in spring. Periodic drying out of freshwater bodies in summer probably does not affect the species' occurrence. Educational workshops and habitat conservation are recommended for reducing human-viper conflict.

Keywords: Cyprus, Blunt-nosed viper, Morphology, Body condition index, Ecology, Water, Snakebite, Conservation.

2.1 Introduction

In the Near and Middle East, true vipers (Viperinae) are of significant medical importance [1–5]. The blunt-nosed viper *Macrovipera lebetina* (Linnaeus, 1758) for instance has been a cause of serious snakebite accidents from Cyprus and Eastern Anatolia to Northwest India [1, 6–11], while its venom is of high value for drug research and development [12–16]. *Macrovipera lebetina* inhabits river valleys, slopes with rocky outcrops and shrubs as well as canyons, gorges, gullies, pine forests, orchards, vineyards, caves and ruins [8, 17]. This large, heavy-bodied viper is an ambush predator of small mammals and birds and is often found in agricultural areas where it feeds on rodents [8]. The nominate subspecies *Macrovipera lebetina lebetina* (Cypriot blunt-nosed viper) is confined to Cyprus [8]. It is the only viper species found on the island and the largest viper in the European Union, growing up to 150 cm in total length (ToL) [17]. Blunt-nosed vipers from the mainland (e.g., *M. l. obtusa*) may even exceed 200 cm in ToL, with the published record being 230 cm [18]. However, the average ToL is between 80 and 100 cm for most *M. lebetina* populations [19]. According to Ščerbak & Böhme (2005), the largest blunt-nosed viper subspecies (*M. l. turanica* in Central Asia) reaches a ToL of 180 cm. The maximum weight of *M. lebetina* is 2,700 g [8].

Despite the special status of *M. l. lebetina* within the European viper fauna, little information is available on the natural history of this subspecies. Furthermore, no body mass data have been published yet on the Cypriot blunt-nosed viper. Although snake body mass (or weight) is rarely reported in the literature, it is considered the best measure of body size in life history studies and often a more useful proxy than length [20]. In this study, the snake body condition index was calculated as a measure of thickness based on *M. l. lebetina* body weight and length [21, 22]. As published data on sex-specific morphological differences in *M. lebetina* are scarce and sometimes contradictory, comparing different morphological characteristics between male and female blunt-nosed vipers can contribute to filling this knowledge gap. Based on the literature [8, 17], we formulated and tested three hypotheses: (1) On average, adult males are larger in snout-vent length (SVL) than adult females. (2) The ratio of tail length to snout-vent length (TaL/SVL) is smaller in adult males than in adult females. (3) In late summer (after the mating season), adult females are thinner than in spring (during the mating season).

Reptiles in semi-arid regions are often confronted with annual droughts. The resulting periodic water scarcity affects their spatial distribution, foraging ecology and body condition. Water availability is an important factor for survival as it may also determine the presence or absence of prey species and shelter from overheating and natural enemies [23, 24]. As water bodies are critical for sustaining both wildlife and human civilization, they have been facilitating contact between people and reptiles for millennia. In some areas, this contact has contributed to the problematic relationship between humans and venomous snakes. During the summer months, when many natural water bodies dry out in Cyprus, *M. l. lebetina* is frequently observed close to water [17, 25] and can even be found in swimming pools. Bites have occurred at waterholes [17]. Although a better understanding of *M. l. lebetina* distribution patterns and habitat requirements can contribute to minimizing human-viper conflicts, little is known about the importance of water for the spatial ecology of this species. In this study we therefore also investigated the relationship between water occurrence and blunt-nosed viper presence in Cyprus, via analysis of distances of the vipers to the nearest body of artificial and (or) natural freshwater.

2.2 Methods

2.2.1 Data collection

Data collection took place during three field surveys in northern Paphos district, Cyprus (ca. 35°02' N, 32°26' E), from 24 March to 4 June 2014, 27 August to 29 September 2015 and 21 September to 3 October 2017. This coastal lowland area is characterized by agriculture in the Polis Chrysochous valley, bordering Akamas Peninsula in the west and the forested foothills of the Troodos Mountains in the east (**Figure 2.1**, see [26, 27]). Daily mean temperatures range from 12.1 °C in January to 27.6 °C in July and August, with maximum temperatures of ≥40 °C during summer. Mean annual precipitation is 394.2 mm, of which 380.9 mm fall between October and April, and 13.3 mm between May and September [28].

Potential viper habitats in Polis municipality and surroundings were systematically examined via a transect survey [29]. Sixteen transects were placed across different potential viper habitats classified as (1) edges along streambanks and water pools, (2) edges along grain fields, orchards and pastures, (3) agrarian landscape mosaic with ruins of a deserted village, and[1] (4) shrubby slopes (maquis and garrigue habitat) in eastern Akamas Peninsula. Over each habitat, 1,500 m of transect line was spread, divided into three 500 m long sections. Additionally, field trips were made to nearby gorges (Androlikou, Petratis and Avakas) and dammed reservoirs (Evretou, Argaka, Agia Marina and Kannaviou), as well as to western Akamas Peninsula and the Troodos Mountains. Road-cruising was applied as a further survey method. Diurnal transect surveys amounted to 8 hours[1] daily throughout spring (starting at 8:00 AM), and nocturnal surveys to 4 hours[1] daily in late summer (starting at 8:00 PM).[1] During late summer, daily diurnal survey

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time was reduced to 4 hours[2] in the morning and early evening time. Three to four days were needed to examine all 16 transects (including field data collection), before repeating the process. Transect lines were walked in both directions and carefully checked for blunt-nosed vipers within a range of 20 m. At every viper observation point, the date, time, climatic and GPS data were collected and the shortest distance to the nearest water body was measured. Distances were measured with a 30 m tape in the field or otherwise on the computer with the Google Maps distance calculator. Swimming pools and other man-made reservoirs supplied by piped water were defined as artificial water bodies, whereas streams, natural springs, reservoirs and lakes were considered natural water bodies.

Live individuals of *M. l. lebetina* were caught for the collection of biometric data. Body weight was measured to the nearest gram (mean of five measurements) using a Beurer digital scale. Snout-vent length, tail length (TaL) and ToL were measured to the nearest 0.5 cm in live individuals and to the nearest 1 mm in dead ones. For statistical purposes, SVL, TaL and ToL of dead specimens were later rounded to the nearest 0.5 cm. Head length (HL) and head width (HW) were measured to the nearest 1 mm. Ventral, subcaudal and dorsal scales were counted, the latter at mid-body. The sex was determined via examination of the tail base and cloaca in live specimens, and via probing of the hemipenes in dead individuals [17]. Vipers below 70 cm ToL were considered sub-adult [11]. To avoid ambiguity in sex determination and risk of injury, juveniles were not sexed. Following Benson (1999), a digital photograph of the anal plate of each specimen was taken for individual recognition [30]. After being kept in captivity for up to 24 h, live individuals were relocated to their native areas, or, if captured in residential areas, to the nearest appropriate habitat. Four specimens (SNM-BS N-56085 to N-56088) found dead in good condition were collected and stored in 90% ethanol. They were later transferred to Braunschweig State Natural History Museum (Germany) and dissected for a stomach content analysis. In addition to these morphological and ecological data, semistructured interviews on human interaction with *M. l. lebetina* were conducted with staff of Cypriot institutions and local people related to environmental management, outdoor labor and medicine.

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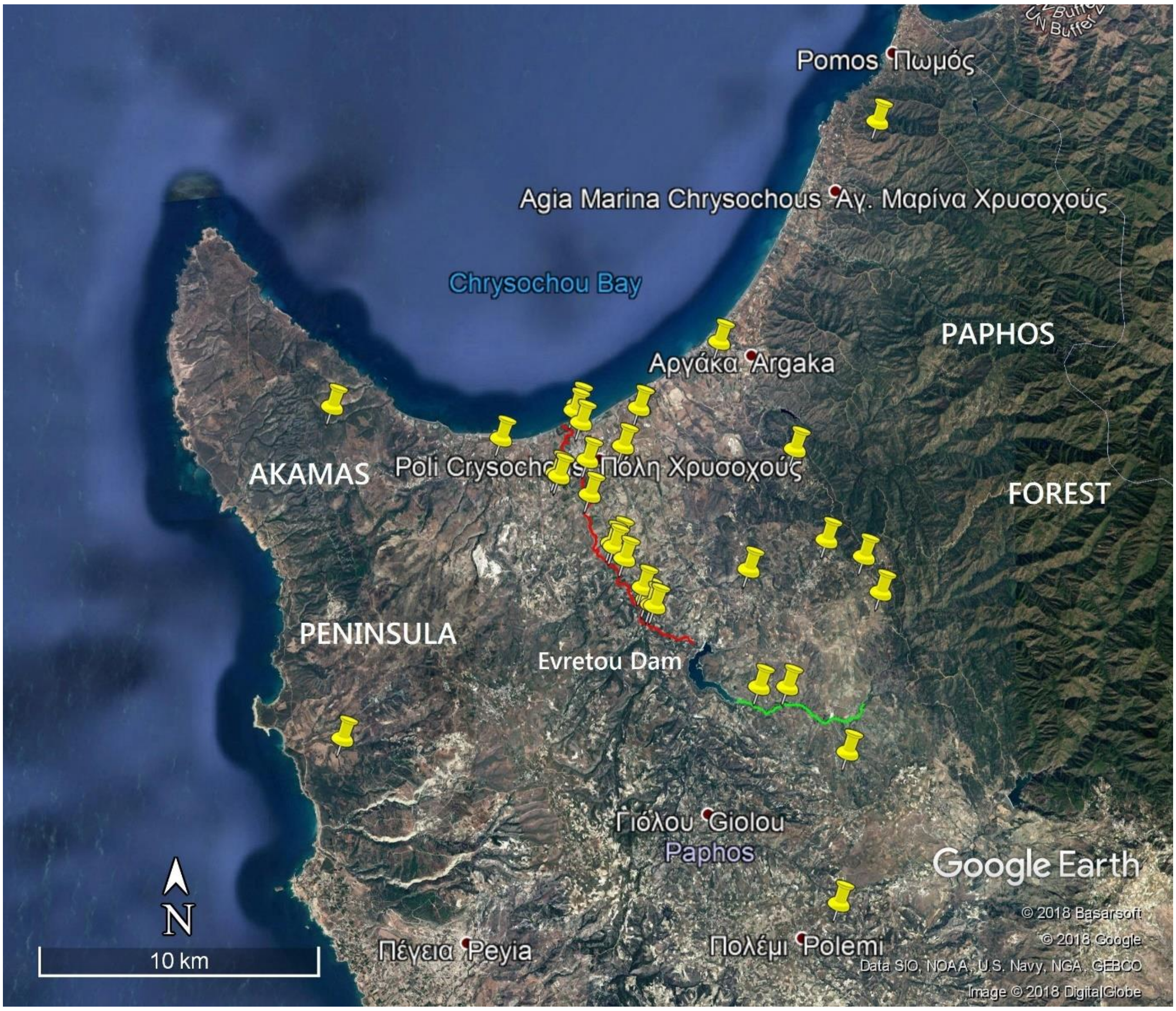

Figure 2.1. The study area in northern Paphos district: Polis Chrysochous municipality (35°02' N, 32°26' E) and surroundings, bordered by Akamas Peninsula in the west and Paphos Forest (Troodos Mountains) in the east. The Chrysochous River is highlighted in red. The lower section of the Stavros tis Psokas River, which flows into the Evretou Reservoir, is highlighted in green. The yellow pin markers represent the locations of the recorded *M. l. lebetina* specimens from this study, with one individual from near Kakopetria (Nicosia district) not included.

2.2.2 Statistical analysis

For all collected morphometric measurements, the mean, standard deviation (SD) and coefficient of variation (CV) were calculated. As a body condition index (BCI) for each snake, Fulton's index (K) was calculated using the formula $K = \frac{Weight}{SVL^3} \text{ x } 10^5$ [21, 22]. Prior to statistical analysis, all distance and morphometric data were tested for normality using the Shapiro-Wilk test. Due to non-normality of the distribution, the Mann-Whitney U (MWU) test[3] was applied to compare the nearest distances to water in the spring of 2014 with those taken in

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late summer of 2015. For the comparison of the nearest distances to artificial and natural water bodies in the spring of 2014 (late summer of 2015), the Wilcoxon matched pairs test was applied. Relationships between morphometric characteristics were examined via regression and correlation analysis. We assumed that the relationship between SVL and weight (W) could be described by the exponential regression equation $W = aSVL^b$, where a and b are constant [31]. The coefficients a and b were estimated for the linearized form of the equation (after log-transformation of both sides of the equation) $\log W = \log a + bSVL$, using the least squares method. The coefficient of determination (R^2) was used as a measure of the fraction of weight variation that can be explained by SVL. The correlation coefficient R was used as a measure of the strength of the linear relationship between SVL and TaL, SVL and HL, SVL and HW, and between HL and HW. Morphometric properties and the BCI of adult males and females were compared using the unpaired *t*-test for normally distributed variables or the MWU test[4] for non-normally distributed variables. For all tests, results were considered to be significant when $P < 0.05$. All statistical analyses were performed using the software Statistica 13.2 (StatSoft, Inc., USA).

2.3 Results

2.3.1 Habitat

Macrovipera l. lebetina was observed at edges of agricultural plantations, grain fields, gardens and streambanks, on grassy slopes, and inside dried-out streambeds. Besides shrubs and thick grassy vegetation, habitats with confirmed blunt-nosed viper presence contained a variety of microstructures such as crevices and caves, animal dens, stone slabs, rock piles, rock walls, and ruins of buildings and other constructions. Ten blunt-nosed vipers were recorded in private gardens (April 2014, July and September 2015), two in a building next to a swimming pool (3 and 19 May 2014) and a killed (newborn) viper at a building entrance door (21 September 2015). An adult female was found inside a pickup truck into which it had crawled when the vehicle had briefly stopped on a dirt road (23 September 2015). Common plant species of surveyed *M. l. lebetina* habitats in Paphos district included oriental alder (*Alnus orientalis*), giant reed (*Arundo donax*), common fig tree (*Ficus carica*), Phoenician juniper (*Juniperus phoenicea*), olive tree (*Olea europaea*), lentisk (*Pistacia lentiscus*), oriental plane (*Platanus orientalis*), holy bramble (*Rubus ulmifolius sanctus*) and the exotic eucalypt (*Eucalyptus* spp.).

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[4] May deviate from the published version (Jestrzemski & Kuzyakova 2018).

2.3.2 Food habits

The stomach of an adult female blunt-nosed viper (SVL 109.0 cm, TaL 14.8 cm, weight 1,225 g; SNM-BS N-56086) contained remains of a tawny pipit (*Anthus campestris*). This snake was found killed on a country road (10 April 2014). A live adult female (SVL 86.5 cm, TaL 11.5 cm) caught on 1 May 2014 (11:07 AM) at the edge of a stream bank next to an olive tree plantation regurgitated a half-digested, adult brown rat (*Rattus norvegicus*) of 270 g after 5 hours[5] in captivity (**Figure 2.2f**). After regurgitation, the body weight of the snake had dropped from 1,123 g to 835 g. The skull of a broad-toothed field mouse (*Apodemus mystacinus*) was found in the stomach of a killed adult male viper (SVL 92.3 cm, TaL 13.2 cm, weight 730 g; SNM-BS N-56087) lying on a small village road (12 May 2014). A killed male found on 17 September 2015 (SVL 117.3 cm, TaL 14.5 cm, weight 1,302 g) contained remains from a rodent, most likely a brown rat.

2.3.3 Shedding locations

Two blunt-nosed viper shedding localities were recorded during the field survey. On 1 April 2014, the slough of a large adult viper (about 1.2 m ToL) was found at the edge of an orange tree plantation and grazing pastures interspersed with almond trees. The slough was in the grass next to a pile of wooden debris. Two adult females (SVL = 65.5 and 93.0 cm) were later observed 33 m (6 May 2014) and 119 m (2 September 2015) away, respectively. The distance of the slough to the next water body (an artificial, permanent reservoir) was 137 m. The slough remnant of another large adult blunt-nosed viper (including a 15.5 cm long tail section) was discovered inside a periodically dry streambed with several small water pools on 27 September 2015. In the same month (6 and 29 September), two large vipers were recorded 76 m and 67 m upstream from this shedding location. The individual of 6 September was an adult male (SVL 116.5 cm, TaL 14.5 cm).

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Figure 2.2. a Dorsal view of a neonate blunt-nosed viper (SVL: 20.5 cm, TaL: 3 cm, weight: 10 g) with a 5 cm scale (11 September 2015). The skin is partially shed. **b** Dorsal view of an adult male blunt-nosed viper (SVL: 116.5 cm, TaL: 14.5 cm, weight: 1,441 g) with a 5 cm scale. **c** The individual from 2b in a periodically dry streambed where it was found in ambush position on 6 September 2015, 10:05 PM.[5] **d** Young adult female blunt-nosed viper (SVL: 65.5 cm, TaL: 9 cm, weight: 279 g) basking well-camouflaged at the edge of cropland after rainfall (6 May 2014, 3:45 PM).[5] **e** Adult male blunt-nosed viper from Akamas Peninsula (SVL: 92.5 cm, TaL: 12.5 cm, weight: 749 g), found in maquis and garrigue shrubland (28 September 2017, 8:45 PM).[5] **f** Adult female blunt-nosed viper (SVL: 86.5 cm, TaL: 11.5 cm, weight: 835 g) with a regurgitated brown rat (*Rattus norvegicus*) of 270 g. Found moving along a stream bank (**Figure 2.3a**). Photos: D. Jestrzemski.

2.3.4 Bite accidents by *Macrovipera lebetina* in Cyprus

During the field study, news was received of at least four incidences of human envenoming following snakebite in the study area. In one case (on 15 September 2015), a woman was bitten on her foot by a juvenile blunt-nosed viper next to her swimming pool (S. Paphitis pers. comm. 17 September 2015). As a survey of snakebite epidemiology was beyond the scope of the present study, it is possible that additional people in the area might have been bitten by vipers during the study period. Livestock and hunting dogs are bitten by *M. l. lebetina* every year (H.-J. Wiedl pers. comm. 29 March 2014; K. Kailis pers. comm. 15 April 2014; H. Demetriades pers. comm. 29 May 2014). In summer, bite accidents happen especially when goats and sheep concentrate around waterholes frequented by vipers (H. Hadjistyllis pers. comm. 6 June 2014; **Figure 2.3b**).

2.3.5 Conservation status

During the 2014 survey (between 3 April and 26 May), 13 blunt-nosed vipers were found killed on roads in Paphos district (Polis area) with injuries including smashed heads, necks and cloacae. In two cases, a string was attached to the body of the snake (**Figure 2.3e**). On 17 September 2015, a large dead male was found underneath the edge of a road bridge in a dried-out streambed, with its skull smashed and its stomach content protruding from its open gut. Interviews confirmed that *M. l. lebetina* is feared and persecuted by local people both in populated areas and in the wild. Road traffic is another threat. People involved in outdoor activities (farmers, forestry employees, game wardens, hunters, mosquito control workers and shepherds) encounter vipers more often in comparison with other occupational groups. The widespread public aversion to *M. l. lebetina* has so far prevented legal protection of this species in Cyprus ([17]; E. Erotokritou, H. Nicolaou pers. comm. 28 May 2014). Additionally, wild viper habitat is continuously destroyed by real-estate development, an ongoing trend in coastal areas (personal observation[6], **Figure 2.3f**). Man-made wildfires occur every year. In June 2016, 751 ha[6] of vegetation were burned down in the Argaka area (H. Nicolaou pers. comm. 17 March 2018).

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[6] May deviate from the published version (Jestrzemski & Kuzyakova 2018).

Figure 2.3. a Richly structured stream bank with adjacent agricultural areas: habitat of the blunt-nosed viper. **b** Rocky slopes with shrubs and ruins: blunt-nosed viper habitat used for sheep and goat grazing. **c** Juvenile blunt-nosed viper caught by children at noon (12:40 PM) in a residential swimming pool in central Paphos district (13 September 2015). **d** Signatures of mosquito control workers at a stream show that this riparian area – habitat of *M. l. lebetina* – is regularly visited by people. **e** Blunt-nosed viper killed and dragged on the road with a string attached to the snake's neck (14 April 2014). **f** Destruction of wild blunt-nosed viper habitat for real-estate development on Akamas Peninsula (1 October 2017). Loss of pristine habitats is a common problem in coastal areas of Cyprus. Photos: D. Jestrzemski.

2.3.6 Morphometric measurements

Morphometric data (**Table 2.1**) were collected on 34 vipers (10 adult males, 16 adult females and eight unsexed juveniles). Of these, 29 were alive and five (three males and two females) dead. No individuals were recaptured. Blunt-nosed viper rounded total lengths and weights (n = 34) ranged from 23.5 cm (10 g) for juveniles to 133.0 cm (1,456 g) for a killed adult male (SNM-BS N-56085). The three largest males exceeded 130 cm ToL whereas the largest female (SNM-BS N-56086) had a rounded ToL of 124.0 cm. Rounded SVL ranged from 20.5 cm to 119.5 cm (**Figure 2.4**). While the two heaviest males exceeded 1,400 g body weight, the heaviest female, a gravid individual, weighed 1,228 g (**Figure 2.5**). The lowest (66) and highest (146) BCI[7] was observed in adult females, whereas the range of K was much narrower among adult males (80–114) and juveniles (80–125). The mean ratio of TaL to ToL was 11.85 ± 1.18% for adult males (n = 10) and 10.80 ± 1.91% for adult females (n = 16). The coefficient of variation was highest for the variable weight, ranging from 46% in adult females to 113% in juveniles. **Figure 2.6** represents the relationship between natural logarithms of weight and natural logarithms of SVL for all examined vipers by a fitted regression line. The coefficient of determination R^2 amounted to 0.99. In comparison, **Figure 2.7** demonstrates a relationship between original data with a fitted exponential function whose coefficients were estimated based on the linear regression approach. In adult females, the relationship between weight and SVL was much weaker ($R^2 = 0.73$) than in juveniles and adult males ($R^2 = 0.97$ and $R^2 = 0.99$, respectively) (**Table 2.2**). Adult females also showed the weakest relationship between SVL and TaL, SVL and HL, SVL and HW, and between HL and HW (**Table 2.3**). The correlation between all characteristics was significant at the 0.05 level.[7] For TaL, only undamaged tails were considered. All correlation coefficients were significant at the 0.05 level. According to the *t*-test, SVL, HW and weight were not significantly different between adult males and females at the 0.05 level, although *P* was relatively low: *P* values were calculated as 0.060, 0.087 and 0.097 for average SVL, HW and weight, respectively. Adult males were significantly longer in average TaL ($P = 0.005$), average ToL ($P = 0.047$) and average HL ($P = 0.016$). No significant sex-specific differences were found for HL/SVL, HL/HW and BCI (**Table 2.4**). The MWU test[7] (**Table 2.5**) did not show a significant sex-specific difference for the ratios of TaL/SVL ($P = 0.285$) and TaL/ToL ($P = 0.285$). These results indicate that adult females did not have longer tails than adult males relative to their body length. Adult females observed in the late summer of 2015 (n = 5) had a significantly lower mean BCI than those found in the spring of 2014 (n = 11) ($P = 0.0007$). Among adult males, the mean BCI was significantly lower

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[7] May deviate from the published version (Jestrzemski & Kuzyakova 2018).

in the four longest individuals (SVL ≥ 100 cm) than in the six smaller ones (SVL < 100 cm) (*P* = 0.013). For measurements involving TaL, only individuals with undamaged tails were considered.

Table 2.1. Morphometric characteristics of juvenile and adult blunt-nosed vipers from Cyprus (SVL, TaL and ToL rounded to the nearest 0.5 cm).

Sex / Re-productive condition	Measurement	n	Range	Mean ± SD	Coefficient of Variation (%)
All	**SVL (cm)**	34	20.5 – 119.5	76.54 ± 28.30	36.97
	TaL (cm)	34	3.0 – 15.0	9.76 ± 3.51	35.93
	ToL (cm)	34	23.5 – 133.0	86.32 ± 31.49	36.47
	HL (mm)	32	16 – 54	40.59 ± 10.89	26.82
	HW (mm)	32	10 – 44	30.47 ± 9.21	30.24
	Weight (g)	34	10 – 1,456	584.65 ± 432.05	73.90
	TaL/SVL	34	0.07 – 0.16	0.13 ± 0.02	15.65
	TaL/ToL	34	0.06 – 0.14	0.12 ± 0.02	14.28
	HL/SVL	32	0.04 – 0.10	0.06 ± 0.01	22.76
	HL/HW	32	1.18 – 1.70	1.36 ± 0.12	8.51
	BCI (K)	34	66 – 146	101.06 ± 18.41	18.21
Adult males	**SVL (cm)**	10	71.0 – 119.5	96.75 ± 18.93	19.57
	TaL (cm)	10	10.0 – 14.5	12.80 ± 1.40	10.93
	ToL (cm)	10	81.0 – 133.0	109.60 ± 20.24	18.47
	HL (mm)	10	40 – 54	48.60 ± 5.52	11.36
	HW (mm)	10	31 – 44	36.70 ± 4.67	12.72
	Weight (g)	10	346 – 1,456	894.20 ± 420.79	47.06
	TaL/SVL	10	0.11 – 0.16	0.13 ± 0.02	11.17
	TaL/ToL	10	0.10 – 0.14	0.12 ± 0.01	9.92
	HL/SVL	10	0.05 – 0.06	0.05 ± 0.00	9.25
	HL/HW	10	1.23 – 1.42	1.33 ± 0.06	4.48
	BCI (K)	10	80 – 114	93.40 ± 9.99	10.70
Adult females	**SVL (cm)**	16	65.5 – 109.0	84.66 ± 12.47	14.73
	TaL (cm)	16	5.5 – 15.0	10.22 ± 2.30	22.53
	ToL (cm)	16	74.5 – 124.0	94.88 ± 13.74	14.48
	HL (mm)	14	36 – 48	43.71 ± 3.67	8.39
	HW (mm)	14	28 – 40	33.64 ± 3.69	10.97
	Weight (g)	16	279 – 1,228	649.88 ± 300.90	46.30
	TaL/SVL	16	0.07 – 0.15	0.12 ± 0.02	19.20

	TaL/ToL	16	0.06 – 0.13	0.11 ± 0.02	17.65
	HL/SVL	14	0.04 – 0.06	0.05 ± 0.00	7.04
	HL/HW	14	1.18 – 1.47	1.30 ± 0.08	6.01
	BCI (K)	16	66 - 146	103.00 ± 22.43	21.77
Juveniles	**SVL (cm)**	8	20.5 – 59.5	35.06 ± 16.74	47.75
	TaL (cm)	8	3.0 – 8.5	5.06 ± 2.29	45.24
	ToL (cm)	8	23.5 – 68.0	40.13 ± 19.03	47.42
	HL (mm)	8	16 – 34	25.13 ± 8.72	34.73
	HW (mm)	8	10 – 24	17.13 ± 6.60	38.54
	Weight (g)	8	10 – 218	67.25 ± 76.04	113.06
	TaL/SVL	8	0.13 – 0.16	0.15 ± 0.01	4.58
	TaL/ToL	8	0.12 – 0.14	0.13 ± 0.01	3.99
	HL/SVL	8	0.06 – 0.10	0.08 ± 0.01	18.69
	HL/HW	8	1.33 – 1.70	1.49 ± 0.12	8.33
	BCI (K)	8	80 – 125	106.75 ± 16.18	15.15
Adults	**SVL (cm)**	26	65.5 – 119.5	89.31 ± 16.07	18.00
	TaL (cm)	26	5.5 – 15.0	11.21 ± 2.35	20.96
	ToL (cm)	26	74.5 – 133.0	100.54 ± 17.73	17.63
	HL (mm)	24	36 – 54	45.75 ± 5.06	11.06
	HW (mm)	24	28 – 44	34.92 ± 4.31	12.35
	Weight (g)	26	279 – 1,456	743.85 ± 364.37	48.98
	TaL/SVL	26	0.07 – 0.16	0.13 ± 0.02	16.77
	TaL/ToL	26	0.06 – 0.14	0.11 ± 0.02	15.33
	HL/SVL	24	0.04 – 0.06	0.05 ± 0.00	7.90
	HL/HW	24	1.18 – 1.47	1.31 ± 0.07	5.37
	BCI (K)	26	66 – 146	99.31 ± 18.98	19.12

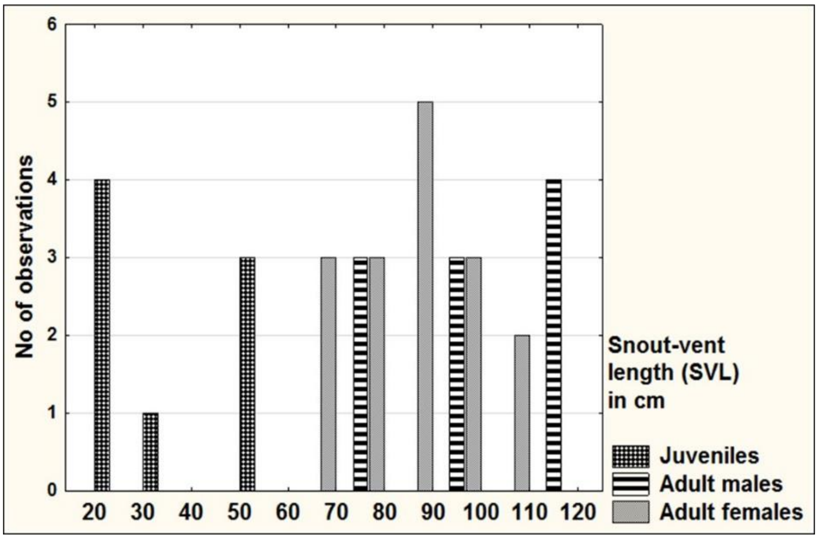

Figure 2.4. Distribution of SVL among 34 blunt-nosed vipers from Cyprus.

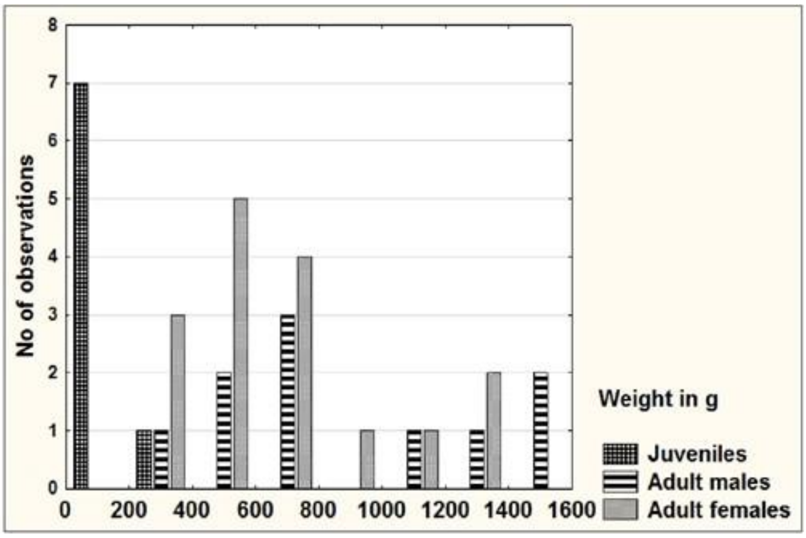

Figure 2.5. Distribution of weight among 34 blunt-nosed vipers from Cyprus.

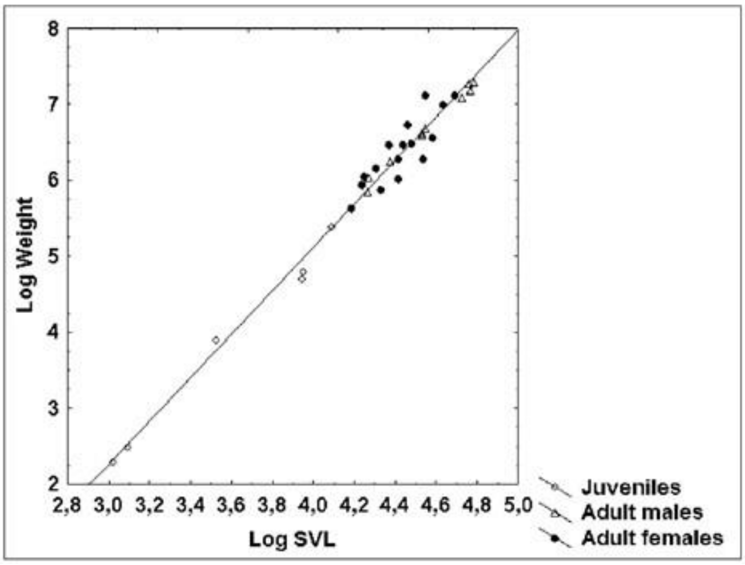

Figure 2.6. Relationship between log-transformed SVL and log-transformed weight in 34 blunt-nosed vipers from Cyprus: $Y = -6.32 + 2.86*X$. $R^2 = 0.99$

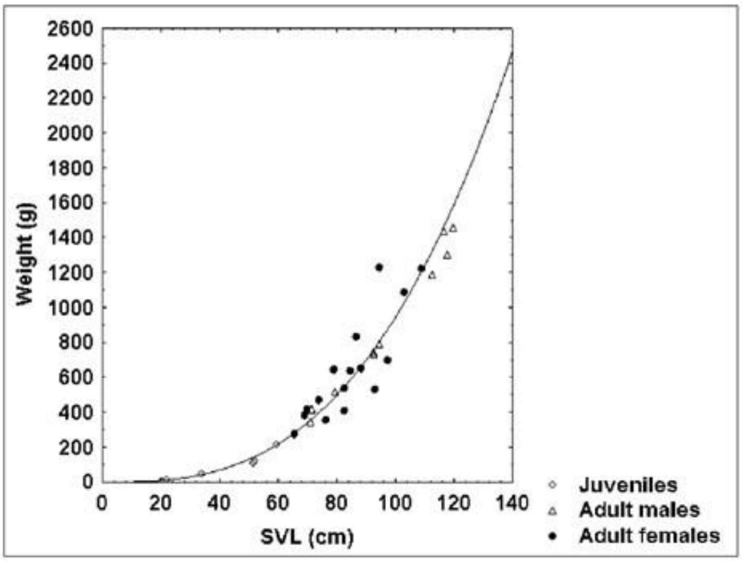

Figure 2.7. Allometric relationship between SVL and weight in 34 blunt-nosed vipers from Cyprus: $Y = \exp.(-6.32)*X^{2.86}$

Table 2.2. Sample sizes, parameter and coefficient of determination (R^2) of the regression: $\log W = \log a + b \cdot \log SVL$ of blunt-nosed vipers from Cyprus.

	N	R^2	a	b
SVL vs. weight (all)	34	0.80	-6.318	2.860
SVL vs. weight (juveniles)	8	0.97	-6.003	2.754
SVL vs. weight (adult males)	10	0.99	-4.961	2.557
SVL vs. weight (adult females)	16	0.73	-5.231	2.623

Table 2.3. Correlation coefficients (R) between morphometric characteristics of blunt-nosed vipers from Cyprus (significant *P* values bold).

	N	R	$P_{\text{Correlation}}$
SVL vs. TaL (all)	33	0.93	**0.000**
SVL vs. TaL (juveniles)	8	0.997	**0.000**
SVL vs. TaL (adult males)	10	0.89	**0.001**
SVL vs. TaL (adult females)	15	0.58	**0.024**
SVL vs. HL (all)	32	0.97	**0.000**
SVL vs. HL (juveniles)	8	0.896	**0.003**
SVL vs. HL (adult males)	10	0.97	**0.000**
SVL vs. HL (adult females)	14	0.85	**0.000**
SVL vs. HW (all)	32	0.96	**0.000**
SVL vs. HW (juveniles)	8	0.93	**0.001**
SVL vs. HW (adult males)	10	0.93	**0.000**
SVL vs. HW (adult females)	14	0.72	**0.004**
HL vs. HW (all)	32	0.98	**0.000**
HL vs. HW (juveniles)	8	0.98	**0.000**
HL vs. HW (adult males)	10	0.93	**0.000**
HL vs. HW (adult females)	14	0.83	**0.000**

Table 2.4. Results of the *t*-test for comparison of normally distributed morphometric characteristics of blunt-nosed vipers from Cyprus (significant *P* values bold).

Measurement	**n adult males**	**n adult females**	***P***
SVL (cm)	10	16	0.060
TaL (cm)	10	15	**0.005**
ToL (cm)	10	15	**0.047**
HL (mm)	10	14	**0.016**
HW (mm)	10	14	0.087
Weight (g)	10	16	0.097
HL/SVL	10	14	0.518
HL/HW	10	14	0.449
BCI (K)	10	16	0.216

Table 2.5. Results of the Mann-Whitney U test for comparison of not normally distributed morphometric characteristics of blunt-nosed vipers from Cyprus.

Measurement	**n adult males**	**n adult females**	**exact *P***
TaL/SVL	10	15	0.285
TaL/ToL	10	15	0.285

2.3.7 Distance to water

Of 38 total field observations, 36 blunt-nosed vipers were recorded with corresponding distances to the nearest body of artificial and (or) natural freshwater in 2014 (n = 19) and in 2015 (n = 17) (**Table 2.6**). For a single observation in 2015, only the nearest distance to artificial water was recorded, decreasing the number of viper observations with both the nearest distance to artificial and to natural water bodies available by the value of 1 (2015: n = 16). Out of the 36 observed vipers, 24 individuals could be captured for data collection, whereas 11 vipers either escaped or were killed by local people. In one case, a viper slough was treated as a single individual. At the site where a large male was observed, another large viper was recorded 21 days later, as were remains of a slough belonging to a similarly sized individual. To exclude the possibility of double-counting, all three observations were treated as one individual. Assumption of normality was rejected for all distances of *M. l. lebetina* to the nearest water bodies. The MWU test[8] showed no significant difference between the distances of *M. l. lebetina* to the nearest water bodies in the spring of 2014 and late summer of 2015 (*P* = 0.537). Likewise, the distances of *M. l. lebetina* to natural water bodies in the spring of 2014 did not vary significantly from those in the late summer of 2015 (*P* = 0.497). There was also no significant difference between the distances of *M. l. lebetina* to artificial water bodies in the spring of 2014 and in the late summer of 2015 (*P* = 0.635). Equally, the Wilcoxon matched pairs test could not prove a significant difference between the distances of *M. l. lebetina* to natural and artificial water bodies in the spring of 2014 (*P* = 0.295) and in the late summer of 2015 (*P* = 0.717).

Table 2.6. Recorded distances of blunt-nosed vipers to the nearest water body (Paphos district, Cyprus).

Distances of blunt-nosed vipers	**n**	**Range (meters)**	**Mean ± SD**
To nearest water body (Spring 2014)	19	3 – 511	103.53 ± 143.82
To nearest natural water body (Spring 2014)	19	3 – 2111	485.95 ± 596.28
To nearest artificial water body (Spring 2014)	19	10 – 1092	211.74 ± 248.14
To nearest water body (Summer 2015)	17	0 – 1740	181.59 ± 416.38
To nearest natural water body (Summer 2015)	16	0 – 2853	864.50 ± 1,038.65
To nearest artificial water body (Summer 2015)	17	0 – 2051	564.24 ± 675.22

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[8] May deviate from the published version (Jestrzemski & Kuzyakova 2018).

2.4 Discussion

2.4.1 Habitat preferences

The summer season is a critical period for populations of *M. lebetina*. Female blunt-nosed vipers not only need to find suitable refuges for oviposition, incubation and clutch protection, but also must cope with postpartum weight loss. Newborn vipers face a diverse range of predators [8]. Due to their small body size, they are also more threatened by dehydration than adults [32]. Against this background, it is not surprising that Cypriot blunt-nosed vipers are often found close to freshwater [25, 33], particularly during the hot dry summer months [17]. Water preference has also been observed among *M. l. turanica* in Uzbekistan [34].

However, the present study could not prove a seasonal preference of *M. l. lebetina* for freshwater bodies. At the same time, the frequent observations of rats[9] in all surveyed blunt-nosed viper habitats indicated sufficient prey availability in the study area. In fact, agricultural areas and other man-made landscapes with plenty of crops and organic waste offer conditions favorable to rat populations in Cyprus [25, 35, 36], which are large [37] and may attract blunt-nosed vipers even if freshwater is not available [38]. Besides ingesting the water contained in their prey animals, vipers can survive in xeric environments by drinking water from rain showers and mists. They can also harvest water from structural features of their environment such as rock surfaces after rain, or by dorsoventrally flattening their body to collect water droplets on their skin [39–41]. Snakes can further reduce their dehydration rate via resting in a coiled instead of stretched position [32]. Habitat structures allowing for thermoregulation and thermal protection have been proven critical for *M. l. obtusa*, and blunt-nosed vipers do not inhabit areas lacking these features [42].

In our current study, structural diversity in all surveyed habitats indicated an abundance of microhabitats providing shelter, humidity and thermoregulation to blunt-nosed vipers, also in areas void of freshwater bodies during summer. A 1993–1998 radio-telemetry study of the Cyclades blunt-nosed viper (*Macrovipera schweizeri*), now considered a subspecies of *M. lebetina* [43], found that this snake was not primarily attracted to water bodies but to structures that attracted their avian prey. These structures changed from water pools in spring to trees in autumn, when the pools had dried out. In both seasons, stream beds with riparian vegetation were important. *Macrovipera schweizeri* was absent from biotopes without large bushes as well as from densely vegetated habitats [44].

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[9] May deviate from the published version (Jestrzemski & Kuzyakova 2018).

Our findings suggest that prey availability and protective microhabitat structures such as large bushes and rocks are critically important for preserving Cypriot blunt-nosed viper populations, whereas the annual drying out of water bodies in late summer does not seem to prevent *M. l. lebetina* from inhabiting areas. However, droughts have become more frequent on Cyprus [17], and their long-term impact on Cypriot snake species has not yet been studied (S. Zotos pers. comm. 12 March 2018).

2.4.2 Shedding sites

Shedding increases dehydration in snakes [32]. As Cypriot blunt-nosed vipers slough four times a year, with two sloughings taking place during the summer months [17], the selection of suitable shedding sites is an important decision for the healthy development of *M. l. lebetina* throughout the year. Prior to molting, blunt-nosed vipers seek contact with water by bathing or lying in the rain to facilitate the sloughing of the old skin [8]. Water bodies may thus be particularly attractive for *M. l. lebetina* in pre-shed condition and may influence the selection of shedding locations, together with other factors (e.g., prey availability) that are communicated via chemical cues. The two blunt-nosed viper sloughs recorded in early April 2014 and late September 2015 were situated in habitats with year-round water availability, diversity of microstructures and abundance of rats.[10] Based on observations of North American rattlesnakes (*Crotalus oreganus*), Loughran et al. (2015) pointed out that different social interactions (e.g., pairing and mating) occurred around shedding sites and that these places had a similar importance for the social structure of snakes as communal hibernacula [45]. This implies that water bodies are potentially important for facilitating interspecific contact in snakes, particularly in more arid environments such as Cyprus.

2.4.3 Home range

Even though no data on *M. l. lebetina* movements are available, distances of 2–3 km to water bodies (as recorded for some individuals during this survey) may still fall within the home range of this species. Nilson et al. (1999) found that the home range of the smaller *M. schweizeri* was up to 20 ha,[10] with one female covering a distance of 5 km within 12 months [44]. A radio-telemetry study in Cyprus would be highly useful for obtaining more precise information about *M. l. lebetina* habitat use.

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[10] May deviate from the published version (Jestrzemski & Kuzyakova 2018).

2.4.4 Artificial and natural water bodies

The annual drying-up of many natural water bodies in summer may imply that Cypriot blunt-nosed vipers prefer man-made, permanent water bodies during this season, but not necessarily during the rainier spring. Yet this study could neither confirm a preference for artificial over natural water bodies during late summer, nor could it provide evidence that vipers are found closer to artificial water bodies in late summer than in spring. These findings indicate that swimming pools are not more attractive for *M. l. lebetina* than natural pools during late summer, highlighting the importance of natural water bodies for blunt-nosed viper conservation. Since water bodies provide significant advantages for blunt-nosed vipers such as ambushing for prey [17], water uptake and thermoregulation during heat periods, swimming pool users still face an increased risk of viper encounters during summer.

The viper bite incident in Latchi in September 2015 highlights the necessity of adequate protection measures. These include illuminating the entire pool area after sunset, wearing protective footwear, and cleaning debris from the surroundings, as blunt-nosed vipers (especially juveniles) may hide underneath larger, flat-surfaced objects such as wooden boards and flower pots (personal observation).[11] Vipers can be kept away from properties by solid fences whereas nets will indiscriminately kill various species of reptiles and other wildlife by entanglement [46]. The idea of developing effective snake repellents is promising, but commercially available chemicals such as naphthalene and sulfur have not proven effective in field trials [47].

2.4.5 Anthropogenic impact and conservation

The ongoing anthropogenic destruction of wild snake habitats for real-estate and industrial development in Cyprus [17] may threaten the survival of wild *M. l. lebetina* populations and increase the likelihood of human-viper confrontations in the future. Habitat loss due to land transformation for tourism is a general threat to snake species on Eastern Mediterranean islands [48]. Wildfires, which regularly occur in Cyprus, may render habitats unsuitable for snakes and their prey organisms (e.g. birds) for up to 10 years. Together with surface mining, wildfires are a major threat to blunt-nosed viper habitats on the island of Milos [44]. Road traffic is a threat to *M. lebetina* throughout the species' range [8]. Although *M. l. lebetina* is listed in Appendix II of the Berne convention (which contains "strictly protected fauna species"), it is the only snake species not protected by law in Cyprus [17], but at the same time the most pursued one. As all surveyed water bodies were checked at least monthly by mosquito control workers, the

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[11] May deviate from the published version (Jestrzemski & Kuzyakova 2018).

local viper populations were exposed to continuous anthropogenic pressure (**Figure 2.3d**). This ongoing human encroachment of riparian habitats coupled with the consequent killing of blunt-nosed vipers may greatly reduce their populations along streams and can be regarded as another major threat to the species in Cyprus (H.-J. Wiedl pers. comm. 29 March 2014). Pursuit by hunters must be considered as a significant threat, too. In Cyprus, feral cats commonly prey on reptiles, including *Laudakia stellio* and *Dolichophis jugularis* [17]. Feral cat predation on young vipers also poses a threat to the reproduction of *M. schweizeri* on Milos [44]. Consequently, their impact on *M. l. lebetina* should be investigated.

Workshops for mosquito control workers and other (occupational) outdoor groups such as hunters, shepherds, farmers, forestry employees and game wardens could help to raise awareness of non-lethal methods of preventing human-viper conflict such as snake deterrence or translocation. Workshops should also be offered to rural communities and schools. Public education could be further aided by the establishment of a national snakebite database for Cyprus, which so far is not available (E. Erotokritou pers. comm. 12 October 2017).

Valuable snake habitats such as wild riparian areas and rocky slopes with confirmed populations of *M. l. lebetina* should be placed under protection, with prohibition of land transformation (e.g., for real-estate development), and strict regulations concerning further interventions by man (e.g., grazing). This could be achieved by designating new areas for the Natura 2000 network of the European Union. In all conservation areas, hunting should be prohibited. Wetlands are also important habitats of other endangered Cypriot reptiles such as *Hierophis cypriensis* (Cyprus whip snake), *Natrix natrix cypriaca* (grass snake) and *Mauremys rivulata* (Balkan terrapin) [17].

2.4.6 Allometric relationship between snout-vent length and weight

The allometric relationship between SVL and weight in *M. l. lebetina* (n = 34) can be modelled as an exponential curve (**Figure 2.7**) fitted to the scatterplot of SVL vs. weight. According to the equation, a very large Cypriot blunt-nosed viper of 135 cm SVL would weigh 2,227 g, which is not far from the reported maximum body weight of mainland blunt-nosed vipers according to Ščerbak & Böhme (2005): 2,700 g for males and 2,000 g for females [8]. However, Sochurek (1979) reports that he obtained a large male blunt-nosed viper of more than 3 kg body weight from the USSR around the year 1950 [49]. Out of 23 blunt-nosed vipers collected in Iran (Khorasan province), the heaviest male (SVL 125.5 cm, TaL 17.0 cm) and female (SVL 113.0 cm, TaL 14.0 cm) each weighed 1,180 g ([50], A. Nasoori pers. comm. 15 January 2018). While Cypriot blunt-nosed vipers can reach a ToL of 150 cm [17], mainland subspecies (e.g., *M. l. obtusa*) may grow up to 218 cm [51]; however, one individual of 230 cm was recorded [18]. Bannikov et al. (1977) state a maximum ToL of 160 cm [52], whereas Sochurek (1979) mentions specimens of up to 190 cm ToL [49].

2.4.7 Variability of body condition

The highest coefficient of variation among all variables in this study was determined for weight (from 46% in adult females to 113% in juveniles). It corresponds to the identified positive allometric relationship between weight and the linear measures of the blunt-nosed viper's body. The relatively low R^2 determination coefficient of the regression ln weight vs. ln SVL among adult female blunt-nosed vipers (0.73) shows that the body weight of females was not only influenced by their SVL but also by other factors. In line with our hypothesis, adult *M. l. lebetina* females caught in September were significantly thinner than those captured in spring ($P = 0.001$). This fluctuation of body mass might be explained by postpartum weight loss, provided that the females examined in late summer had reproduced in that year. After oviposition, blunt-nosed vipers (*M. l. obtusa*) may lose up to 52% of their weight (mean: 35%) [53]. Snake body condition is also influenced by the size and time of the last meal, and by diseases or parasites.

Adult male blunt-nosed vipers are more likely to thrive during late summer than during spring, as they neither have to search for females nor engage in combat with male rivals anymore [17]. However, the low number of adult males recorded during this study in late summer (three compared to seven males in spring) prevented a seasonal comparison within males. Although our sample of adult male vipers was small ($n = 10$), the significantly lower body condition index of the four largest adult males compared to the six smaller ones was striking ($P = 0.013$), since it suggests that male blunt-nosed vipers may become thinner with age. This hypothesis would be consistent with the trend of the scatterplot of SVL vs. BCI (**Figure 2.8**) showing that the highest BCI values were observed among small and medium-sized vipers (SVL < 100 cm). Large blunt-nosed vipers may lose weight due to the accumulation of endoparasites in snakes with increasing age, and the consequent loss of appetite and weight [54–56]. Zinyakova (1967) detected 25 species of endoparasites in *M. l. turanica* from the Soviet Union [57], while Murvanidze et al. (2008) listed seven species of helminths inhabiting Georgian *M. l. obtusa* [58]. Of 20 Iranian blunt-nosed vipers (*M. l. obtusa*) examined by Nasiri et al. (2014), 16 were infested with parasites [59]. As our sample included only two females exceeding 100 cm SVL, a comparison between larger and smaller adult females was impossible (14:2 ratio). Our findings indicate that adult female blunt-nosed vipers are in a more vulnerable physical condition in late summer than in spring, and that old age possibly has an adverse effect on the body condition index of *M. l. lebetina*. However, a much larger dataset is needed to provide solid statistical evidence.

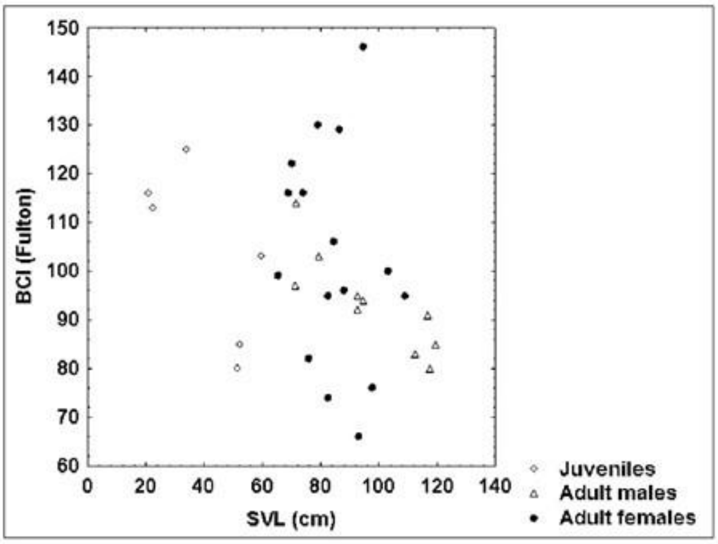

Figure 2.8. Relationship between SVL and BCI in 34 blunt-nosed vipers from Cyprus: R = 0.40

2.4.8 Sex-specific differences

Although we could not prove that adult male blunt-nosed vipers exceeded adult females in body size and weight (*P*-values of the respective *t*-test amounted to 0.06 for SVL and 0.097 for weight), our results suggested that they were longer and heavier. This apparent dimorphism was further supported by the significantly longer TaL (P = 0.005), ToL (P = 0.047) and HL (P = 0.016) of adult males. This trend is also shown by the scatterplots of SVL vs. TaL (**Figure 2.9**), SVL vs. HL (**Figure 2.10**) and HL vs. HW (**Figure 2.11**). As the ratio of TaL to SVL and of TaL to ToL in adult individuals was not significantly different between sexes, we could not confirm our hypothesis that males had shorter tails than females (see [17]). This indicates that sex-specific morphological differences in *M. l. lebetina* are still a controversial issue. However, our findings of the ratio TaL/ToL were similar to those of other authors [50], who recorded a mean ratio of 11.89 ± 1.56% among 15 adult males (our study: 11.85 ± 1.18%) and a mean ratio of 11.10 ± 0.59% in eight adult females of Iranian *M. l. obtusa* (our study: 10.80 ± 1.91%). Larger sample sizes are required to obtain more powerful test results on sex-specific differences.

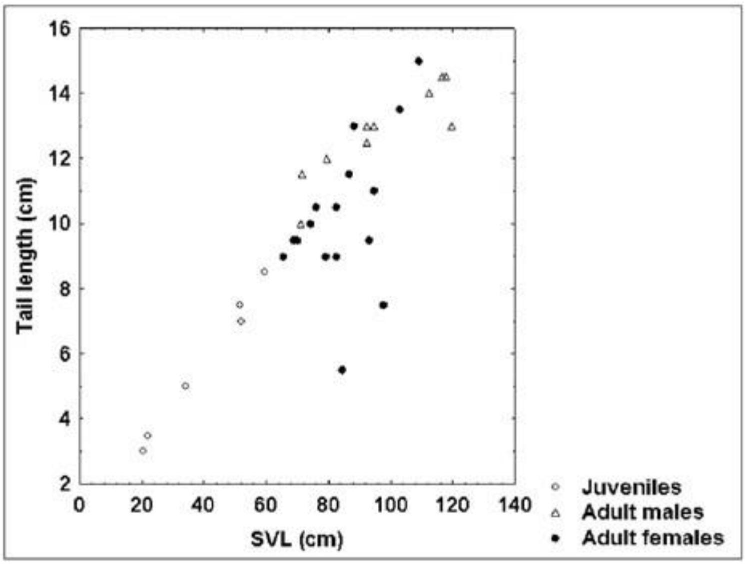

Figure 2.9. Relationship between SVL and TaL in 34 blunt-nosed vipers from Cyprus: R = 0.89

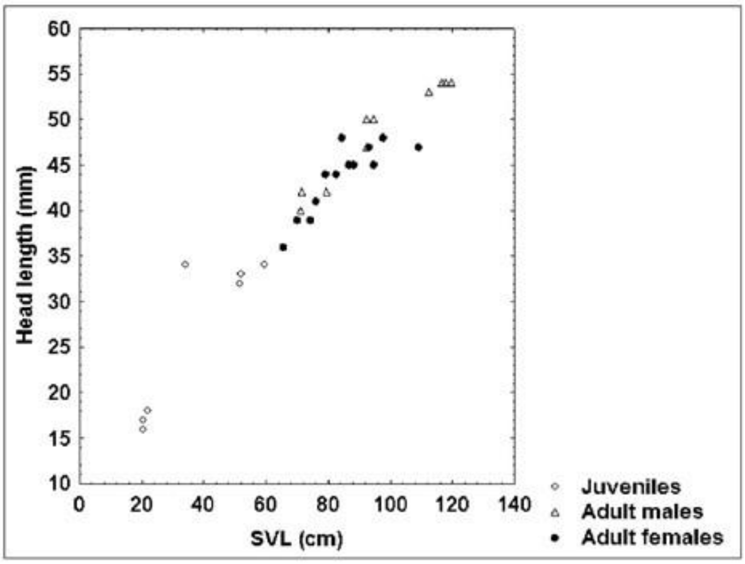

Figure 2.10. Relationship between SVL and HL in 34 blunt-nosed vipers from Cyprus: R = 0.97

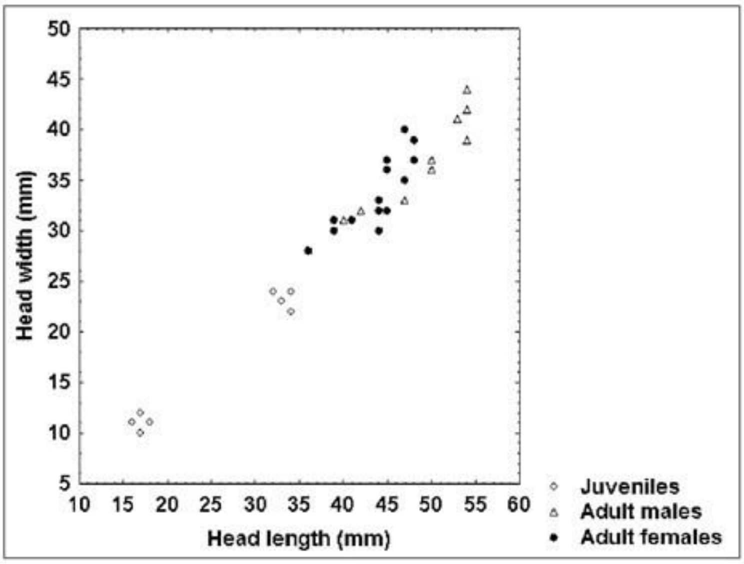

Figure 2.11. Relationship between HL and HW in 34 blunt-nosed vipers from Cyprus: R = 0.98

2.4.9 Size comparison with other large Holarctic vipers

The enormous differences in body size between blunt-nosed vipers from the Cyclades and Cyprus [8, 17, 44, 60] demonstrate the morphological variability within *M. lebetina*. It is the only European viper reaching 150 cm ToL and 2,000 g body mass (or more), which is comparable to the body dimensions of large North American rattlesnake species (*Crotalus* spp.) (**Table 2.7**). Some of the latter (e.g., *C. atrox*) are found in similar biomes (dry steppe, semi-arid desert) as *M. lebetina*. In the Mediterranean basin, Moorish vipers (*Daboia mauritanica*) and desert vipers (*D. deserti*) reach similar dimensions (180 cm and 160 cm maximum ToL, respectively), whereas Palestine (*D. palaestinae*) and Ottoman vipers (*Montivipera xanthina*) commonly do not grow much larger than 1 m [61]. However, exceptionally long individuals may reach 136 cm ToL (1,227 g body mass) in *D. palaestinae* (S. Meiri pers. comm. 1 May 2017) and 143.5 cm ToL (1,284 g body mass) in *M. xanthina nilsoni* ([62]; A. Cattaneo pers. comm. 2 August 2017). Maximum recorded body weights of *D. palaestinae* were 1,640 g (131 cm ToL) and 1,800 g (128 cm ToL), respectively, the latter being a gravid female (S. Meiri, unpublished data). As large viper species are primarily found in the Americas, Africa, the Middle East and South Asia, the enormous body dimensions of *M. l. lebetina* highlight its morphological and evolutionary uniqueness within the European viper fauna.

Table 2.7. Snout-vent length, weight and BCI[12] of *M. l. lebetina* compared to large North American rattlesnake species (*Crotalus* spp., USA).

General information [31, 61]					
Scientific name	*M. lebetina*	*C. adamanteus*	*C. atrox*	*C. horridus*	*C. oreganus*
Common name	Blunt-nosed viper	Eastern diamondback rattlesnake	Western diamondback rattlesnake	Timber rattlesnake	Western rattlesnake
Distribution	Eurasia to Central and South Asia	Southeastern USA	Southwestern North America	Eastern to Central USA	Western North America
Maximum ToL (cm)	150 (Cyprus); 218-230 (mainland)	244	213	189	163
Specimen data					
Origin	Wild, Cyprus	Captive, North Carolina	Wild, Oklahoma	Wild, Virginia	Wild, California
SVL (cm)	116.5	168.8	152.0	140.7	109.0
TaL (cm)	14.5	16.0	-	11.3	9.0
Weight (g)	1,441	4,850 (“4.85 kg”)	2,776	3,509	1,150
BCI (K)	91	101	79	126	89
Reference	This study	[63]	[64]	S. Goetz pers. comm. 29 January 2018	B. Putman pers. comm. 19 January 2018

2.5 Conclusions

We conclude that adult males of *M. l. lebetina* exceed adult females in head length, tail length and total length. Adult female blunt-nosed vipers are probably in a more vulnerable physical condition in late summer than in spring. Old age possibly has an adverse effect on the body condition index of *M. l. lebetina*. The annual drying out of freshwater bodies in late summer most likely is not a decisive factor for the occurrence of the species on Cyprus. It must be assumed that prey availability and protective microhabitat structures such as large bushes and rocks are of crucial importance for preserving blunt-nosed viper populations in Cyprus. A radio-telemetry study would greatly improve the understanding of the spatial ecology of *M. l. lebetina*. Workshops should be conducted for rural communities, schools and outdoor groups

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[12] May deviate from the published version (Jestrzemski & Kuzyakova 2018).

to raise awareness of non-lethal ways of removing blunt-nosed vipers from areas inhabited or frequented by people. The establishment of a national snakebite database for Cyprus would contribute to improving public education. Wild snake habitats with confirmed populations of *M. l. lebetina* should be protected from anthropogenic modifications such as real-estate development, grazing and hunting.

2.6 Abbreviations

°C: Degrees Celsius; °E: Eastern latitude; °N: Northern latitude; AM: Time before noon; BCI (K): Fulton's body condition index; cm: Centimeters; CV: Coefficient of variation; g: Grams; ha: Hectares;[13] HL/HW: Ratio of head length to head width; HL/SVL: Ratio of head length to snout-vent length; HL: Head length; HW: Head width; kg: Kilograms; m: Meters; mm: 1) millimeters of length, 2) millimeters of precipitation; n: Sample size; *P*: Probability value; PM: Time after noon; R: Correlation coefficient; R^2: Coefficient of determination; SD: Standard deviation; SVL: Snout-vent length; TaL/SVL: Ratio of tail length to snout-vent length; TaL/ToL: Ratio of tail length to total length; TaL: Tail length; ToL: Total length; W: Weight in grams.

2.7 Acknowledgements

We acknowledge support by the German Research Foundation and the Open Access Publication Fund of Göttingen University. Also, we thank Ulrich Joger, Stefan Schütz, Spyros Sfenthourakis, Savvas Zotos, Nikolaus Stümpel, Volker Schrempf, Hans-Jörg Wiedl, Felix Baier, Ulrich Kuch and Frank Gessler for insightful discussions and encouragement. We are immensely grateful to Elena Erotokritou and Elena Stylianopoulou (Department of Environment, Cyprus) for granting research permits. Great thanks also go to Elias Christodoulou and his family for their hospitality during the visits of DJ to Cyprus. The fieldwork was kindly supported by Volker Schrempf, Hans-Jörg Wiedl, Marios and Periklis Theodorou, Savvas Zotos, Elena Erotokritou, Haris Nicolaou, Spyros Sfenthourakis, Caroline Penman, Chandani Ekanayaka, Eftichia Demetriade, Konrad Mebert, Demetris Christofi, Fidias Charalambous, Demetris Pavlou, Pawel Sikorski, Stelios Paphitis, Harris Demetriades and Koula Michael, as well as by Christaki and Lucy (Peristerona), and the interview partners in Cyprus. Many thanks go to Takis Tsintides for the determination of plant species and to Joachim Jenrich, who analyzed all blunt-nosed viper stomach contents. Verena Tobert helped with the transport of samples. We are indebted to Shai Meiri for sharing morphological data of *D.*

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[13] May deviate from the published version (Jestrzemski & Kuzyakova 2018).

palaestinae and to Augusto Cattaneo for morphological data of *M. xanthina nilsoni*. We also thank Ali Nasoori (*M. lebetina*), Breanne Putman (*C. oreganus oreganus*), George Pisani (*C. atrox*), Rulon Clark and Scott Goetz (*C. horridus*) for further specimen data. Additional information on blunt-nosed vipers was provided by Mario Schweiger, Dieter Glandt and André Schmidt.

2.8 Funding

The fieldwork in Cyprus was generously supported by the Mohamed bin Zayed Species Conservation Fund, the German Society for Herpetology and Herpetoculture (via the Hans-Schiemenz Fonds), the Department of Forest Zoology and Forest Conservation (University of Göttingen) and the Nature and Biodiversity Conservation Union (NABU, Germany).

2.9 Authors' contributions

DJ designed the study, carried out the fieldwork and discussed the results with IK, who also conducted the statistical analysis. Both authors wrote, read and approved the manuscript.

2.10 Availability of data and materials

The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

2.11 Ethics approval and consent to participate

Approval for this study was granted by the Department of Environment of Cyprus (Ministry of Agriculture, Rural Development and Environment; Reference number 02.15.001.003, 04.05.2002.005.006).

2.12 Competing interests

The authors declare that they have no competing interests.

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Chapter 3

Updated morphometric characteristics of the Cypriot blunt-nosed viper (*Macrovipera lebetina lebetina*), based on an enlarged dataset (2014-2022)

Abstract Chapter 3

Background: The Cypriot blunt-nosed viper (*Macrovipera lebetina lebetina*) is Europe's largest and heaviest viper. Yet, morphometric data of blunt-nosed vipers are rarely published, although body weight is considered an ideal body size measure in snakes. Following up on Jestrzemski & Kuzyakova (2018), this chapter aims at presenting an enlarged morphometric dataset of *M. l. lebetina* and repeating the statistical analyses with enlarged sub-samples, to critically re-evaluate the previous assumptions and test new ones.

Methods: In 2022, new morphometric data of *M. l. lebetina* were collected and the enlarged dataset used for re-examining the relationships between morphometric characters via regression and correlation analysis. Again, adult males and females were morphometrically compared, as well as adult males of different size classes.

Results: Morphometric characteristics were collected from 46 wild blunt-nosed vipers (16 adult males, 21 adult females, nine unsexed juveniles). The rounded total lengths (ToL) ranged from 23.5 cm to 133.5 cm and body weights from 10 g to 1,456 g. The relationship between snout-vent length (SVL) and weight was much weaker in adult females than in adult males and juveniles, as well as between SVL and tail length (TaL), head width (HW) and body condition index (BCI), and there was no significant correlation between SVL and TaL (SVL and BCI) in adult females. Adult males significantly exceeded adult females in SVL, TaL, ToL and HL, but had a significantly lower mean BCI. In the six largest adult males, the mean BCI was significantly lower than in the eight smaller ones, while the mean ratio TaL/SVL was significantly lower in the eight largest males than in the eight smaller ones.

Conclusions: The ratios TaL/SVL, TaL/ToL, HL/SVL and HL/HW are unsuitable indicators for sex determination in adult *M. l. lebetina*. Adult males of *M. l. lebetina* exceed adult females in SVL, TaL, ToL and HL, while adult females tend to have a higher BCI. In large adult males, body condition appears to decline with increasing body size due to deteriorating health with age, while tail growth in adult males seems to decrease with age as well.

Keywords: Cyprus, Blunt-nosed viper, Morphometric data, Sex-specific differences, Body condition index, Relative tail length.

3.1 Introduction

The blunt-nosed viper (*Macrovipera lebetina*) is a medically important, front-fanged venomous snake species complex native to the Middle East. Despite its large body size and their medical importance [1, 2, 3], published literature on its morphology is rare and mostly anecdotical [4]. Published body weight data of *M. lebetina* ssp. are particularly scarce, although snake body mass is considered the best measure of body size in life history studies and often a more useful proxy than length. Furthermore, published information on sex-specific morphological differences in *M. lebetina* are sometimes contradictory and often lack supporting data [5]. Against this background, Jestrzemski & Kuzyakova (2018) presented for the first time systematically collected morphometric data of the Cypriot blunt-nosed viper (*Macrovipera l. lebetina*), calculating a body condition index as a measure of snake thickness [6]. While relationships between morphometric characteristics were examined via regression and a correlation analysis, a morphometric comparison between adult males and females was conducted by applying the unpaired *t*-test for normally distributed variables, and the Mann-Whitney U (MWU) test for non-normally distributed variables.

Hypothesis testing focused on the examination of possible sex-specific differences in snout-vent length (SVL), and in relative tail length (TaL/SVL), while the BCI was compared between females during and after the breeding season. Furthermore, the BCI was compared between large-sized and medium- to small-sized adult males. The morphometrical dataset was based on 34 individuals (10 males, 16 females and eight unsexed juveniles) collected during three field visits to Cyprus in 2014, 2015 and 2017. Of all measured vipers, five adults (two females, three males) were well-preserved specimens found dead (i.e., freshly killed). However, the data subsets used for testing the hypotheses were relatively small.

Sex-specific differences were compared between 10 adult males and 14 (four characteristics), 15 (two characteristics) and 16 (three characteristics) adult females, respectively. For the comparison of BCI within adult females, five individuals caught in spring (during the breeding season) were compared to 11 individuals captured in late summer (after the breeding season). With merely four and six individuals respectively, the samples for the comparison of BCI within males were even smaller.

In this regard, the question arises how the results of these tests would change with larger sample sizes. During a fourth field trip to Cyprus in May 2022, new morphometric data from wild-caught blunt-nosed vipers were obtained. Therefore, the aim of this chapter is to present an enlarged morphometric dataset of Cypriot *M. l. lebetina*, and to update the morphometric analyses (regression, correlation and morphometric comparisons) with enlarged sub-samples.

Thus, with the bigger dataset, two hypotheses presented by Jestrzemski & Kuzyakova (2018) were tested again:

(1) Adult males exceed adult females in mean SVL.

(2) The mean ratio TaL/SVL is smaller in adult males than in adult females.

However, the hypothesis assuming female blunt-nosed vipers to be thinner after the mating season was not investigated again, since it would have required the systematic collection of females during continuous spring and late summer seasons. Since vipers were collected only in the spring of 2014 and 2022, and in the late summer of 2015 and 2017, the missing data collection in the year 2016, and from 2018 to 2021, prevented standardized sampling conditions, and thus the further investigation of this research question. Instead, based on the results of Jestrzemski & Kuzyakova (2018), two further hypotheses were formulated and tested:

(3) The mean BCI of large adult males (SVL >= 100 cm) is lower compared to smaller adult males, since older vipers experience more infestation by parasites, and are generally stronger affected by diseases, with adverse consequences for their health and thus their body condition.

(4) The mean ratio TaL/SVL of large adult males (SVL >= 100 cm) is lower compared to smaller adult males, since the latter face higher sexual selective pressure than larger adults, and thus experience faster tail growth during their life stage.

3.2 Methods

3.2.1 Data collection

In June 2022, the dataset from Jestrzemski & Kuzyakova (2018) was increased by the morphometric measurements of 12 blunt-nosed vipers collected during a field trip by Daniel Jestrzemski (DJ) to Cyprus from 12 May to 1 June 2022. As part of this research, 13 live blunt-nosed vipers collected in Paphos district were tested for their chemo-ecological preferences, as an indicator of their interactions with a sympatric colubrid snake species (*Dolichophis jugularis*). From all of these 13 individuals, as well as from a dead specimen of *M. l. lebetina* (found killed in an orange plantation), morphometric characteristics (SVL, TaL, head length (HL), head width (HW) and weight) were collected, following the same scheme as described by Jestrzemski & Kuzyakova (2018). However, two individuals were excluded from the final dataset, as they were held in captivity since 28 April 2019 and 17 September 2020, respectively, and thus their morphometric characteristics could not be compared to those of their wild counterparts considered for the study. For another four vipers, only the weight data were excluded, as they had been held in captivity without food for 30 (25, 19, 35) days prior to weighing. Hence, their weights would not be representative of their original condition when captured in the wild (**Table 3.1**). After the experiments, all wild-caught blunt-nosed vipers were released back to the wild.

3.2.2 Statistical analysis

As part of the data analysis, the mean, standard deviation (SD) and coefficient of variation (CV) were calculated for all collected morphometric measurements. Fulton's index (K) was calculated using the formula $K = Weight/SVL^3 \times 10^5$ [7, 8] as a body condition index (BCI) for each snake. Following the methodology of Jestrzemski & Kuzyakova (2018), the relationships between morphometric characteristics were examined via a regression and correlation analysis. For the regression analysis, it was again assumed that the relationship between SVL and weight (W) was best described by the exponential regression equation $W = aSVL^b$, where a and b are constant [9]. After log-transformation of both sides of the equation (natural logarithms), the linearized form $\log W = \log a + bSVL$ was obtained, from which the coefficients a and b were estimated (least squares method). Again, the coefficient of determination (R^2) was used as a measure of the fraction of weight variation that can be explained by SVL. For the correlation analysis, the correlation coefficient R was applied as a measure of the strength of the linear relationship between SVL and TaL, SVL and HL, SVL and HW, and between HL and HW. Thus, both R^2 and R were applied for describing the relationship between the examined variables. Prior to statistical analysis, all morphometric data were tested for normality using the Shapiro-Wilk test. Morphometric properties and the BCI of adult males and females were compared using the unpaired *t*-test for normally distributed variables and the MWU test for non-normally distributed variables. For comparing the mean BCI and mean TaL/SVL of large with small to medium-sized adult males, we defined "large" as the SVL measuring two thirds of the maximum total length (ToL) for *M. l. lebetina* stated in literature [5]. Hence, with a maximum ToL of 150 cm for *M. l. lebetina*, the threshold SVL value was 100 cm. For all tests, results were considered significant when $P < 0.05$. All statistical analyses were performed using the software Statistica 14 (StatSoft, USA).

3.3 Results

Morphometric data (**Table 3.2**) were collected from 46 wild vipers (16 adult males, 21 adult females and nine unsexed juveniles). Of these, 40 were alive and six (four males and two females) dead. No individuals were recaptured. Blunt-nosed viper rounded total lengths (n = 46) and weights (n = 42) ranged from 23.5 cm (10 g) for juveniles to 133.5 cm (1,456 g) for a killed adult male (SNM-BS N-56085). The four largest males exceeded 130 cm ToL, whereas the largest female (SNM-BS N-56086) had a rounded ToL of 124.0 cm. Rounded SVL ranged from 20.5 cm to 121.5 cm (**Figure 3.1**). While the three heaviest males exceeded 1,400 g body weight (in one case, even 1,500 g), the heaviest female, a gravid individual, weighed 1,228 g (**Table 3.1, Figure 3.2**). The lowest (66) and highest (146) BCI (K) was observed in adult females, whereas the range of K was much narrower among adult males (80–114) and juveniles (80–125). The mean ratio of TaL to ToL was 11.53 ± 1.41% for adult males (n = 16) and 10.52 ± 2.19% for adult females (n = 21). The coefficient of variation was highest for the variable weight, and ranged from 43% in adult females to 106% in juveniles.

Table 3.1. Morphometric characteristics (with SVL, TaL and ToL rounded to the nearest 0.5 cm) of four live blunt-nosed vipers (two males and two females caught in 2022) excluded from weight-related data, and of two male captive-held blunt-nosed vipers (measured in 2022) fully excluded from the morphometric analysis.

	Live vipers collected in spring 2022				**Live vipers in long-term captivity**	
	Male 1	**Male 2**	**Female 1**	**Female 2**	**Male 1**	**Male 2**
Days in captivity before measurement	25	19	30	35	1128	620
Date of measurement (2022)	29 May	29 May	29 May	31 May	30 May	30 May
Diet in captivity	None	None	None	None	1 mouse / 2 weeks	1 mouse / 2 weeks
SVL (cm)	107.0	121.5	109.0	93.5	121.0	90.0
TaL (cm)	14.0	12.0	13.0	13.0	15.0	13.0
ToL (cm)	121.0	133.5	122.0	106.5	136.0	103.0
HL (mm)	56	54	53	49	59	47
HW (mm)	38	46	38	38	40	34
Weight (g)	1,012	1,540	1,085	418	805	683
TaL/SVL	0.13	0.10	0.12	0.14	0.12	0.14
TaL/ToL	0.12	0.09	0.11	0.12	0.11	0.13
HL/SVL	0.05	0.04	0.05	0.05	0.05	0.05
HL/HW	1.47	1.17	1.39	1.29	1.48	1.38
HW/SVL	0.04	0.04	0.03	0.04	0.03	0.04
BCI (K)	83	86	84	51	45	94

Table 3.2. Updated morphometric characteristics of wild juvenile and adult blunt-nosed vipers from Cyprus (rounded to the nearest 0.5 cm). Values which increased in 2022 are marked in bold.

Sex / Re-productive condition	**Measurement**	**n**	**Range**	**Mean ± SD**	**Coefficient of Variation (%)**
All	**SVL (cm)**	46	20.5 – **121.5**	80.89 ± 27.39	33.87
	TaL (cm)	46	3.0 – 15.0	10.05 ± 3.39	33.72
	ToL (cm)	46	23.5 – **133.5**	90.95 ± 30.27	33.28
	HL (mm)	44	16 – **56**	42.73 ± 10.68	24.99
	HW (mm)	44	10 – **46**	32.02 ± 8.88	27.72
	Weight (g)	42	10 – 1,456	608.60 ± 416.67	68.46
	TaL/SVL	46	**0.05** – 0.16	0.13 ± 0.02	18.18
	TaL/ToL	46	**0.04** – 0.14	0.11 ± 0.02	16.73
	HL/SVL	44	0.04 – 0.10	0.06 ± 0.01	20.75
	HL/HW	44	**1.17** – 1.70	1.36 ± 0.11	8.16
	HW/SVL	44	0.03 – 0.06	0.04 ± 0.01	15.35
	BCI (K)	42	66 – 146	100.71 ± 17.50	17.38
Adult males	**SVL (cm)**	16	71.0 – **121.5**	97.94 ± 17.59	17.96
	TaL (cm)	16	10.0 – 14.5	12.56 ± 1.54	12.24
	ToL (cm)	16	81.0 – **133.5**	110.50 ± 18.60	16.84
	HL (mm)	16	40 – **56**	49.56 ± 5.48	11.05
	HW (mm)	16	**29 – 46**	37.00 ± 4.82	13.02
	Weight (g)	14	**295** – 1,456	852.36 ± 386.99	45.40
	TaL/SVL	16	**0.10** – 0.16	0.13 ± 0.02	13.67
	TaL/ToL	16	**0.09** – 0.14	0.12 ± 0.01	12.20
	HL/SVL	16	**0.04** – 0.06	0.05 ± 0.00	8.54
	HL/HW	16	**1.17 – 1.50**	1.34 ± 0.09	6.38
	HW/SVL	16	0.03 – 0.04	0.04 ± 0.003	8.35
	BCI (K)	14	80 – 114	91.86 ± 9.15	9.96
Adult females	**SVL (cm)**	21	65.5 – 109.0	87.33 ± 12.52	14.33
	TaL (cm)	21	**4.5** – 15.0	10.24 ± 2.53	24.69
	ToL (cm)	21	74.5 – 124.0	97.57 ± 13.66	14.00
	HL (mm)	19	36 – **53**	45.21 ± 4.28	9.46
	HW (mm)	19	28 – 40	34.79 ± 3.72	10.69
	Weight (g)	19	279 – 1,228	685.37 ± 293.48	42.82
	TaL/SVL	21	**0.05** – 0.15	0.12 ± 0.03	22.40
	TaL/ToL	21	**0.04** – 0.13	0.11 ± 0.02	20.84
	HL/SVL	19	0.04 – 0.06	0.05 ± 0.00	6.82
	HL/HW	19	1.18 – 1.47	1.30 ± 0.07	5.71

	HW/SVL	19	0.03 – 0.04	0.04 ± 0.003	8.31
	BCI (K)	19	66 – 146	104.21 ± 20.99	20.15
Juveniles	**SVL (cm)**	9	20.5 – 59.5	35.56 ± 15.73	44.24
	TaL (cm)	9	3.0 – 8.5	5.17 ± 2.17	41.90
	ToL (cm)	9	23.5 – 68.0	40.72 ± 17.89	43.92
	HL (mm)	9	16 – 34	25.33 ± 8.19	32.31
	HW (mm)	9	10 – 24	17.33 ± 6.20	35.80
	Weight (g)	9	10 – 218	67.33 ± 71.12	105.63
	TaL/SVL	9	0.13 – 0.16	0.15 ± 0.01	4.46
	TaL/ToL	9	0.12 – 0.14	0.13 ± 0.00	3.89
	HL/SVL	9	0.06 – 0.10	0.08 ± 0.01	18.01
	HL/HW	9	1.33 – 1.70	1.48 ± 0.12	8.00
	HW/SVL	9	0.03 – 0.06	0.05 ± 0.01	14.72
	BCI (K)	9	80 – 125	107.11 ± 15.17	14.16
Adults	**SVL (cm)**	37	65.5 – **121.5**	91.92 ± 15.63	17.00
	TaL (cm)	37	**4.5** – 15.0	11.24 ± 2.43	21.60
	ToL (cm)	37	74.5 – **133.5**	103.16 ± 17.03	16.51
	HL (mm)	35	36 – **56**	47.20 ± 5.27	11.16
	HW (mm)	35	28 – **46**	35.80 ± 4.34	12.12
	Weight (g)	33	279 – 1,456	756.21 ± 341.05	45.10
	TaL/SVL	37	**0.05** – 0.16	0.12 ± 0.02	19.17
	TaL/ToL	37	**0.04** – 0.14	0.11 ± 0.02	17.68
	HL/SVL	35	0.04 – 0.06	0.05 ± 0.00	7.30
	HL/HW	35	**1.17 – 1.50**	1.32 ± 0.08	6.15
	HW/SVL	35	0.03 – 0.05	0.04 ± 0.003	8.54
	BCI (K)	33	66 – 146	98.97 ± 17.90	18.08

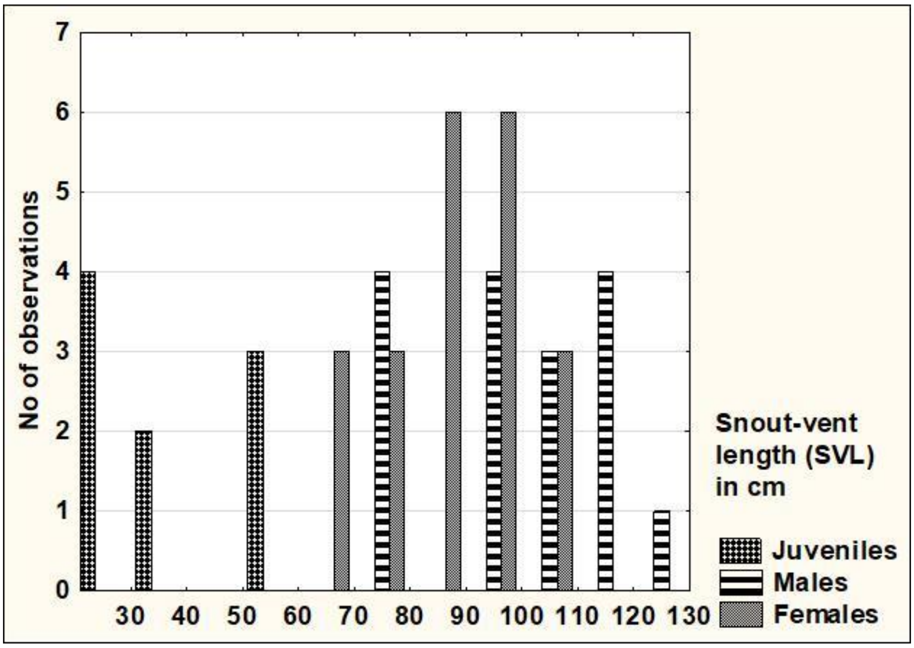

Figure 3.1. Distribution of snout-vent length (SVL) for 46 wild blunt-nosed vipers from Cyprus.

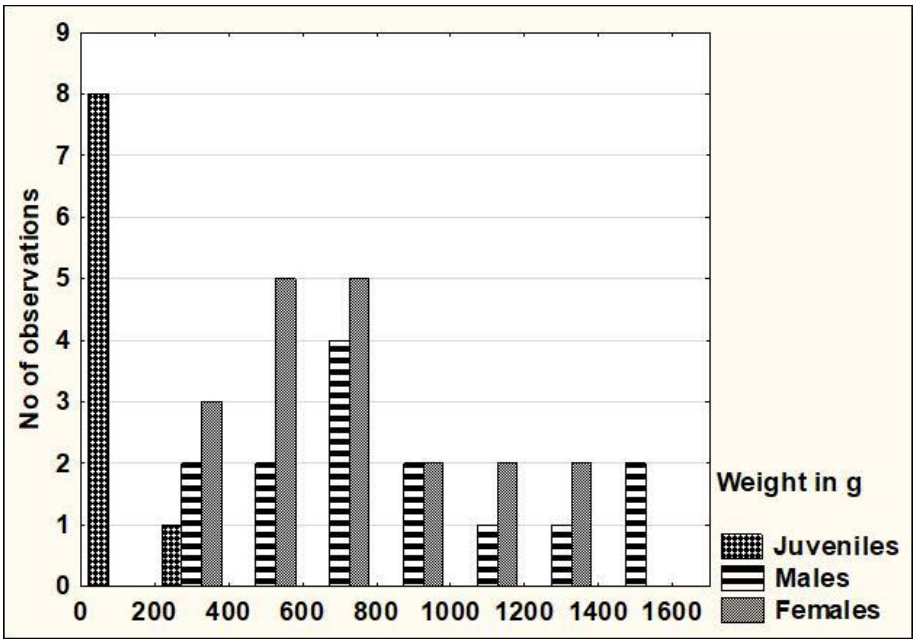

Figure 3.2. Distribution of weight for 42 wild blunt-nosed vipers from Cyprus.

For the investigation of relationships between morphometric characteristics (regression analysis), the relationship between log-transformed SVL and log-transformed weight for 42 wild blunt-nosed vipers from Cyprus (natural logarithms) can be described by $Y = -6.33 + 2.86*X$ ($R^2 = 0.99$). In contrast, the allometric relationship between SVL and weight for 42 wild blunt-nosed vipers from Cyprus is described by $Y = \exp(-6.33)* X^{2.86}$ ($R^2 = 0.93$). **Figure 3.3** shows the relationship between natural logarithms of SVL and natural logarithms of weight for all examined (i.e., freshly weighed) wild vipers, together with a fitted regression line. The coefficient of determination R^2 is 0.99. In adult females, the logarithmic relationship between weight and SVL was much weaker ($R^2 = 0.77$) than in juveniles and adult males ($R^2 = 0.99$ and $R^2 = 0.98$ respectively) (**Table 3.3**). In contrast, **Figure 3.4** shows the allometric relationship between SVL and weight with a fitted exponential function based on the linear regression approach.

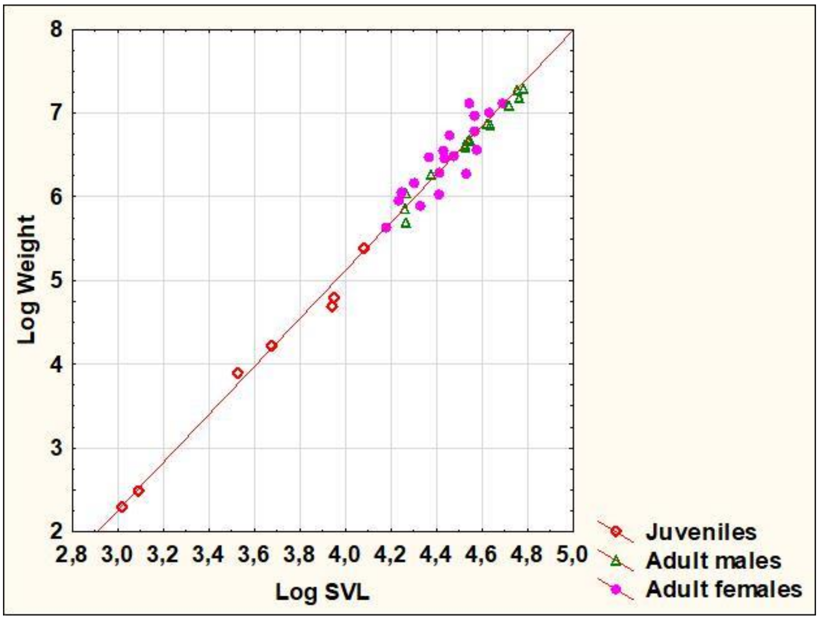

Figure 3.3. Relationship between log-transformed SVL and log-transformed weight (natural logarithms) in 42 blunt-nosed vipers from Cyprus: $Y = -6.33 + 2.86*X$. $R^2 = 0.99$

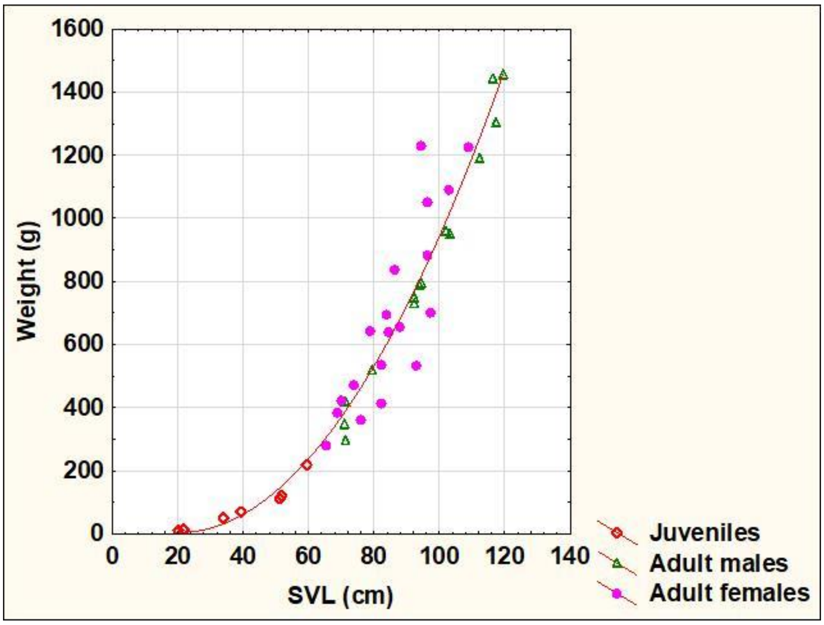

Figure 3.4. Allometric relationship between SVL and weight in 42 blunt-nosed vipers from Cyprus: $Y = \exp(-6.33)* X^{2.86}$

Table 3.3. Sample sizes, parameter and coefficient of determination (R^2) of the regression "logW = loga + b*logSVL" of blunt-nosed vipers from Cyprus (2018 vs 2022, natural logarithms). Also added is R^2 (left) of the linearized regression "Y = exp(-6.33)* $X^{2.86}$"

			SVL^3 vs Weight	**logSVL vs logWeight**		
	Year	**N**	**R^2**	**R^2**	**a**	**b**
SVL vs weight (all)	**2022**	**42**	**0.93**	**0.99**	**-6.33**	**2.86**
	2018	34	0.80	0.99	-6.32	2.86
SVL vs weight (Males)	**2022**	**14**	**0.98**	**0.98**	**-5.67**	**2.71**
	2018	10	0.99	0.99	-4.96	2.56
SVL vs weight (Females)	**2022**	**19**	**0.74**	**0.77**	**-5.55**	**2.70**
	2018	16	0.73	0.76	-5.23	2.62
SVL vs weight (Juveniles)	**2022**	**9**	**0.97**	**0.99**	**-6.03**	**2.77**
	2018	8	0.97	0.99	-6.00	2.75
SVL vs weight (Adult)	**2022**	**33**	**0.87**	**0.87**	**-5.29**	**2.63**
	2018	-	-	-	-	-

Adult females also showed the weakest relationship between SVL and TaL (R = 0.37), SVL and HL (R = 0.88), SVL and HW (R = 0.75), between HL and HW (R = 0.84), and between SVL and BCI (R = 0.18) (**Table 3.4**). For females, the correlation between SVL and TaL (*P* = 0.095) and between SVL and BCI (*P* = 0.456) was not significant. The significance level for all correlations was 0.05.

Table 3.4. Correlation coefficients (R) between morphometric characteristics of blunt-nosed vipers from Cyprus (significant *P* values bold): 2018 vs 2022.

	Year	**N**	**R**	***P*correlation**
SVL vs TaL (all)	**2022**	**46**	**0.83**	**0.000**
	2018	33	0.93	**0.000**
SVL vs TaL (Males)	**2022**	**16**	**0.64**	**0.008**
	2018	10	0.89	**0.001**
SVL vs TaL (Females)	**2022**	**21**	**0.37**	0.095
	2018	15	0.58	**0.024**
SVL vs TaL (Juveniles)	**2022**	**9**	**0.995**	**0.000**
	2018	8	0.997	**0.000**
SVL vs TaL (Adults)	**2022**	**37**	**0.53**	**0.001**
	2018	-	-	-
	Year	**N**	**R**	***P*correlation**
SVL vs HL (all)	**2022**	**44**	**0.97**	**0.000**
	2018	32	0.97	**0.000**

SVL vs HL (Males)	**2022**	**16**	**0.94**	**0.000**
	2018	10	0.97	**0.000**
SVL vs HL (Females)	**2022**	**19**	**0.88**	**0.000**
	2018	14	0.85	**0.000**
SVL vs HL (Juveniles)	**2022**	**9**	**0.897**	**0.001**
	2018	8	0.896	**0.003**
SVL vs HL (Adults)	**2022**	**35**	**0.92**	**0.000**
	2018	-	-	-
	Year	**N**	**R**	***P*correlation**
SVL vs HW (all)	**2022**	**44**	**0.96**	**0.000**
	2018	32	0.96	**0.000**
SVL vs HW (Males)	**2022**	**16**	**0.93**	**0.000**
	2018	10	0.93	**0.000**
SVL vs HW (Females)	**2022**	**19**	**0.75**	**0.000**
	2018	14	0.72	**0.004**
SVL vs HW (Juveniles)	**2022**	**9**	**0.94**	**0.000**
	2018	8	0.93	**0.001**
SVL vs HW (Adults)	**2022**	**35**	**0.86**	**0.000**
	2018	-	-	-
	Year	**N**	**R**	***P*correlation**
HL vs HW (all)	**2022**	**44**	**0.97**	**0.000**
	2018	32	0.98	**0.000**
HL vs HW (Males)	**2022**	**16**	**0.85**	**0.000**
	2018	10	0.93	**0.000**
HL vs HW (Females)	**2022**	**19**	**0.84**	**0.000**
	2018	14	0.83	**0.000**
HL vs HW (Juveniles)	**2022**	**9**	**0.98**	**0.000**
	2018	8	0.98	**0.000**
HL vs HW (Adults)	**2022**	**35**	**0.85**	**0.000**
	2018	-	-	-
	Year	**N**	**R**	***P*correlation**
SVL vs BCI (all)	**2022**	**42**	**0.39**	**0.011**
	2018	34	0.40	**0.018**
SVL vs BCI (Males)	**2022**	**14**	**0.60**	**0.023**
	2018	10	0.86	**0.001**
SVL vs BCI (Females)	**2022**	**19**	**0.18**	0.456
	2018	16	0.21	0.429
SVL vs BCI (Juveniles)	**2022**	**9**	**0.73**	**0.025**
	2018	8	0.74	**0.035**
SVL vs BCI (Adults)	**2022**	**33**	**0.35**	**0.045**
	2018	-	-	-

According to the unpaired *t*-test, adult males were significantly longer in SVL (*P* = 0.039), TaL (*P* = 0.003) and ToL (*P* = 0.0199) than females and showed a significantly lower BCI (*P* = 0.048) than the other sex. As Levene's test was significant for BCI, the variable was tested again with separate variance estimates, and the resulting, significant *P* value was equal to *P* in the *t*-test applied before (0.0483). No significant sex-specific differences were found for HW, relative head length (HL/SVL), relative head width (HW/SVL) and weight (**Table 3.5**).

Table 3.5. Results of the *t*-test for comparison of normally distributed morphometric characteristics of male (M) and female (F) blunt-nosed vipers from Cyprus (significant *P* values bold).

Variable/ Characteristic	**Year**	**N males**	**N females**	***P***	**Results of 2018 vs 2022**
SVL (cm)	2022	16	21	**0.0390**	**2022: significant**
SVL (cm)	2018	10	16	0.0603	2018: not significant
TaL (cm)	2022	16	21	**0.0026**	**2022: significant**
TaL (cm)	2018	10	15	**0.0049**	**2018: significant**
ToL (cm)	2022	16	21	**0.0199**	**2022: significant**
ToL (cm)	2018	10	14	**0.047**	**2018: significant**
HL (mm)	2022	-	-	**-**	**Not normally distributed in 2022: significant MWU test**
HL (mm)	2018	10	14	**0.0159**	**Normally distributed in 2018: significant *t*-test**
HW (mm)	2022	16	19	0.1352	2022: not significant
HW (mm)	2018	10	14	0.0868	2018: not significant
HL/SVL	2022	16	19	0.5775	2022: not significant
HL/SVL	2018	10	14	0.5179	2018: not significant
HL/HW	2022	16	19	0.1339	2022: not significant
HL/HW	2018	10	14	0.4488	2018: not significant
HW/SVL	2022	16	19	0.1081	2022: not significant
HW/SVL	2018	-	-	-	-
Weight (g)	2022	14	19	0.1680	2022: not significant
Weight (g)	2018	10	16	0.0968	2018: not significant
BCI (K)	2022	14	19	**0.0483**	**2022: significant**
BCI (K)	2018	10	16	0.2163	2018: not significant

The non-normally distributed variables HL (males), TaL/SVL (females) and TaL/ToL (females) were examined with the MWU test (**Table 3.6**), which showed a significant sex-specific difference for HL (*P* = 0.011), but not for the ratios TaL/SVL (*P* = 0.195) and TaL/ToL (*P* = 0.195). These results indicate that the tested adult females had shorter head lengths but did not exceed the adult males in relative tail length.

Table 3.6. Results of the Mann-Whitney U test for comparison of not normally distributed morphometric characteristics of blunt-nosed vipers from Cyprus (2018 vs 2022, significant *P* values bold).

Variable/ Characteristic	Year	N males	N females	exact *P*	Results of 2018 vs 2022
HL	2022	16	19	**0.0111**	**Not normally distributed in 2022: significant MWU test**
HL	2018	-	-	-	**Normally distributed in 2018: significant *t*-test**
TaL/SVL	2022	16	21	0.1951	2022: not significant
TaL/SVL	2018	10	15	0.285	2018: not significant
TaL/ToL	2022	16	21	0.1951	2022: not significant
TaL/ToL	2018	10	15	0.285	2018: not significant

Within adult males, the mean BCI was significantly lower in the six largest individuals (SVL ≥ 100 cm) than in the eight smaller ones with SVL < 100 cm (*P* = 0.026) (**Table 3.7**).

Table 3.7. Results of the *t*-test for comparing the mean BCI of large (SVL >= 100 cm) and small to medium-sized (SVL < 100 cm) wild adult males of *M. l. lebetina* (significant *P* value bold).

Variable/ Character	N males SVL < 100 cm	Mean BCI for 1st group	N males SVL >= 100 cm	Mean BCI for 2nd group	*P*	*P* Levene's test
BCI (K)	8	**96.4**	6	**85.8**	**0.0258**	0.3073

Furthermore, the mean ratio TaL/SVL was significantly lower in the eight largest individuals (SVL ≥ 100 cm) than in the eight smaller ones with SVL < 100 cm (*P* = 0.003) (**Table 3.8**).

Table 3.8. Results of the *t*-test for comparing the mean ratio TaL/SVL of large (SVL >= 100 cm) and small to medium-sized (SVL < 100 cm) wild adult males of *M. l. lebetina* (significant *P* value bold).

Variable/ Character	N males SVL < 100 cm	Mean TaL/SVL for 1st group	N males SVL >= 100 cm	Mean TaL/SVL for 2nd group	*P*	*P* Levene's test
TaL/SVL	8	**0.143**	8	**0.119**	**0.0031**	0.1547

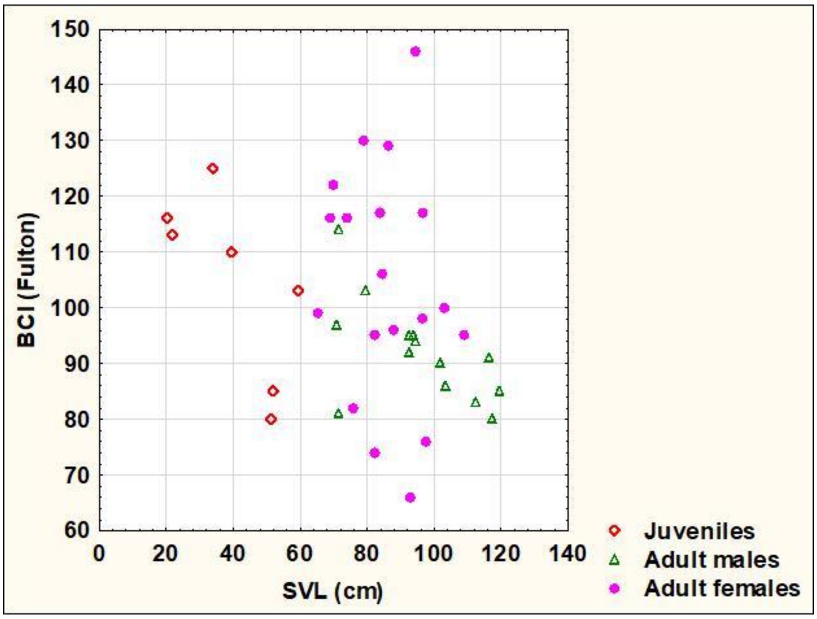

Figure 3.5. Relationship between SVL and BCI in 42 blunt-nosed vipers from Cyprus: R = 0.39

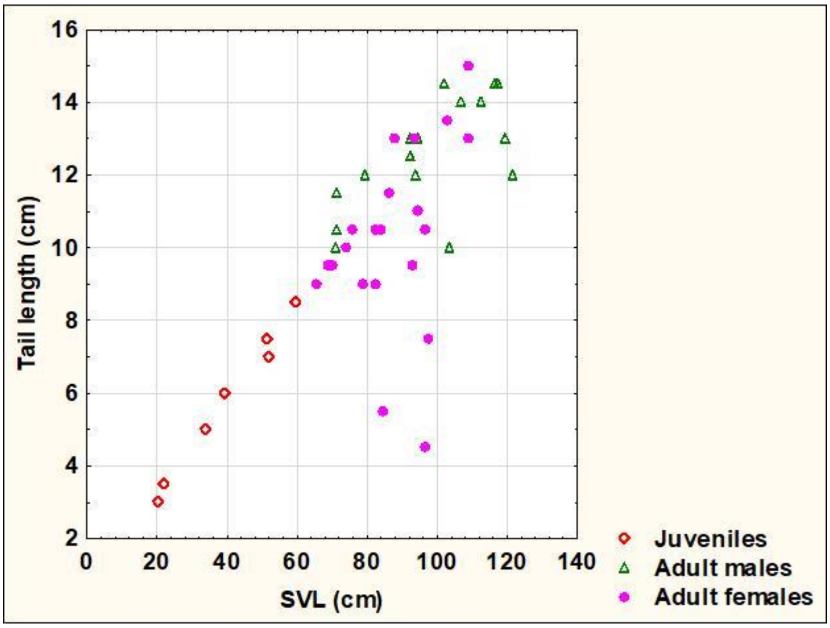

Figure 3.6. Relationship between SVL and TaL in 46 blunt-nosed vipers from Cyprus: R = 0.83

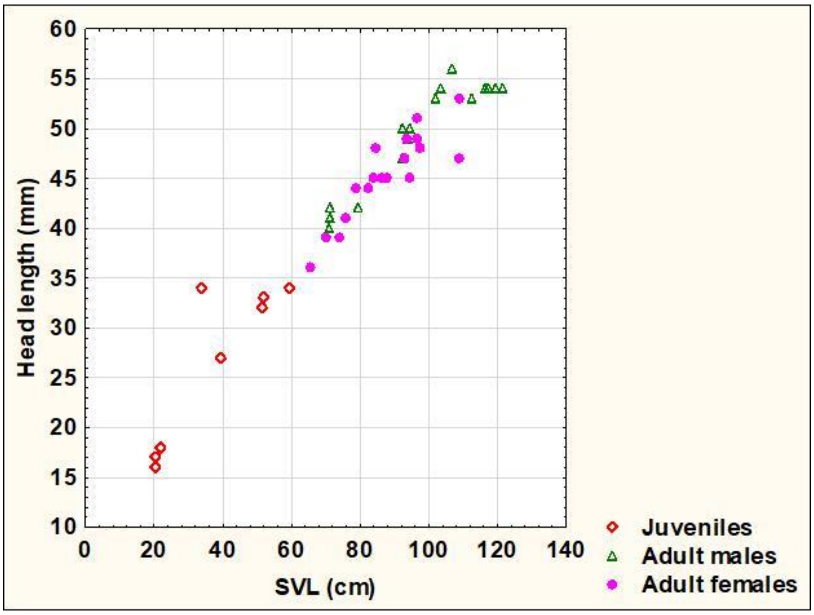

Figure 3.7. Relationship between SVL and HL in 44 blunt-nosed vipers from Cyprus: R = 0.97

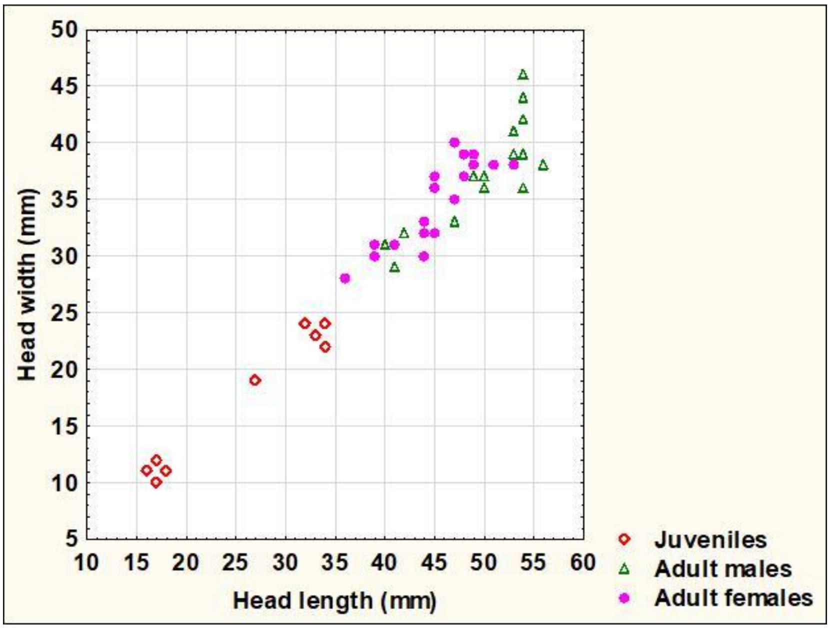

Figure 3.8. Relationship between HL and HW in 44 blunt-nosed vipers from Cyprus: R = 0.97

3.4 Discussion

3.4.1 Change in sex ratio and allometric length-weight relationship with increasing dataset

The overall dataset increased from 34 (Jestrzemski & Kuzyakova 2018) to 46 individuals, including 16 adult males (2018: 10 males), 21 adult females (2018: 16 females) and nine juveniles (2018: eight juveniles). Accordingly, from 2018 to 2022, the male-female ratio increased from 62.5% (10:16) to 76.2% (16:21) for the variables SVL, TaL, ToL, TaL/SVL and TaL/ToL, from 71.4% (10:14) to 84.2% (16:19) for HL, HW, HL/SVL and HL/HW, and from 62.5% (10:16) to 73.7% (14:19) for weight and BCI. Hence, with its larger size (an increase by 35%) and more balanced sex ratio, the new dataset is more suitable for investigating sex-specific differences in *M. l. lebetina*. The updated allometric relationship between SVL and weight in *M. l. lebetina* (n = 42) is modeled as an exponential curve fitted to the scatterplot of SVL vs. weight (**Figure 3.4**). The new equation (2022: $Y = \exp(-6.33)*X^{2.86}$) is very similar to the previous one (2018: $Y = \exp(-6.32)*X^{2.86}$). Thus, a very large blunt-nosed viper (135 cm SVL) would weigh 2,239 g, whereas based on the 2018 equation, its weight would amount to 2,227 g.

3.4.2 Correlation analysis

The results of the correlation analysis of the new dataset show that, like in Jestrzemski & Kuzyakova (2018), the relationship between morphometric variables was weaker in adult females than in adult males and juveniles. While the correlation coefficients (R) for SVL vs HW (0.75), HL vs HW (0.84) and HL vs BCI (0.18) from 2022 were similar to the results of 2018 (R = 0.72, R = 0.83 and R = 0.21, respectively), they were much lower for SVL vs TaL, with R = 0.37 in 2022 (Pcorrelation = 0.095), as compared to R = 0.58 in 2018 (Pcorrelation = 0.024). Consequently, there was no linear relationship between SVL and the variables TaL and BCI in adult *M. l. lebetina* females (2022), which indicates that the ratio between TaL and SVL is an unsuitable indicator for sex determination in adult *M. l. lebetina*.

3.4.3 Sex-specific differences

Both in the enlarged dataset (2022) and in the old dataset (Jestrzemski & Kuzyakova 2018), significant sex-specific differences could be found for the normally distributed variables TaL and ToL, while in the 2022 dataset additionally the normally distributed variables SVL (P = 0.039) and BCI (P = 0.048) differed significantly between the sexes. Furthermore, HL differed significantly between the sexes again (P = 0.011), although this variable was distributed non-normally in the 2022 dataset, and thus tested with the MWU test (exact P = 0.011). Since the sex-specific differences for SVL were already relatively high in 2018 (but not significant, as P = 0.060 then), it is rather unsurprising that they were significant in the enlarged dataset (2022), with the difference between the old and new P value being 0.021. Therefore, the hypothesis that adult males exceed adult females in mean SVL can be confirmed. While the 2018 dataset hinted at possible sex-specific differences for HW (P = 0.087) and weight (P = 0.097), the enlarged dataset (2022) did not provide any more hints towards such differences, as the *P* values increased (*P* = 0.135 and *P* = 0.168, respectively). As the differences in the ratios HL/SVL (*P* = 0.578 in 2022) and HL/HW (*P* = 0.134 in 2022) remained insignificant between both sexes, relative head length and relative head width can also be excluded as a reliable indicator for sex determination in *M. l. lebetina*. Equally, the non-normally distributed ratios TaL/SVL (Exact *P* = 0.195 in 2022) and TaL/ToL (Exact *P* = 0.195 in 2022) did not differ significantly between the sexes, which also shows the unsuitability of relative tail length as criterion for *M. l. lebetina* sex determination. Hence, like in Jestrzemski & Kuzyakova (2018), the hypothesis that the ratio TaL/SVL is smaller in adult males than in adult females could not be confirmed.

3.4.4 Morphometric comparison of Cypriot *M. l. lebetina* with Iranian *M. l. obtusa, M. l. cernovi* and *M. razii*

The comprehensive morphological analysis of the genus *Macrovipera* in Iran, published by Oraie at el. (2018), enables a direct comparison between selected morphometric characters (mean values of SVL, TaL/SVL, HL/SVL and HW/SVL) of *M. l. lebetina* (this study) and those of Iranian *M. l. obtusa*, *M. l. cernovi* and of the newly described, Iranian endemic blunt-nosed viper species *M. razii* (**Table 3.9**). The mean SVL values of *M. l. lebetina* (87 cm in females and 98 cm in males) were lower than those of *M. l. cernovi*, *M. l. obtusa* and *M. razii*. For females, *M. l. cernovi* showed the highest mean SVL (105 cm), followed by *M. l. obtusa* (96 cm) and *M. razii* (92 cm), while the highest mean SVL values for males were found in *M. razii* (108 cm), *M. l. cernovi* (107 cm) and *M. l. obtusa* (101 cm). For all examined subspecies datasets, the mean SVL was constantly higher in males than in females. The mean relative tail length (TaL/SVL) was slightly lower in *M. l. lebetina* females (12%) than in *M. l. cernovi* (14%), *M. l. obtusa* (13%) and *M. razii* (13%) females. However, it was almost equal in males, with 13% in *M. l. lebetina*, compared to *M. l. cernovi* (14%), *M. l. obtusa* (14%) and *M. razii* (13%). Equally, the mean ratio HL/SVL was similar between the subspecies, with each 5% in *M. l. lebetina* females and males, each 4% in *M. l. obtusa* females and males, and 5% (4%) both in *M. l. cernovi* and *M. razii* females and males. With 4% in both sexes, *M. l. lebetina* had a higher mean ratio HW/SVL than the Iranian *Macrovipera* taxa (each 3% for both females and males). These findings indicate that Cypriot blunt-nosed vipers grow to smaller body lengths than their close relatives from the Eurasian mainland [see 4], while their heads are likely broader in relation to their body size.

Table 3.9. Comparison of selected morphometric characteristics (mean values of SVL, TaL/SVL, HL/SVL, HW/SVL) of 37 wild adult *M. l. lebetina* (this study), with those of alcohol-preserved blunt-nosed vipers from Iran (*M. l. obtusa*: N = 45; *M. l. cernovi*: N = 37; *M. razii*: N = 27) [10]. For simplification, all SVL minimum and maximum values were rounded to the nearest 0.5 cm.

M. l. lebetina **(Cyprus, measured in 2014, 2015, 2017 and 2022)**

	Sex	N	SVL (cm)	TaL/SVL	N	HL/SVL	HW/SVL
Mean± SD	**F**	**21**	**87.33** ± 12.52	**0.12** ± 0.03	**19**	**0.05** ± 0.00	**0.04** ± 0.003
Min-Max	**F**	**21**	65.5 – 109.0	0.05 – 0.15	**19**	0.04 – 0.06	0.03 – 0.04
Mean± SD	**M**	**16**	**97.94** ± 17.59	**0.13** ± 0.02	**16**	**0.05** ± 0.00	**0.04** ± 0.003
Min-Max	**M**	**16**	71.0 – 121.5	0.10 – 0.16	**19**	0.04 – 0.06	0.03 – 0.04

M. l. obtusa **(Iran)**

	Sex	N	SVL (cm)	TaL/SVL	HL/SVL	HW/SVL
Mean ± SD	**F**	**30**	**96.17** ± 17.14	**0.13** ± 0.01	**0.04** ± 0.01	**0.03** ± 0.004
Min-Max	**F**	**30**	66.0 – 137.0	0.10 – 0.15	0.02 – 0.05	0.02 – 0.04
Mean ± SD	**M**	**15**	**101.30** ± 18.74	**0.14** ± 0.09	**0.04** ± 0.003	**0.03** ± 0.003
Min-Max	**M**	**15**	72.0 – 126.0	0.12 – 0.16	0.04 – 0.05	0.02 – 0.04

M. l. cernovi **(Iran)**

	Sex	N	SVL (cm)	TaL/SVL	HL/SVL	HW/SVL
Mean ± SD	**F**	**22**	**105.25** ± 13.77	**0.14** ± 0.02	**0.05** ± 0.002	**0.03** ± 0.003
Min-Max	**F**	**22**	77.0 – 127.0	0.12 – 0.18	0.04 – 0.05	0.03 – 0.04
Mean ± SD	**M**	**15**	**106.53** ± 19.23	**0.14** ± 0.01	**0.04** ± 0.004	**0.03** ± 0.01
Min-Max	**M**	**15**	69.5 – 129.0	0.12 – 0.16	0.03 – 0.05	0.02 – 0.04

M. razii **(Iran)**

	Sex	N	SVL (cm)	TaL/SVL	HL/SVL	HW/SVL
Mean ± SD	**F**	**13**	**92.11** ± 17.73	**0.13** ± 0.01	**0.05** ± 0.003	**0.03** ± 0.002
Min-Max	**F**	**13**	55.0 – 115.5	0.11 – 0.16	0.04 – 0.05	0.02 – 0.03
Mean ± SD	**M**	**14**	**108.14** ± 18.51	**0.13** ± 0.02	**0.04** ± 0.003	**0.03** ± 0.004
Min-Max	**M**	**14**	66.0 – 136.0	0.10 – 0.16	0.04 – 0.05	0.02 – 0.04

3.4.5 Limitations of the morphometric comparison between Cypriot and Iranian blunt-nosed vipers

Since the Iranian specimens listed by Oraie et al. (2018) range from 55 cm (*M. razii*) to 137 cm SVL (*M. l. obtusa*) [10], one can assume that only specimens considered adult were included in the list. If so, it is yet unclear which minimum length the authors set for the status “adult”. Additionally, it is not clear whether the Iranian specimens were collected from the wild, or also from captivity. Furthermore, one has to consider that all Cypriot *M. l. lebetina* were measured to the nearest 0.5 cm (SVL, TaL, ToL), and to the nearest 1 mm (HL, HW), respectively, whereas for all Iranian *M. l. obtusa*, *M. l. cernovi* and *M. razii*, morphometric characters were measured to the nearest 0.01 mm (SVL, TaL, HL, HW, head height (HH), snout length (SL), and distances between selected facial scales). Consequently, the rounded values from the Cypriot subspecies are less precise, with a higher deviation from the actual metric values than for the Iranian taxa.

3.4.6 Decreasing body condition with age in *M. l. lebetina* and *Daboia palaestinae*

Like in Jestrzemski & Kuzyakova (2018), the differences between small to medium-sized and large-sized male *M. l. lebetina* (**Table 3.7**) were significant in the enlarged dataset ($P = 0.0258$), thus confirming my hypothesis that the mean BCI of large adult males is lower compared to smaller and medium-sized adult males. This trend hints at a decreasing body condition index due to deteriorating health with age in wild Cypriot blunt-nosed vipers. The same trend (decreasing BCI with increasing SVL) was also observed in *Daboia palaestinae,* another large, medically highly important viperine species native to the Eastern Mediterranean (**Table 3.10**). Notably, with a much larger dataset being available for *D. palaestinae* (186 adult males, as compared to the 14 wild males of *M. l. lebetina* for which weight data could be obtained), the differences between both SVL subgroups were even more significant ($P = 0.0048$) in the Palestine vipers. For the test, the same definition of “large” as used for *M. l. lebetina* (SVL >= 2/3 of the maximum ToL stated in literature), was applied for *D. palaestinae*. Thus, with a maximum ToL of 136 cm [11], the threshold SVL value for *D. palaestinae* was 90.5 cm. Interestingly, the mean BCI values were generally much lower for male *D. palaestinae* than for male *M. l. lebetina* (e.g., 66 vs 96 in group 1, and 59 vs 86 in group 2), which reflects the morphological variation in large viperine species of the Middle East.

Table 3.10. Results of the unpaired *t*-test for comparing the mean BCI of 107 small to medium-sized adult males of *D. palaestinae* from Israel (71.0 cm >= SVL < 90.5 cm) with 79 large (SVL >= 90.5 cm) adult males (significant *P* value bold). Dataset provided by S. Meiri (pers. comm., 19 November 2022).

Variable/ Character	**N males SVL 71-90.5 cm**	**Mean for 1st group**	**N males SVL >= 90.5 cm**	**Mean for 2nd group**	***P***	***P* Levene's test**
BCI (K)	107	**66**	79	**59**	**0.0048**	0.2206

3.4.7 Decreasing relative tail length (TaL/SVL) with age in *M. l. lebetina* and *D. palaestinae*

Relative tail length in snakes is commonly higher in males, which is unsurprising, as the male tail serves for the accommodation of the hemipenes and retractor muscles [12]. Thus, the importance of the tail for the male reproductive system may implicate that tail growth increases when males reach sexual maturity, since they start participating in courtship and mating, and thus need fully developed reproductive organs. One may further assume that, once the development of the reproductive system is completed, reproductive organ growth and thus also tail growth will decrease. These assumptions may explain the significantly higher relative tail length in smaller (younger) males of *M. l. lebetina* as compared to larger (older) individuals. However, Jadin & King (2012) investigated the ontogenetic effects on the hemipenial morphology in captive-bred garter snakes (*Thamnophis radix*) by comparing nine juveniles with 12 adults and found hemipenial length to be strongly correlated with tail length (r = 0.93), without indication of accelerated growth periods in young adult males. Since only few ontogenetic studies of hemipenial morphology exist, the correlation between reproductive system development and relative tail length in male snakes remains elusive for most species [13]. This problem could be elucidated by an in-depth investigation of relative tail length in male blunt-nosed vipers, based on a larger dataset including individuals of all life stages. Another approach is that longer tails in male snakes may be of advantage during mating, when males align their bodies with that of the female, stimulate the female cloaca with their tails, and try to insert a hemipenis. When several males court a female in this way, they often try to push aside and displace the tails of their rivals [12, 14]. In this regard, relatively longer tails may be of advantage for younger (smaller) males to increase their chances against older (larger) competitors. Yet, tail length may be of less importance in species exhibiting male combat (e.g., *M. l. lebetina*), where only a single male copulates with a given female and thus no tail wrestling will occur [see 12]. Furthermore, even in the red-sided garter snake (*Thamnophis sirtalis*), a species exhibiting mating aggregations and tail wresting, younger adult males had smaller hemipenes and obtained less matings than their larger counterparts [15], which hints at SVL as a much more important factor for male mating success than increased tail growth periods during early adulthood. Anyway, the trend of relatively longer tails in younger males was also confirmed by a dataset of 186 males of *D. palaestinae*, where mean relative tail length was significantly higher in younger adult males (**Table 3.11**).

Table 3.11. Results of the MWU test for comparing the mean ratio TaL/SVL between 107 small to medium-sized adult males of *D. palaestinae* from Israel (71.0 cm >= SVL < 90.5 cm) and 79 large (SVL >= 90.5 cm) adult males (significant *P* value bold). Dataset provided by S. Meiri (pers. comm., 19 November 2022).

Variable/ Character	N males SVL 71-90.5 cm	Mean for 1st group	N males SVL >= 90.5 cm	Mean for 2nd group	U	*P*
TaL/SVL	107	0.142	79	0.137	2804	**0.0001**

3.5 Conclusions

With a larger sample size and a more balanced sex ratio, the new dataset is more suitable for investigating sex-specific differences in *M. l. lebetina*. Although these test results will inevitably further change with increasing sample size, the comparison between the 2018 and 2022 datasets implicates that variables which are significantly different between the sexes, with *P* values much below 0.05, can be considered as relatively solid indicators for sex determination, as they are likely to remain significant also in larger samples. The significant sex-specific differences for the variables SVL, TaL, ToL, HL and BCI indicate that these morphometric characters generally seem to differ between *M. l. lebetina* males and females, and thus should be further investigated for their application in sex determination. Thus, while we can conclude that adult males exceed adult females in mean SVL (Hypothesis 1), there seem to be no sex-specific differences for the mean ratio TaL/SVL (Hypothesis 2), nor for the mean ratios TaL/ToL, HL/SVL and HL/HW. Likewise, there appears to be no linear relationship between SVL and TaL in adult females. Therefore, relative head length, relative head width and relative tail length can be considered as unsuitable indicators for sex determination in *M. l. lebetina*. The lower mean BCI of large adult males as compared to smaller ones (Hypothesis 3) hints at a generally worsening body condition in male blunt-nosed vipers with older age. While the observed higher mean relative tail length in smaller adult males (Hypothesis 4) may implicate a higher sexual selective pressure or increased tail growth during this life stage of *M. l. lebetina*, this aspect is yet elusive and requires in-depth research. The observation of a higher mean BCI and higher mean relative tail length in smaller adult males of *D. palaestinae* (as compared to larger males) implicates that this trend may affect several viper species.

3.6 Abbreviations

BCI (K): Fulton’s body condition index; cm: Centimeters; CV: Coefficient of variation; g: Grams; HL/HW: Ratio of head length to head width; HL/SVL: Ratio of head length to snout-vent length; HL: Head length; HW: Head width; kg: Kilograms; m: Meters; mm: Millimeters of length; n: Sample size; *P*: Probability value; R: Correlation coefficient; R^2 : Coefficient of determination; SD: Standard deviation; SVL: Snout-vent length; TaL/SVL: Ratio of tail length to snout-vent length; TaL/ToL: Ratio of tail length to total length; TaL: Tail length; ToL: Total length; W: Weight in grams.

3.1 References Chapter 3

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Chapter 4

Comparative Venom Proteomics of Iranian, *Macrovipera lebetina cernovi*, and Cypriot, *Macrovipera lebetina lebetina*, Giant Vipers

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Published in: Toxins, 2022, 14(10), 716
https://doi.org/10.3390/toxins14100716
Chapter version: as published in Toxins, with only formal corrections.

Abstract Chapter 4

Envenoming by *Macrovipera lebetina* ssp.[14] causes severe life-threatening difficulties for people living in North Africa and the Middle East. To better understand the pathophysiology of envenoming and improve patient management, knowledge about the venom components of the subspecies is essential. Here, the venom proteomes of *Macrovipera lebetina lebetina* from Cyprus and *Macrovipera lebetina cernovi* from Iran were characterized using RP-HPLC separation of the crude venom proteins, SDS-PAGE of fractionated proteins, and LC-MS/MS of peptides obtained from in-gel tryptic digestion of protein bands. Moreover, we also used high-resolution shot-gun proteomics to gain more reliable identification, where the whole venom proteomes were subjected directly to in-solution digestion before LC-HR-MS/MS. The data revealed that both venoms consisted of at least 18 protein families, of which snake venom Zn2+-dependent metalloprotease (SVMP), serine protease, disintegrin, phospholipase A2, C-type lectin-like, and L-amino acid oxidase together accounted for more than 80% of the venoms' protein contents. Although the two viper venoms shared mostly similar protein classes, the relative occurrences of these toxins were different in each snake subspecies. For instance, the P-I class of SVMP toxins was found to be more abundant than the P-III class[14] in the venoms of *M. l. cernovi* compared to *M. l. lebetina*, which gives hint at a more potent myonecrotic effect and minor systemic hemorrhage following envenoming by *M. l. cernovi* than *M. l. lebetina*. Moreover, single-shot proteomics also revealed many proteins with low abundance (<1%) within the venoms, such as aminopeptidase, hyaluronidase, glutaminyl-peptide, cyclotransferase, cystatin, phospholipase B, and vascular endothelial growth factor. Our study extends the in-depth understanding of the venom complexity of *M. lebetina* ssp.,[14] particularly regarding toxin families associated with envenoming pathogenesis and those hard-detected protein classes expressed in trace amounts.

Keywords: *Macrovipera lebetina cernovi*, *Macrovipera lebetina lebetina,* venom, proteomics, mass spectrometry, Iran, Cyprus.

Key Contribution: The study describes the first comparative characterization of the venom composition from the two subspecies *M. l. lebetina* and *M. l. cernovi*, using two different proteomic approaches and revealing the presence of a large quantity of SVMP, PLA2, SVSP, CTL-like and DISI toxins, most likely playing a key role in envenoming of medical importance. The overall toxin profiles deliver a molecular aspect for understanding the clinical

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consequences following *M. lebetina* envenomings, such as local pain, edema, necrosis, and systemic manifestations, including hemorrhage, hypotension, coagulopathy and thrombocytopenia.

4.1 Introduction

Venomous snakes, belonging to the family Viperidae (true vipers and pit vipers), include the most medically relevant species which cause the majority of snakebite envenomings and fatalities in humans and their domestic animals [1]. Venoms produced by these snakes contain potent toxins that play a crucial role in the pathophysiology of victims of envenoming. The clinically most relevant effects of viperid bites are[15] usually complex, involving local tissue damage such as edema and blistering, dermo- and myonecrosis, and systemic alterations like hemorrhage, coagulopathy, cardiovascular disturbances, and renal damage [1].

Members of the family Viperidae are distributed across Europe, Africa, and Asia, including the genera *Cerastes, Daboia*, *Eristicophis, Macrovipera, Montivipera, Pseudocerastes,* and *Vipera* [2]. The genus *Macrovipera* (giant Palearctic vipers) comprises[15] three species named *M. schweizeri* (found on the Greek islands of the Cyclades Archipelago), *M. razii* (native to Southern and Central Iran), and *M. lebetina* (widely distributed from Eastern Europe to Central Asia and the Middle East) [3]. *Macrovipera lebetina* is further divided into the five subspecies *M. l. cernovi* (Iran, Afghanistan, Pakistan, India, Turkmenistan, and Uzbekistan), *M. l. obtusa* (Turkey, Russia, Syria, Iran, Iraq, Armenia, Azerbaijan, Russia, Georgia, Lebanon, Kazakhstan, and Jordan), *M. l. turanica* (Turkmenistan, Tajikistan, and Uzbekistan), *M. l. transmediterranea* (Tunisia and Algeria), and *M. l. lebetina* (Cyprus) [4,5]. They are responsible for a significant number of envenomings[15] annually in Africa and Western Asia. Since they can deliver significant amounts of venom, they have also gained a more dangerous reputation. Envenoming by *M. lebetina* ssp.[15] can cause severe life-threatening complications, e.g., haemorrhage, inflammation, dermo- or myonecrosis, merged with long-term musculoskeletal disabilities [6–8]. Furthermore, venoms of all subspecies have shown extremely potent procoagulant activity on human plasma by activating Factor X and V enzymes of the coagulation cascade [9,10]. However, information is insufficient regarding their detailed venom compositions and clinical manifestations after envenoming caused by most of these widely distributed giant vipers.

So far, proteomic studies have provided considerable information on the proteome contents and relative abundances of toxin families in a few *M. lebetina* ssp.,[15] including Tunisian *M. l. transmediterranea* [11], Armenian *M. l. obtusa* [12], and Russian *M. l. obtusa* [13]. The results

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demonstrated apparent quantitative and qualitative differences between the venom compositions of these subspecies. However, toxin families belonging to snake venom Zn2+-dependent metalloprotease (SVMP), snake venom serine protease (SVSP), disintegrin (DISI), phospholipase A2 (PLA2), and C-type lectin-like (CTL-like) protein comprised the most dominant components of their venoms. Such potent biologically active toxins in their venoms can explain some of the leading local and systemic manifestations (e.g., edema, cutaneous necrosis, bleeding, and coagulopathies) following *M. lebetina* envenoming. Hence, much is still unknown about the venom proteome of *M. lebetina* ssp.[16]

Currently, the administration of an appropriate antivenom remains the only effective treatment for snakebite envenoming. However, antivenom efficacy is limited to snake species whose venoms were used in manufacture due to inter- and intraspecific variations of venom components [14]. These venom variations may result from, e.g., evolutionary history, climatic factors, ontogeny, or adaptation toward different prey, among others [15]. Therefore, most available antivenoms are limited to distinct geographical regions and may not effectively neutralize the venom toxins of other species and subspecies of snakes in different locales for therapeutic purposes. For this reason, knowledge of snake venom composition and related variation among conspecific populations can provide important information for predicting the likely efficacy of an existing antivenom, along with influencing the design of more effective immunizing mixtures for future antivenom production [16].

In this study, we performed comprehensive venom proteomics of two medically important *M. lebetina* subspecies, *M. l. lebetina* from Cyprus and *M. l. cernovi* from Iran (**Figure 4.1**), which have not yet been thoroughly investigated. The subspecies *M. l. lebetina* or Cypriot blunt-nosed viper is exclusively limited to Cyprus and is found all over the country mostly on agricultural fields and rocky slopes [17]. Adult blunt-nosed vipers were recorded to feed on birds (e.g., *Anthus campestris*) and mammals (e.g., *Rattus norvegicus* and *Apodemus mystacinus*) [16]. The venom of *M. l. lebetina* was found to be highly potent, with an LD50 value of 7.58 mg/kg, after intraperitoneal administration in mice [18]. The viper is the only medically important snake species in Cyprus [19], with more than fifteen annual snakebite cases recorded on average [20]. Human envenoming caused by the[16] venom of *M. l. lebetina* resulted in serious life-threating complications, such as swelling and edema at the bite site, and tissue necrosis following hypotension shock, hemorrhage, and melanoderma [21–23]. Approximately 2,800 km away from the Cypriot *M. l. lebetina*, the subspecies *M. l. cernovi* occupies a unique habitat around the Kopet Dagh mountain range [24]. The viper is found in the diverse desert and montane-

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steppe biotopes and even occurs in the mountains up to 1,500 m above sea level [25]. *Macrovipera l. cernovi* mainly feeds upon small birds (e.g., *Alectoris chukar* and *Galerida cristata*) and rodents (e.g., *Rhombomys opimus*) (personal observation), similar to its sister subspecies in Cyprus. With a massive body size and large venom glands, *M. l. cernovi* can release high volumes of bioactive venom. Human patients following snake bites by Iranian *M. l. cernovi* experienced significant systemic alterations such as a reduction in platelets and higher creatine kinase levels in serum among the local common symptoms [25].

Considering the previous studies of *M. lebetina* ssp.[17] venom, the present study aimed to unravel the proteomic details of the venom of Iranian *M. lebetina cernovi* and Cypriot *M. lebetina lebetina,* and to enhance our knowledge of the geographical variability that is potentially present in the venom of *M. lebetina*. Hopefully, the results will provide in-depth insights into the diversity of *M. lebetina* venom, influencing the design of more effective immunizing mixtures for future antivenom production and clinical application in the region.

Figure 4.1. Geographical map of the collection sites. Two female specimens of *M. l. lebetina* were used for milking, which were the F2 generation of blunt-nosed vipers originally caught nearby the cities of Paphos and Polis (Paphos district, Republic of Cyprus). In comparison, two female specimens of *M. l. cernovi* were collected and milked from Khorasan Razavi, Iran. Map provided by using the “leafletR” package [26].

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4.2 Materials and methods

4.2.1 Venoms

The venom sample of *M. l. cernovi* was sourced from two[18] adult female specimens collected from Khorasan Razavi, Iran, whereas the *M. l. lebetina* sample was collected from two[18] adult female specimens (F2 generation of blunt-nosed vipers) originally caught nearby the cities of Paphos and Polis (Paphos distinct, Cyprus). The venoms of each subspecies were pooled, lyophilized immediately and stored at −20 °C until used for further analysis. The samples were provided by SMK and SJ under permission numbers of 40,401,008 (issued by the Department of the Environment of Iran) and 02.15.001.003, 04.05.2002.005.006 (issued by the Department of Environment of Cyprus), respectively.

4.2.2 Chemicals

All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, MO, USA), and VWR chemicals (VWR international, Darmstadt, Germany). RapiGest SF surfactant was purchased from Waters (Waters Corporation, MA, USA). The mass spec grade Tryp-sin/Lys-C mix was purchased from Promega (Promega, Mannheim, Germany). Zip Tip C18 was purchased from Millipore (Bedford, MA, USA).

4.2.3 Protein concentration estimation

The protein concentrations were determined before each proteomic analysis using a standard Bradford assay (Biorad, Hercules, CA, USA), with bovine serum albumin (BSA) as a reference. Absorbance was measured spectrophotometrically at 595 nm on a BioTek Synergy 2 plate reader (BioTek, Winooski, VT, USA) with Gen5 software (version 2.01).

4.2.4 HPLC and SCS-PAGE separation

Five milligrams of crude venoms were dissolved in 500 µL of 5% acetonitrile/water (ACN/H_2O) containing 0.1% trifluoroacetic acid (TFA) and were centrifuged at 12,000× *g* at 4°C for 5 minutes (Beckman Coulter, Krefeld, Germany) to remove debris. Fifty micrograms of proteins from each sample were separated using a bioZen™ LC Column (3.6 µm, 50 × 2.1 mm, pore size of 200 Å; Intact XB-C8, Phenomenex, CA, USA) connected to a Dionex UltiMate 3000 RSLC HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic analyses were performed at a flow rate of 800 µL/minute using H_2O with

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0.1% trifluoroacetic acid (TFA) as mobile phase A and ACN with 0.1% TFA as mobile phase B. The gradient elution profile was applied as follows: isocratic (5% B) for 5 minutes (min),[19] followed by 5–45% B over 70 min, 40–70% B over 20 min, and re-equilibration (5% B). The absorbance of fractions was measured at wavelengths of 215 nm, and the fractions were collected manually. The collected fractions were vacuum-dried and further applied to non-reducing and reducing SDS-PAGE, using 15% acrylamide gels with a pre-stained protein ladder (Thermo Fisher Scientific, Dreieich, Germany). The gels were stained with Coomassie Brilliant Blue G-250 (Merck, Darmstadt, Germany), and the protein bands were excised and subjected to in-gel tryptic digestion. The scanned gel picture was analyzed for the relative density of protein spots using ImageJ software (https://imagej.nih.gov/ij/list.html (accessed 1 March 2022)).[19]

4.2.5 LC-MS/MS analysis

Equal amounts of the whole venoms were diluted with 50 mM ammonium bicarbonate buffer, containing 0.1% RapiGest, and incubated for 15 min in a thermomixer at 80°C (Eppendorf Thermomixer C, Hamburg, Germany) to complete proteome solubilization. The venom and SDS-PAGE band samples were reduced and alkylated with 100 mM dithiothreitol (DTT) at 56°C for 15 min, and 200 mM iodoacetamide (IAA) at room temperature (dark place) for 30 min, respectively. The digestion was performed with mass spectrometry grade Trypsin/Lys-C mix (1:25 enzyme to proteins ratio) at 37°C. The reaction was stopped after 16 hours**Fehler! Textmarke nicht definiert.** by adding formic acid to 1.5% and incubating at 37°C for 10 min prior to centrifugation. The peptide samples were desalted before the LC-MS/MS measurement by using ZipTip C18 and then concentrated using Eppendorf Concentrator Plus (Eppendorf, Hamburg, Germany). The resulting peptides were separated using a Dionex UltiMate 3000 RSLC UHPLC system (Thermo Fisher Scientific) on a Kinetex C18 (2.1 mm × 100 mm, 2.6 µm 100 Å particle size) column (Phenomenex, CA, USA) coupled to a Q Exactive HF-X benchtop Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) by heated electrospray ionization (HESI-II) ion source (Thermo Fisher Scientific).

For each sample, three technical replicates were performed. The chromatographic analysis was performed at 250 µL/min flow rate with water/0.1% formic acid (mobile phase A) and acetonitrile/0.1% formic acid (mobile phase B). The gradient elution of 120 min was applied as follows: isocratically (2% B) for 5 min, followed by 2-40% B over 100 min, 40-50% B over 5 min, 50–98% B over 2 min, and re-equilibration in 2% B. The mass spectrometer was operated

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in data-dependent acquisition mode (top-15 DDA) with the following parameters in full MS scans: mass range of m/z 350 to 1650, mass resolution of 120,000, automatic gain control (AGC) target of 3 × 10^6, injection time (IT) of 50 ms, and MS/MS scans with mass resolution of 30,000, AGC target of 1 × 10^5, IT of 55 ms, isolation window Δ(m/z) 1.3, normalized collision energy (NCE) of 28, and dynamic exclusion of 30 s. Top-down mass measurements were applied to the fractions not revealed in the SDS-PAGE as follows: fractions were loaded onto a Jupiter C18 (4.6 mm × 250 mm, 3 µm 300 Å particle size) column (Phenomenex, Torrance, CA, USA) independently. A Dionex UltiMate 3000 RSLC UHPLC system (Thermo Fisher Scientific, Bremen, Germany) was interfaced with a Q Exactive HF-X benchtop Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) using HESI-II ion source (Thermo Fisher Scientific). Chromatographic analysis was performed at a flow rate of 400 µL/min, using water/0.1% FA (mobile phase A) and ACN/0.1% FA (mobile phase B). The gradient of 90 min was applied as follows: isocratically (2% B) for 5 min, 2-55% B over 85 min, and re-equilibration in 2% B. The mass spectrometer was operated in data-dependent acquisition (top-5 DDA) with the following parameters in full MS scans: mass range m/z 350–1650, mass resolution of 120,000 (@ m/z 200), AGC target of 1 × 10^6, IT of 100 ms and MS/MS scans: mass resolution 30,000 (@ m/z 200), AGC target of 1 × 10^5, IT of 120 ms, isolation window Δ(m/z) 1.3, dynamic exclusion 30 s, and normalized collision energy (NCE) of 30.

4.2.6 Data analysis

The raw files were searched against the UniProt protein database that was taxonomically set to the Viperidae (taxon ID # 8689) databases (downloaded on 1 March 2021) using Proteome Discoverer (PD) software suite, version 2.4 (Thermo Fisher Scientific) for bottom-up proteomics, and TopPIC Suite, version 2.0 [68]. For PD, two search engines, SEQUEST and MS Amanda, were used with the peptide precursor and fragment ion mass tolerance set to 10 and 0.5 ppm, respectively. The parameters were assigned to a maximum of two missed cleavage sites of trypsin digestion and a minimum peptide length of 6. The dynamic modification was set to oxidation (+15.995 Da [M]) and static modification to carbamidomethyl (+57.021 Da [C]). Percolator node was used to validate identified peptide-spectrum matches (PSMs) and filter the data with parameters of a strict target FDR (false discovery rate) of 0.01 and a relaxed target FDR of 0.05. The MaxQuant contaminant database was used to mark contaminants in the results file, and high confidence proteins in the master group with at least two unique peptides were considered. In TopPIC software, the signal-to-noise ratio was set to 2, the precursor window size set to Δm/z = 4, mass error tolerance was set to 10 ppm, and the decoy database was used to filter the spectrum with FDR cut-off of 0.01. For shot-gun proteomics, the relative quantitation of identified proteins from the PD output was calculated based on the normalized spectral abundance factor (NSAF) [69,70] manually.

4.3 Results

4.3.1 An overview of proteomic strategies for profiling venoms of *M. l. cernovi* and *M. l. lebetina*

We applied different analytical approaches to comprehensively profile the venom proteomes of *M. l. cernovi* (Iran) and *M. l. lebetina* (Cyprus). We initially utilized the classical venomics method, starting with separating the venom proteome using reversed-phase ultrahigh-performance liquid chromatography (RP-UHPLC) and then manually collecting the detected chromatographic peaks. We next subjected the fractions to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis for further separation based on molecular weights under non-reduced and reduced conditions (**Figure 4.2A,B** and Figure 4.S1, Supplementary Materials). The chromatographic fractions were identified by subsequent liquid chromatography tandem-mass spectrometry (LC-MS/MS) analyses of peptides obtained from in-gel tryptic digestion of SDS-PAGE protein bands. In addition, we applied intact mass measurement for the fractions that did not appear in SDS-PAGE. Since snake venoms contain a significant amount of low-abundance[20] proteins and low molecular weight peptide classes, they are mostly lost during the fraction collecting and in-gel digestion processes. In this case, for more profound identification, we also used single-shot proteomics, where the proteomes of whole venoms were subjected directly to in-solution digestion. We separated all resulting peptides online (RP-UHPLC) in this method and analyzed them via electrospray ionization high-resolution tandem mass spectrometry (ESI-HR-MS/MS).

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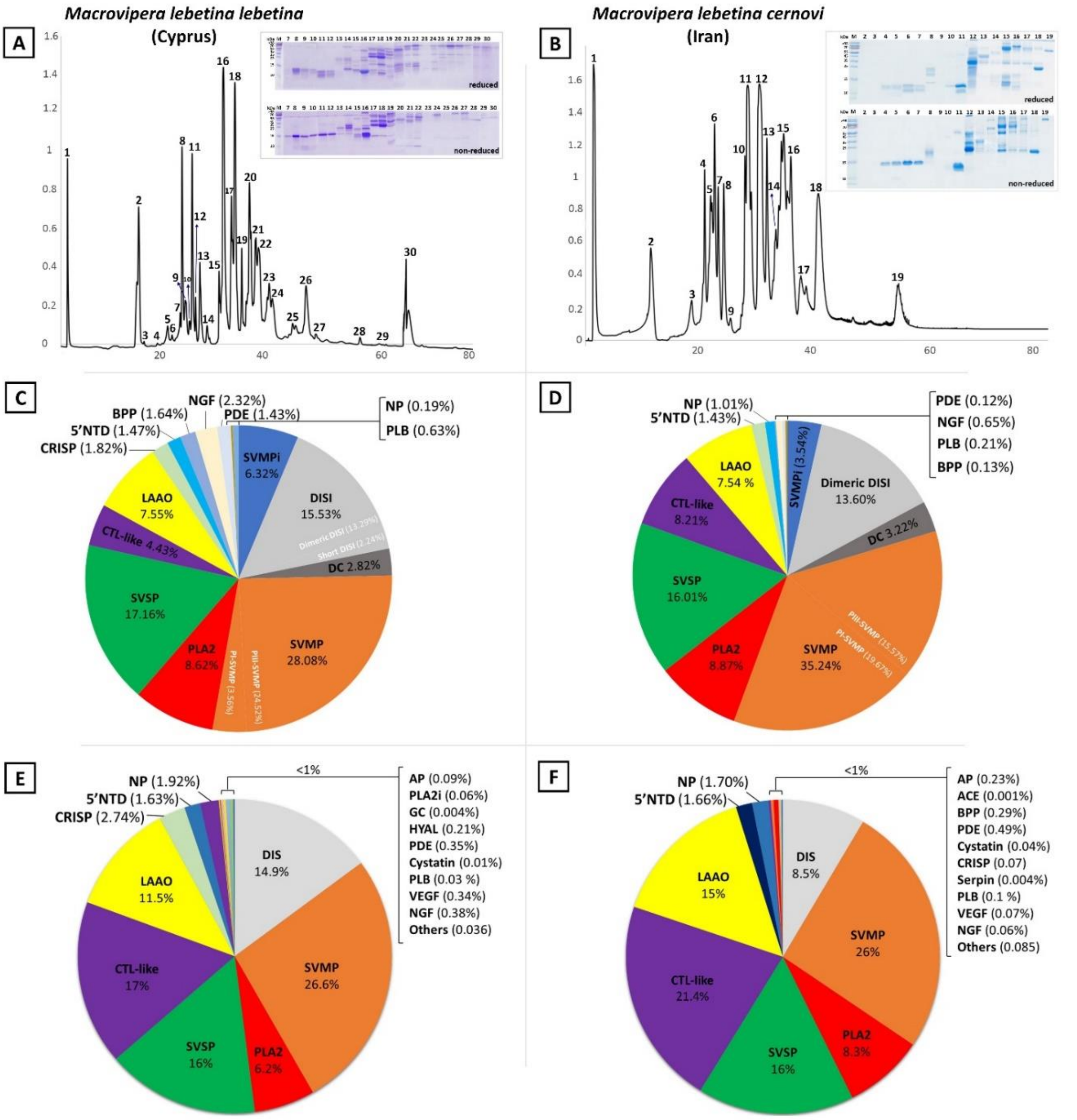

Figure 4.2. Venomic analysis of *M. l. lebetina* from Cyprus and *M. l. cernovi* from Iran. (A) Reverse-phase chromatographic separation of the venom proteome of *M. l. lebetina*, and (B) *M. l. cernovi*. The chromatographic fractions of both venoms were collected manually and analyzed by SDS-PAGE in both, reduced (upper section) and non-reduced (lower section), condition. (C, E) The pie charts indicate the identified protein families and their relative abundances (as percentage of total detected proteins) within the venom of *M. l. lebetina*, and (D, F) *M. l. cernovi* as determined either from the peak areas of reverse-phase chromatography with the help of SDS-PAGE protein bands (C, D) or based on the normalized spectral abundance factor from single-shot whole venom bottom-up proteomics (E, F). Acronyms: SVMP, snake venom Zn2+-metalloproteinase; PLA2, phospholipase A2; SVSP, snake venom serine protease; CTL-like, Snaclec, C-type lectin-like protein; LAAO, l-amino acid oxidases; DIS, disintegrin; CRISP, cysteine-rich secretory protein; NGF, nerve growth factor; VEGF, vascular endothelial growth factor; PDE, phosphodiesterase; AP, aminopeptidase; ACE, angiotensin-converting enzyme; BPP, bradykinin-potentiating peptides; PLB, phospholipase B; PLA2i, phospholipase A2 inhibitor; HYAL, hyaluronidase; NP, natriuretic peptide; 5′NTD, 5′-nucleotidase; DC, disintegrin-like/cysteine-rich fragment of PIII-SVMP; SVMPi, snake venom metalloproteinase inhibitor; NGF, nerve growth factor.

The recorded data were searched against a Viperidae database, and peptides from each LC fraction, related gel bands, and whole venoms were identified independently. Supplementary Table 4.S1 presents the features of molecular mass, MS/MS-derived sequence, protein annotation, and quantification of each reverse-phase eluted fraction. HPLC peak-area calculations as a percentage of total venom proteins (for in-gel protein digestion) and the normalized spectral abundance factor (NSAF) in the case of shot-gun proteomics datasets were used for quantitative analysis. Panels C-F of **Figure 4.2** show the composition profiles and relative abundances of protein families from the proteomes of both targeted venoms.

4.3.2 The venom proteome of Cypriot *M. l. lebetina*

Figure 4.2A shows the RP-HPLC elution profile of female Cypriot *M. l. lebetina* venom, and the SDS-PAGE protein pattern of each collected fraction. The chromatogram revealed a high level of venom complexity, considering at least 30 elution peaks. LC-MS/MS analysis of peptides obtained from in-gel digestion and intact analysis of collected fractions identified 15 protein families (**Figure 4.2C**, and Table 4.S1, Supplementary Materials). The most abundant toxin groups included SVMP, SVSP, DISI, PLA2, CTL-like, disintegrin-like/cysteine-rich domain (DC), and L-amino acid oxidase (LAAO), together accounting for more than 80% of the venom's protein content. SVMP (28.08%), DISI (15.53%) and DC (2.82%), with an abundance of ~46%, were the most expressed toxins in the venom. Among the SVMP toxin subfamilies, we detected PIII-SVMP (24.52%) and PI-SVMP (3.56%) subfamilies in the venom of *M. l. lebetina*. DISI as small polypeptides were also present in the venom, possibly produced by C-terminal domain proteolytical cleavage of PII-SVMP precursors or directly encoded as mRNA in the venom [27–29]. The presence of DC in the venom can also result from post-translational processing of PIII-SVMP in venom glands [30]. Other toxin families that accounted for more than 1% of the venom proteins included SVSP (17.16%), PLA2 (8.62%), LAAO (7.55%), snake venom metalloprotease inhibitor (SVMPi; 6.32%), CTL-like protein (4.43%), cysteine-rich secretory protein (CRISP; 1.82%), bradykinin-potentiating peptide (BPP; 1.64%), 5′-nucleotidase (5′NTD; 1.47%), nerve growth factor (NGF; 2.32%), natriuretic peptide (NP; 1.92%) and phosphodiesterase (PDE; 1.43%).

In addition, a single-shot data-dependent acquisition (DDA) experiment of whole digested venom yielded a higher number of identified peptides, resulting in the detection of many low-abundance proteins (less than 1% abundance) among the others in the venom (**Figure 4.2E**). They included aminopeptidase (AP), hyaluronidase (HYAL), glutaminyl-peptide cyclotransferase (GC), cystatin, phospholipase B (PLB), vascular endothelial growth factor

(VEGF), phospholipase A2 inhibitor (PLA2i), and also trace amounts of cellular proteins (others). The combination of two proteomic approaches detected at least 18 protein families in the venom of Cypriot *M. l. lebetina*, and together provided greater insight into the venom components.

4.3.3 The venom proteome of Iranian *M. l. cernovi*

The reversed-phase LC-separated pattern of Iranian *M. l. cernovi* venom uncovered 19 chromatographic peaks (**Figure 4.2B**, and Figure 4.S1, Supplementary Materials). Proteomic analysis of collected fractions resulted in the qualification and quantification of 14 protein families (**Figure 4.2D**, and Table 4.S2, Supplementary Materials). The PI and PIII classes of snake venom Zn2+-dependent metalloproteinase (SVMP) represented 19.67% and 15.57% of the venom content, respectively, and together with proteolytic products of SVMP (DC; 3.22% and DISI; 13.60%) comprised the most abundant gene family expressed as proteins (52.06%) in the Iranian *M. l. cernovi* venom. Other high-abundant protein/peptide families were PLA2 (8.87%), SVSP (16.01), CTL-like protein (8.21%), LAAO (7.54%), 5′NTD (1.43%), SVMPi (3.54%) and NP (1.01%).

Furthermore, the whole digested venom was subjected to direct (shot-gun) LC-MS/MS analysis for further investigation of the composition and complexity of *M. l. cernovi* venom. The result discovered more than 18 protein families, and the method was sensitive to detect the families with less than 1% relative abundance, including AP, angiotensin-converting enzyme (ACE), BPP, PDE, Cyastatin, CRISP, Serpin, PLB, VEGF, NGF and others as illustrated in **Figure 4.2F**.

4.3.4 Comparison between venom proteomes of *M. lebetina* ssp.[21]

Reverse-phase chromatographic profiles of *M. l. cernovi* (Iran) and *M. l. lebetina* (Cyprus) venoms yielded 19 and 30 distinctive eluted peaks, respectively (**Figure 4.2A,B**). Gel electrophoretic patterns of collected fractions from the venoms also displayed that some chromatographic peaks contained multiple protein bands (**Figure 4.2A,B**, and Figure 4.S1, Supplementary Materials), which support the identification of different protein families from each fraction by LC-MS/MS (Tables 4.S1 and 4.S2, Supplementary Materials). Despite the various number of chromatographic peaks, we identified 15 and 14 protein/peptide classes in the venom of *M. l. lebetina* and *M. l. cernovi* using the classical venomics approach (**Figure 4.2C,D**). Furthermore, additional analysis with a shot-gun proteomic strategy resulted in 18

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protein families besides the minor cellular proteins for both venoms (**Figure 4.2E,F**). The result revealed that the two viper venoms share similar protein classes. However, the relative occurrence of these toxins was different in each snake subspecies. They shared proteins belonging to SVMP, PLA2, SVSP, CTL-like, LAAO, CRISP, 5′NTD, BPP-NP, DISI, PDE, NGF, PLB, SVMPi, AP, VEGF, and Cystatin classes. On the contrary, the two venoms each contained trace amounts of unique components such as GC, PLA2i, and Hyal in *M. l. lebetina*, and Serpin in *M. l. cernovi*.

In **Table 4**, we detailed the proteome composition of *M. lebetina* ssp.[22] venoms, investigated so far by different proteomics methodologies. Considerable differences are shown between the expressed toxins and their relative abundances in the venoms of *M. lebetina* subspecies. Although various methods can highly impact qualitative and quantitative outputs, the result demonstrated that the *M. lebetina* ssp.[22] venoms are dominated by five major toxin families: SVMP, PLA2, SVSP, CTL-like, and DISI. Among all expressed toxins, SVMP comprises a significant part of *M. lebetina* venom proteomes, especially in the venom of *M. l. transmediterranea*, with 67%, roughly 2-fold more than the others. Interestingly, a significant difference was also detected among the P-classes of SVMP, revealing that P-I class toxins were more abundant than P-III ones in the venoms of *M. l. cernovi* and *M. l. obtusa* than in *M. l. lebetina* and *M. l. transmediterranea*, and vice versa. Apart from the toxin families abundantly expressed in the venoms of *M. l. cernovi* and *M. l. lebetina*, direct LC-MS/MS analysis of the peptide mixtures generated by in-solution digestion of the whole venoms revealed a considerable number of low-abundant protein classes (<1%). Notably, just a few of them (e.g., Hyal, PDE and VEGF) have been reported in other *M. lebetina* ssp.[22] venoms until now. This probably is due to employing 1D and/or 2D gel electrophoresis/MS approaches for protein identification. As a result, low-abundant protein classes are excluded or less represented in gel patterns, and analysis of the yielded peptides from the digestion of gel spots cannot detect them without pre-gel enrichment.

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Table 4. Comparison of toxin families detected in the venom proteomes of *M. lebetina* subspecies, investigated by almost the same proteomic technique. Overview of the relative abundances of toxin families in the venom of Cypriot *M. l. lebetina* and Iranian *M. l. cernovi* along with published data on Russian *M. l. obtusa* [13], Armenian *M. l. obtusa* [12] and Tunisian *M. l. transmediterranea* [11].

Protein Family	**% of total venom proteins**				
	M. l. lebetina **(Cyprus)**	***M. l. cernovi*** **(Iran)**	***M. l. obtusa*** **(Armenia)**	***M. l. obtusa*** **(Dagestan)**	***M. l. transmediterranea*** **(Tunisia)**
P-I snake venom Zn^{2+}-metalloproteinase	3.56	19.67	27.8	14.6	-
P-III snake venom Zn^{2+}-metalloproteinase	24.52	15.57	4.3	9.4	67
Dimeric disintegrin	13.29	13.6	8.5	5.5	6
Medium disintegrin	-	-	-	<1	-
Short disintegrin	2.24	-	2.8	7.4	<1
Disintegrin/cysteine-rich fragment	2.82	3.22	1.7	0.6	1
Serine proteinase	17.16	16	14.9	23.4	9
Phospholipase A2	8.62	8.87	14.6	13.6	4
C-type lectin-like	4.43	8.21	14.8	8.7	10
L-amino acid oxidase	7.55	7.54	1.7	2	-
Cysteine-rich secretory protein	1.82	<1	2.6	1.1	-
Bradykinin-potentiating peptides/natriuretic peptide	1.83	1.14	5.3	5.6	<1
Hyaluronidase	<1	-	-	<1	-
Phosphodiesterase	1.43	<1	-	<1	-
5′-nucleotidase	1.47	1.43	-	<1	-
Snake venom Zn^{2+}-metalloproteinase inhibitor	6.32	3.54	-	4.8	-
Phospholipase B	-	<1	-	-	-
Nerve growth factor	2.32	<1	-	-	-
Vascular endothelial growth factor	<1	<1	-	-	2

4.4 Discussion

The proteomic analysis of venoms from *M. l. lebetina* and *M. l. cernovi* provides a snapshot of the toxin arsenal of *M. lebetina* members. The data presented here revealed that the protein family compositions of two *M. lebetina* ssp.[23] are more complex than previously reported [31]. The number of identified proteins obtained here exceeded those documented by other *M. lebetina* ssp.[23] [11–13], likely due to combining in-gel and in-solution digests of the venoms and fractions using a more sensitive mass spectrometer. Venom profiles between the analyzed *M. lebetina* ssp.[23] indicate dominant expression of proteins belonging to SVMP, PLA2, SVSP, CTL-like, and DISI families. It is known that some major toxins can play a significant role in the pathophysiology of envenoming, for instance, disrupting the extracellular matrix of the vascular subendothelium (by SVMPs) [32], degradation of fibrinogen (by SVSPs) [33], inducing vascular hyperpermeability (by VGEF) [34], and promoting hypotension (by BPP/NP)

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[35]. However, the in vivo effects of most of the venom components are still poorly understood. Recently, clinical observations of human patients following Cypriot *M. l. lebetina* [19] and Iranian *M. l. cernovi* [25] envenoming have shown common local symptoms like edema and dermal necrosis, which seems mostly related to PLA2 and SVMPs toxins in both venoms [36]. Also, the clinical data of snakebite victims envenomated by *M. l. lebetina* revealed hypotension shock [19], possibly due to BPP/NP actions. In the case report of *M. l. cernovi* bites, the patients had significantly higher[24] creatine kinase (CK) levels [25]. An elevated serum CK level is a relatively common consequence of some viper envenomings, as a result of rhabdomyolysis or skeletal muscle damage, which is a useful marker of myotoxicity [37]. However, caution should be exercised when considering CK as a clinical indicator for early recognition of myotoxicity based on the observation of non-envenomed patients with early in-creased CK [38,39]. Clinical signs of *M. l. cernovi* snakebites furthermore evidenced that the venom induces thrombocytopenia [25], as a consequence of CTL-like toxin, described as the most relevant action of the toxin [40]. CTL-like protein generates platelet aggregation by indirectly modulating the von Willebrand factor (vWF) and promoting thrombocytopenia [41,42]. In addition, the impact of thrombocytopenia can contribute to the extent of systemic hemorrhage induced by SVMPs, as observed in the case of *Bothrops* sp.[24] envenomings [40,43].

Overall, the toxin profile data delivers molecular evidence for understanding the clinical consequences following *M. lebetina* envenomings, such as local pain, edema, necrosis, and systemic manifestations, including hemorrhage, hypotension, coagulopathy and thrombocytopenia. Among all toxins, SVMP constitutes a significant portion of the total proteins in many Viperidae venoms [44,45], including *M. lebetina* venoms. Although SVMP is shown up in different subclasses (PI-, PII-, and PIII-SVMP), the biological function of each subclass can appear diverse from the other [46]. For example, venomic data of *M. lebetina* ssp.[24] unveiled that the P-I class was less abundant than P-III in the venoms of *M. l. lebetina* and *M. l. transmediterranea* than *M. l. cernovi* and *M. l. obtusa*. While PIII-SVMPs induce hemorrhage by disrupting the extracellular matrix of the vascular endothelium, PI-SVMP provoke consumptive coagulopathy by fibrino(geno)lytic activities as well as persistent disruption of muscle tissue [47,48] but lacking from hemorrhagic activity [48–52]. Noteworthy, though myotoxicity was initially thought to be the result of PLA2 actions, there is now evidence that PI-SVMP are also explicitly involved in the disruption of muscle tissue [47]. In addition, proteomic data showed that PLA2 comprise a small part of the venom proteome (8–14%) of *M. lebetina* ssp.[24] Thus, we expect to see a more potent myonecrotic effect and minor systemic

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hemorrhage following envenoming by *M. l. obtusa* and *M. l. cernovi* compared to the two other ones. This is in agreement with the strong hemorrhagic activity of *M. l. transmediterranea* venom [53,54], and the clinical signs of potent myotoxicity in the venoms of *M. l. obtusa* and *M. l. cernovi* [16,25]. However, comprehensive knowledge about the action of venom toxins and their contribution to the clinical features of envenoming in humans has mostly remained unclear and needs to be investigated in future studies.

Several regional antivenoms have been produced using the venom of local *M. lebetina* ssp.[24] during the hyperimmunization process, such as those of the Razi Vaccine & Serum Research Institute (polyvalent snake antivenom), the Institut Pasteur d'Algerie (anti-viperin), the Egyptian Organization for Biological Products & Vaccines (VACSERA) and the Institut Pasteur du Tunis (Gamma-vip) [55]. However, there is a lack of knowledge on the efficacy of these antivenoms against the regional used venoms and even other *M. lebetina* ssp.[24] A recent study showed that venoms of *M. lebetina* ssp.[24] are highly potent in their ability to procoagulate, and their activation patterns of clotting factors are variable biochemically, influencing antivenom cross-neutralization efficacy [10]. Therefore, anti-venoms produced without including the targeted venom are not entirely effective in treating the envenomed patients. Given that *M. l. cernovi, M. l. obtusa* and *M. razii* are distributed in Iran, it is unclear which *Macrovipera* species[24] or subspecies were used for anti-venom production by the producers [6]. In addition, in the case of *M. l. lebetina* envenoming in Cyprus, the Egyptian polyvalent antivenom (VACSERA) is used for snakebite treatment (drawn from a personal conversation with pharmacies in Cyprus). However, it is unclear if *M. l. lebetina* venom was included in the antivenom production. It is appropriate to have polyvalent antivenoms targeting the toxins of all *Macrovipera* venoms instead of a single snake species or subspecies. This may be possible as reported in the case of Inoserp Europe polyvalent antivenom, which showed promising neutralizing potency against the venoms of *M. lebetina* ssp.[24] and *M. schweizeri* [10,56]. The venoms of *M. l. cernovi, M. l. obtusa* and *M. l. lebetina* were found to contain significant quantities of endogenous tripeptide metalloprotease inhibitors (SVMPi). The tripeptides were also detected previously from venoms of different species, e.g., *Trimeresurus mucrosquamatus* [57], *Bothrops asper* [58], *Echis carinatus sochureki* [59], E*chis ocellatus, Cerastes cerastes cerastes* [60], *Deinagkistrodon acutus* [61], *Vipera ammodytes transcaucasiana* and *Vipera ammodytes montandoni* [62], and various species of rattlesnakes [63], confirming their role in reducing the proteolytic activity of SVMPs during storage in the venom gland to prevent self-intoxication [59]. They appear to be among the essential components of snake venoms, which contain significant amounts of SVMP toxins. However, their presence in the venom of other snakes requires intact-mass profiling due to their low-molecular weight (<500 Da), as utilizing gel electrophoresis for venom separation before MS analysis failed to detect them, for example, as seen in the case of *M. l. transmediterranea* venomics. SVMPi-related transcripts were detected from the venom gland transcriptomes of *Echis ocellatus, Cerastes cerastes cerastes* [60] and *Daboia russelii* [6164] encoding tandem-repeated peptides, even containing C-type natriuretic peptide (CNP) and poly-His poly-Gly (pHpG) domains. Although these peptides are separately detectable from proteinaceous components in snake venoms, they are encoded from the same transcript and possibly liberated by post-translational processes. It should be noted that the molecular evolutionary history of SVMPi has not yet been described. In addition to the most abundant proteins, many low-abundance proteins are also detected in the venoms of *M. l. cernovi* and *M. l. lebetina*. However, the actual contribution of most of them to the venom and envenoming is entirely uncertain and needs further research attention.

Considering the variety of structures and functions of snake venom toxins, it is expected that these substances are used as pharmacological tools and as prototypes in drug development [65, 66]. Besides the severe clinical concerns marked in humans following *M. lebetina* envenoming, studies have shown the therapeutic potential of whole venoms and isolated toxins of *M. lebetina* ssp.,[25] particularly the cytotoxic activities against several types of human tumor cells [67]. Nevertheless, the detailed mechanism behind the actions is still largely unknown and needs further research [67].

4.5 Conclusions

The present study revealed the qualitative and quantitative details of the venom proteomes of Iranian *M. l. cernovi* and Cypriot *M. l. lebetina*, employing RP-HPLC, SDS-PAGE, and high-resolution mass spectrometry. The finding showed that both venoms contain complex components which have not been reported previously. SVMP, PLA2, SVSP, CTL-like, LAAO, DISI, and SVMPi are the major protein/peptide classes present[25] in the venoms. By using the shot-gun proteomics strategy, we have also detected the proteins that had not been previously detected in the venom of *M. lebetina* subspecies, e.g., PLB, NGF, PDE, AP, ACE, Serpin, and Cystatin. This information may help to improve the therapeutic management of *M. lebetina* snakebite by predicting the clinical effects of the venoms. It also may influence the design of more effective immunizing mixtures for future antivenom production in the region. However, additional research using samples from different geographical localities is essential to resolve in-depth geographical variation in the venom composition of *M. lebetina* subspecies. In

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addition, further analysis is critical to characterize the in-vivo activities and biological roles of these venom components.

4.6 Supplementary materials

The following materials are available online at www.mdpi.com/xxx/s1, Tables 4S1 and 4S2: List of identified proteins and peptides from *M. l. cernovi* and *M. l. lebetina* venoms. Figure S1: RP-HPLC and SDS-PAGE separation of the venom proteome of *M. l. lebetina*, and *M. l. cernovi*.

4.7 Author contributions

Conceptualization, methodology, software, validation, formal analysis, investigation, P.G.; resources, S.M.K., D.J.; experimental work, P.G., M.D.; data curation, P.G.; writing—original draft preparation, P.G., B.S.; writing—review and editing, P.G., M.D., S.M.K., D.J., B.H., B.S.; visualization, P.G., B.H.; supervision, B.S., P.G.; project administration, B.S.; funding acquisition, B.S. All authors have read and agreed to the published version of the manuscript.

4.8 Funding

Financial support by the Deutsche Forschungsgemeinschaft (DFG) (INST 162/500-1 FUGG) and by the State of Hesse through LOEWE Center DRUID (Novel Drug Targets against Poverty-Related and Neglected Tropical Infectious Diseases) is gratefully acknowledged.

4.9 Data availability statement

The bottom-up proteomic data presented in this study are available in ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org (accessed 1 September 2022))[25] via the PRIDE partner repository with the dataset identifier PXD036466.

4.10 Acknowledgments

D.J. would like to thank Peter van Issem for providing the venom sample of Cypriot *M. l. lebetina* and Frank Gessler (miprolab GmbH) for venom sampling technical support.

4.11 Conflicts of interest

The authors declare no conflicts of interest.

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Chapter 5

Hospital admissions due to snakebite envenoming in the Republic of Cyprus: a 7-year retrospective review

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Published in: Journal of Occupational Medicine and Toxicology (JOMT, 2022), 17:25
https://doi.org/10.1186/s12995-022-00363-1
Chapter version: as published in the JOMT, with only minor formal corrections.

Abstract Chapter 5

Background: Snakebite envenoming is a major neglected tropical disease, lacking data in many countries including Cyprus, a Mediterranean island inhabited by the medically important blunt-nosed viper (*Macrovipera lebetina*). Reviewing the 2013-2019 period, we present first-time epidemiological snakebite data in the Republic of Cyprus.

Methods: We obtained data on snakebite envenoming-related hospital admissions from the Ministry of Health, and population and rainfall data from the Statistical Service of Cyprus and Department of Meteorology websites. Human-viper conflict information was acquired from interviews with 12 representatives of Cypriot institutions.

Results: Between 2013 and 2019, 288 snakebite envenoming cases were admitted to public hospitals, averaging 41 people annually. The minimum was 29 cases (2017) and the maximum was 58 (2015). Snakebite envenoming incidence increased from 4.55 per 100,000 population (2013) to 6.84 (2015), but remained low since 2017 (3.49 in 2019). Between 2000 and 2018, the deaths of one man (73 years), and indirectly, one woman (77 years), were related to snakebite envenoming. While 266 cases (92%) happened between April and October (the blunt-nosed viper activity period), most envenomings occurred in September (cumulative for 2013-2019), with 88 cases (31%). Snakebite incidence peaked in the 60-69 years age group (9.19 per 100,000 population), and was higher in males (6.85) than in females (2.82). Of all admitted patients, 242 (84%) were discharged within four days. Mean hospital stay duration was 2.65 days, with one case of 13 days. Most patients were admitted to the general hospitals in Paphos (51%), Limassol (30%) and Nicosia (11%), which provide secondary healthcare, with the last one providing tertiary healthcare.

Conclusions: Snakebite-related deaths are very rare in the Republic of Cyprus. Most envenoming cases happened in late summer (September). Short hospital stays indicate mostly non-severe clinical courses. The hospital admission data suggest that snakebite envenoming risk is highest in Paphos district. The statistical data hint at males and middle- to older-aged people being at highest risk, whereas from our interview data we assume that outdoor workers are at higher risk than other occupational groups.

Keywords: Snakebite envenoming, Epidemiology, Hospital admissions, Cyprus, Paphos, Blunt-nosed viper.

5.1 Introduction

Cyprus is the third largest Mediterranean island, measuring 224 km in length, 96 km in width and covering 9,251 km², of which 1,733 km² are forested. The distance to the nearest country is 75 km to Turkey in the north [1]. The island comprises three main geographic regions (Troodos Mountains, Keryneia Range and Mesaoria Plain) [1-3]. Cyprus has a subtropical Mediterranean climate with dry, hot summers and mild, humid winters (Köppen climate classifications Csa and BSh) [4]. It is the hottest and driest island of the Mediterranean, and has the most hours of sunshine. Annual precipitation varies from 300-350 mm (Mesaoria) up to 1,200 mm (highest Troodos slopes), with the southwestern coastal districts receiving 450 mm. Due to its distinct dry and rainy seasons, with most rainfall occurring from November to March, Cyprus has no permanent rivers [2, 3]. Zoogeographically, Cyprus is considered part of Southwestern Asia. Three species of venomous snakes exist on the island: the cat snake (*Telescopus fallax*), the eastern Montpellier snake (*Malpolon insignitus*), and the blunt-nosed viper (*Macrovipera lebetina*) (**Figure 5.1**). The cat snake (*T. fallax cyprianus*) is endemic to Cyprus at subspecies level, while the blunt-nosed viper is present on Cyprus with its nominate subspecies (*M. lebetina lebetina*) [2].

Figure 5.1. a Cat snake (*Telescopus fallax*), rear-fanged. **b** Eastern Montpellier snake (*Malpolon insignitus*), rear-fanged. **c** Blunt-nosed viper (*Macrovipera lebetina*), a medically important front-fanged species. **d** The swollen foot of a 35-year-old woman, five days after the bite by a juvenile *M. l. lebetina* in Latchi, Paphos district, Cyprus. Photos: D. Jestrzemski.

While *T. fallax* and *M. insignitus* are rear-fanged venomous colubrids [5], *M. lebetina* is a large, medically important, front-fanged viper and a recognized cause of serious snakebite envenoming across its vast geographic distribution from Cyprus to Kashmir [6-9]. Although severe cases of envenoming by *M. lebetina* have been reported from the 20st century, data of recent envenoming cases are scarce [8]. *Macrovipera l. lebetina* is relatively common in agricultural areas and rocky slopes (**Figure 5.2**), occurring from the coastal lowlands to the Troodos mountains [2, 10]. Similarly, *M. insignitus* and *T. fallax* are found in stony habitats with well-developed vegetation (Phrygiana) [2]. While there are no documented cases of human envenoming by *T. fallax* and *M. insignitus* for Cyprus [2], 16-20 cases of snakebite by *M. l. lebetina* have been recorded annually on the island [11]. Bites to livestock have been reported as well [12]. Blunt-nosed viper venom mainly consists of the protein families Zn2+-metalloproteinases (SVMPs, causing internal bleeding, intravascular clotting, edema, inflammation and necrosis), serine proteinases (SVSPs, impacting physiological functions such as blood coagulation, fibrino(geno)lysis and platelet aggregation), L-amino acid oxidases (LAAOs, inhibiting ADP or collagen-induced platelet aggregation), snake venom hyaluronidase (playing a crucial role in venom circulation and strongly contributing to the envenoming symptomology), phospholipases A2 (PLA2s, with neurotoxic, hemolytic, myotoxic, anticoagulant, antiplatelet and antibacterial effects), C-type lectin-like proteins (CTLs, biologically highly active and strongly affecting platelet aggregation) and disintegrins (strong inhibitors of platelet aggregation) [13].

Local symptoms of *M. lebetina* envenoming include local pain and swelling, bruising, lymphangitic markings, regional lymphadenopathy, necrosis and local blistering. Systemic symptoms comprise thirst, nausea, vomiting, trembling, hypotension, shock, tachycardia, syncope, cold sweating, cardiorespiratory distress and spontaneous bleeding [9]. In the Republic of Cyprus, antivenom administration is based on a routine indication procedure: If a patient reports to be bitten by a snake, the physician evaluates the bitten area for signs of inflammation, swelling and necrosis. If such signs exist, then antivenom is given, otherwise not. On the other hand, if the patient states that the bite was certainly delivered by a dangerously venomous snake species (of which only one exists in Cyprus), then this is judged by the physician in order to give the treatment, or not. Hence, a physician has to be sure that either 1) the snake was venomous, or 2) the clinical course of the snakebite shows a toxic effect on the patient. This is of importance since antivenom treatment might have serious side effects.

Figure 5.2. **a** Goat-grazing in *M. l. lebetina* habitat at the edge of farmland (19 September 2015). In Cyprus, snakebite envenoming is a constant risk to livestock. **b** Habitat of *M. l. lebetina* at the edge of an orange plantation and riparian vegetation, offering favorable conditions for rats and their ophidian predators (8 September 2015). **c** A local man harvesting capers (*Capparis spinosa*) (9 April 2014). As *M. l. lebetina* commonly ambushes prey underneath shrubs, including capers, agricultural workers in Cyprus face an increased risk of snakebite envenoming. **d** *Macrovipera l. lebetina* camouflaged at the edge of farmland and riparian vegetation (9 May 2014). Photos: D. Jestrzemski.

Snakebite envenoming is a cause of significant human morbidity around the globe and was recently ranked as a Category A Neglected Tropical Disease by the WHO [14]. According to Kasturiratne et al. (2008), worldwide 421,000 to 1,841,000 people are envenomed by snakes annually, of which 20,000 to 94,000 die [15]. Snakebite survivors often suffer from chronic morbidity and disability following envenoming, including amputations, blindness, contractures, chronic infections and malignant ulcers [16]. In addition to physical impairments, snakebite survivors commonly experience severe psychological trauma caused by the incident and its health consequences, which forces them to retire from work and causes the loss of the primary source of income for many families [17]. On a global scale, conservative estimates set the burden of snakebite envenoming at 6.07 million disability-adjusted human life years annually [18]. However, snakebite remains notoriously underreported [16]. As snakebite victims often face high treatment costs, enforced borrowing and loss of income, the disease is a significant factor exacerbating poverty [16]. Snakebites also cause damage to livestock, which adds to the economic hardship of poor rural communities [19]. In fact, snakebite primarily affects poor rural communities, who suffer from adverse living conditions that increase the likelihood of human-snake encounters, low income, and limited access to education and healthcare [20-23].

In Europe, snakebite represents a much lower medical emergency than in tropical developing countries [24], although several European viper species, including *Vipera berus*, *V. aspis* and *V. ammodytes*, may cause life-threatening envenomings [25-27]. Apart from vipers, the only other European snakes capable of causing serious, but usually not life-threatening envenomings are the rear-fanged Montpellier snakes in the genus *Malpolon* [5, 27]. From 1970 to 2010, the annual number of snakebite cases in Europe was estimated at 7,992, with four deaths caused by snakebite per year on average. A better knowledge of snakebite epidemiology in Europe is necessary for an improved understanding of therapeutic requirements [24].

In the Eastern Mediterranean region (which includes the countries Greece, Cyprus, Turkey, Syria, Lebanon, Palestine, Israel, Jordan and Egypt) [28], several genera of medically highly important, venomous snake taxa occur, of which only *Macrovipera* (i.e., *M. l. lebetina*) is native to Cyprus. The annual number of snakebite envenomings in North Africa and the Middle East, with a total human population of about 160 million, is estimated between 3,017 and 80,191, and between 43 and 78 deaths annually [15]. The available data on snakebite in the Middle East are highly fragmentary, and for some countries (e.g., Syria) no information is yet available [29].

Despite the medical importance of snakebite envenoming, data on its epidemiology is scarce for most countries, due to a lack of reliable snakebite reporting systems, as well as a low awareness of health authorities regarding the public health importance of snakebite envenoming. In addition, snakebite cases are often reported only internally in institutions or published in newspapers or local journals, precluding international scientific access [30].

The goal of this article is, therefore, to present first-time systematically obtained data on snakebite hospitals admissions in Cyprus, considering annual occurrence, sex and age distribution of the bite victims, and distribution of admissions among Cypriot public hospitals. In this publication, only the Republic of Cyprus (de facto the southern part of the island) is included, since no statistics are available on snakebite envenoming in the Turkish-controlled north part of Cyprus.

5.2 Methods

We retrospectively analyzed data on public hospital admissions due to snakebite envenomings that were provided by the State Health Services Organisation of Cyprus to the Ministry of

Health (Republic of Cyprus). Of all public hospitals, the percentage of coding was around 95% of all inpatients. No data were available for admissions in private hospitals. All snakebite-related admissions were based on the ICD-10 code T63.0 referring to “Contact with snake venom” [31]. For all discharges due to death from snakebite (within the premises of a hospital, as well as outside), a Medical Certificate for Causes of Death was completed and coded within the Health Monitoring Unit, as part of the national Registry of Causes of Deaths. All deaths records were based on coding with Iris software, an automatic system used for coding multiple causes of death, and for selecting the underlying cause [32]. From all seven public Cypriot hospitals, the sex (male or female), age group (in five-year-steps, from 0 to 89 years), length of stay (days spent in hospital) and monthly number of admitted snakebite patients were recorded for the years from 2013-2019.

The data were provided in the form of two Microsoft Excel documents, containing tables and charts. The tables show the number of males and females envenomed by snakebite from 2013-2019, categorized by year and age group. This information is provided both for the overall number of snakebite incidents, and for each of the seven hospitals. Additional information for each hospital included the number of snakebite registrations per month and year, and the length of stay (in whole days) for each snakebite patient. The charts present the number of snakebite incidents in Cyprus from 2013-2019 by year of incidence and sex (male or female), as well as by age group and sex. However, the dataset neither shows the monthly number of admitted male and female snakebite victims, nor the monthly age group distribution, nor the length of stay of the monthly admitted patients. It also does not provide information of the length of stay separately for both sexes, nor for the age groups.

The data were obtained from two small rural hospitals providing primary healthcare (Polis Chrysochous and Kyperounta), from four district general hospitals offering secondary healthcare (Paphos, Limassol, Famagusta and Larnaca) and from Nicosia General hospital, which provides tertiary healthcare [33]. Complex medical cases are referred from the rural hospitals in Polis and Kyperounta to the large general hospitals in Paphos and Limassol, respectively [34]. Population growth data (2001-2019) were obtained from the Statistical Service of Cyprus [35] and rainfall records (2013-2019) from the Department of Meteorology website [36]. To observe trends in the annual occurrence of snakebite in Cyprus, we calculated snakebite incidence (annual number of hospitalized persons due to snakebites per 100,000 inhabitants) from 2013-2019.

Data on human-viper conflict were acquired from semi-structured interviews conducted by Daniel Jestrzemski (DJ) in the spring of 2014 with 12 representatives of Cypriot institutions related to the sectors of health, forestry, agriculture, environment and wildlife management. Of these, 10 interviewees provided information cited in this article (**Table 5**). The interviews took up to 60 minutes time and focused on aspects such as the public perception and management of *M. l. lebetina* and other Cypriot snake species, and on subject-related training by staff of the respective institutions. Interviews further included questions on the ecology, abundance, snakebite epidemiology and public perception of the blunt-nosed viper, its conservation status and possible future approaches to snake management and conservation in Cyprus. Additional questions were suited to the work of the respective institutions, providing the possibility for participants to include individual experiences. All participants expressed their consent for scientific evaluation of the provided information, and for the disclosure of their names. The information from the interviews were summarized in a MS Excel file, by dividing it into the categories "blunt-nosed viper habitat", "blunt-nosed viper species trend on Cyprus", "protection measures for blunt-nosed vipers", "problems caused by blunt-nosed vipers on Cyprus", "threats to the Cypriot blunt-nosed viper" and "recommended measures".

Table 5. Representatives of Cypriot institutions interviewed on the conflict between people and blunt-nosed vipers in Cyprus.

Institution	**Representative**	**Date of interview**
Troodos Visitor Centre (Troodos National Forest Park), Department of Forests, Ministry of Agriculture, Rural Development and Environment	K. Kailis	15 April 2014
Stavros Tis Psokas Forest Station (Paphos Forest), Department of Forests, Ministry of Agriculture, Rural Development and Environment	X. Ioannou	24 April 2014
District Agriculture Office (Polis Chrysochous), Department of Agriculture, Ministry of Agriculture, Rural Development and Environment	A. Pavlou	23 May 2014
Polis Chrysochous Hospital	M. Theodorou	27 May 2014
Department of Forests, Ministry of Agriculture, Rural Development and Environment	H. Nicolaou	28 May 2014
Department of Environment, Ministry of Agriculture, Rural Development and Environment	E. Erotokritou	28 May 2014
Gunsmith and hunting instructor from Skoulli village (Paphos district)	H. Demetriades	30 May 2014
Cyprus Game and Fauna Service	H. Hadjistyllis	6 June 2014
Cyprus Herpetological Society	H.-J. Wiedl, V. Schrempf	29 March 2014, 4 April 2014, 10 April 2014, 17 August 2014

5.3 Results

5.3.1 Snakebite envenoming in the Republic of Cyprus from 2013-2019

From 2013 to 2019, 288 cases of snakebite envenoming were recorded in the Republic of Cyprus. During the seven-year period, an average of 41 people were admitted to public hospitals due to snakebite annually, with a minimum of 29 bites in 2017 and a maximum of 58 in 2015. While most snakebites took place in the years 2014-2016 (47, 58 and 47 bites, respectively), the number was lower for the consecutive three years, with 29 bites occurring in 2017, 37 bites in 2018 and 31 bites in 2019 (**Figure 5.3**). The annual incidence of admissions due to venomous snakebite per 100,000 people in the Republic of Cyprus rose from 4.55 (2013) and 5.55 (2014) to 6.84 (2015), but dropped to 5.50 in 2016. It remained on a lower level since then, with an incidence of 3.36 (2017), 4.22 (2018) and 3.49 (2019). The mean incidence of snakebite (2013-2019) was 4.79.

5.3.2 Deaths related to snakebite from 2000-2019

Two deaths were linked to snakebite in Cyprus during the period from 2000-2019. In July 2004, a 73-year-old man died as a direct result of snakebite envenoming. Consequently, the underlying cause of death was coded X20 [31], referring to “contact with venomous snakes”. The second death occurred in April 2015 in an agricultural field and concerned a 77-year-old woman. Unlike the first case, it was related to multiple causes. These included cardiorespiratory arrest (I469, five hours), arterial hypertension (I10, 10 years), hyperlipidemia (E785, 10 years), postsurgical hypothyroidism (E890, 15 years), arteriosclerosis (I709, 15 years), overexertion (T733, X50), possible heatstroke (T670, X30) and the possibility of snakebite (T630, X20). According to the IRIS analysis, hyperlipidemia (unspecified, E785) was considered as the underlying cause of death, although snakebite could not be excluded as well. For both cases, no patient charts were available, and it is not known whether antivenom was administered.

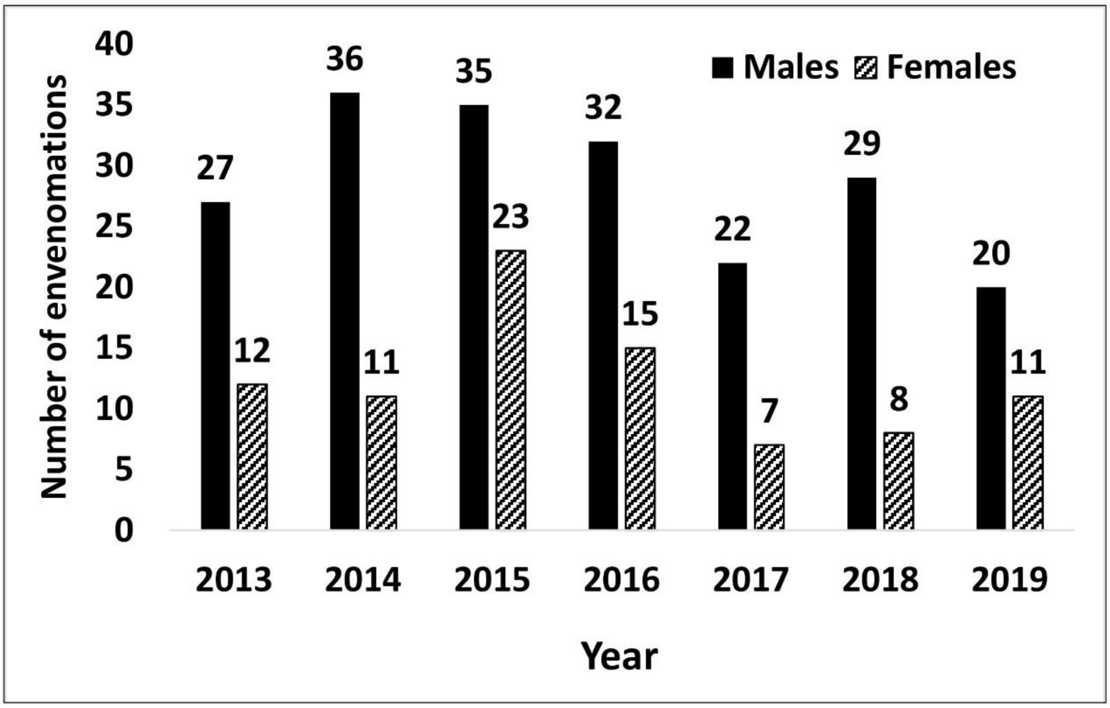

Figure 5.3. Snakebite envenoming in the Republic of Cyprus during the period from 2013-2019, by year of occurrence and sex.

5.3.3 Length of stay in hospital

From a total of 288 patients diagnosed with snakebite envenoming, 90 (31.25%) spent one day in hospital, followed by 64 patients (22.22%) with a two-day stay, 38 patients (13.19%) who spent three days in hospital and 26 patients (9.03%) who were discharged on the same day

(**Figure 5.4**). Of all 288 patients, 242 (84.03%) were discharged after four days or less in hospital. Six people (2.08%) spent longer than 10 days in hospital, namely 11 days (one case), 12 days (five cases) and 13 days (one case). On average, snakebite patients spent 2.65 days in hospital, while the median was two days.

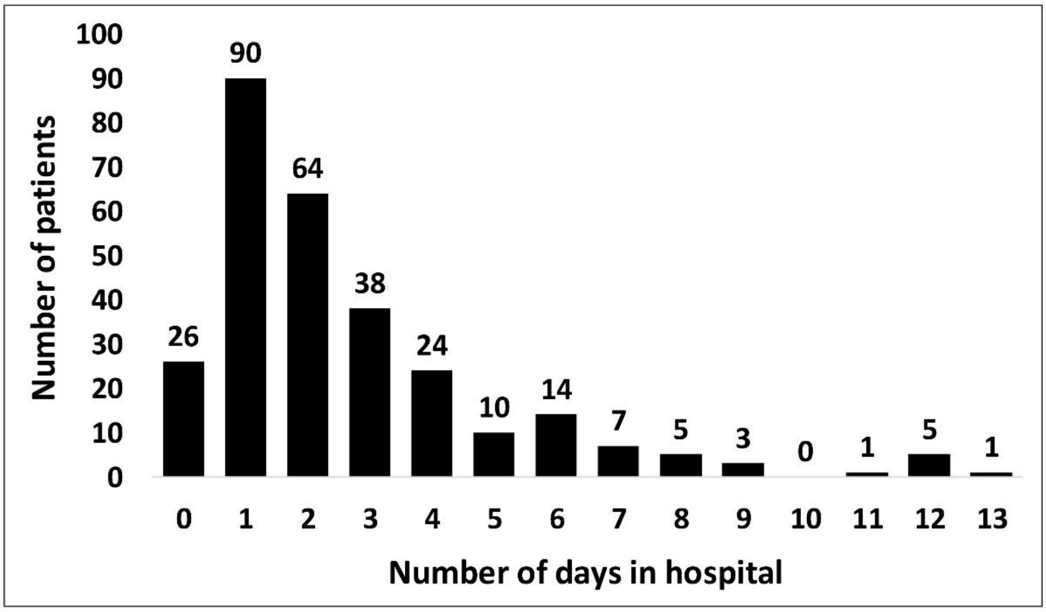

Figure 5.4. Number of days in hospital spent by 288 snakebite victims in the Republic of Cyprus (2013-2019).

5.3.4 Annual occurrence of admissions due to snakebite envenoming in Cyprus

From winter (January to March) to spring (April to June), a steady increase in the average number of snakebite envenoming cases per month could be observed, culminating in late summer (September), and then decreasing towards autumn (October to December) and winter. Consequently, the average number of snakebite envenoming cases per month (2013-2019) ranged from 0.14 in January to 12.57 in September, whereas the monthly average for a whole year was 3.43. Of all 288 cases of envenoming, 266 (92.36%) took place between April and October (**Figure 5.5**), which coincides with the activity period of *M. l. lebetina*. During the colder months (November to March), blunt-nosed vipers hibernate, while they are most active during the mating season (April to May), but also show activity during summer (June to September). Snakebite envenoming culminated during the dry summer season, with 188 cases (65.28%) occurring from June to September (2013-2019). During the peak of the dry season (August and September), 134 incidents (46.5%) took place. During the rainy winter and spring seasons (2013-2019), only 100 cases of envenoming (34.72%) were registered. On average, 27 persons per month were bitten during the dry season, and 14 persons per month during the rainy season. Over the year, most snakebite envenomings took place in September (88 cases in total).

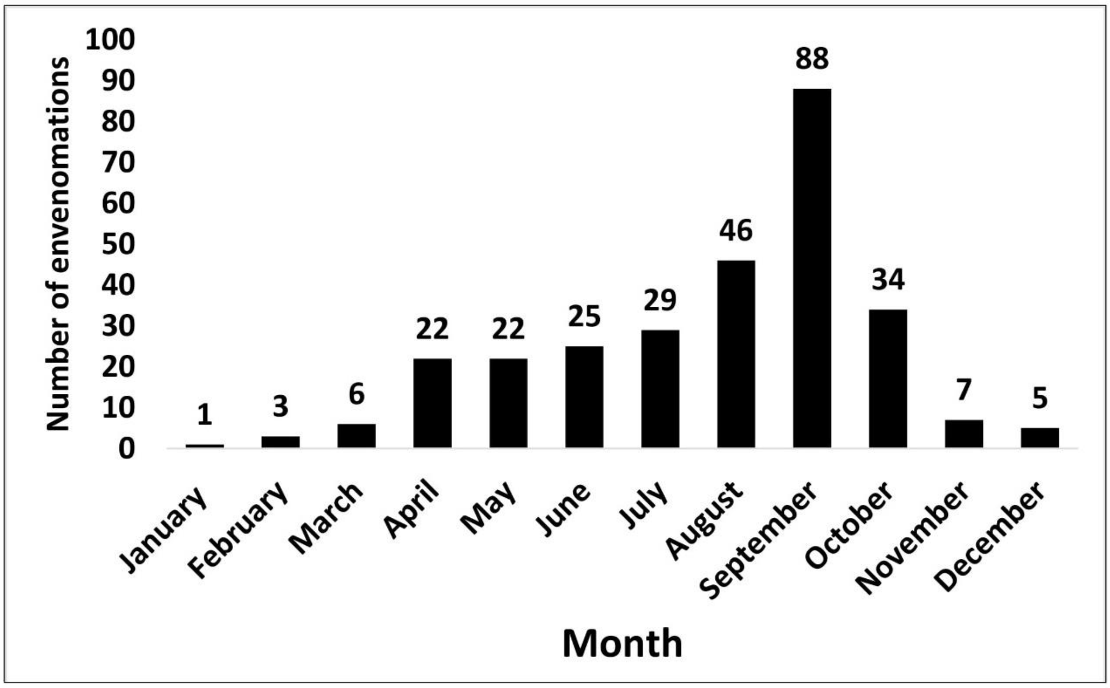

Figure 5.5. Snakebite envenoming in the Republic of Cyprus per month (cumulative for 2013-2019).

5.3.5 Distribution of sex and age groups among admitted snakebite victims in Cyprus

Most patients admitted for snakebite envenoming were males (201 cases or 69.79%), compared to only 87 cases (30.21%) involving females. Correspondingly, of 41 people bitten on average every month (2013-2019), 29 (69.79%) were males and 12 (30.21%) were females. Hence, the average snakebite incidence in Cypriot males (6.85 per 100,000 population) was more than twice as high as the snakebite incidence in females (2.82). The snakebite incidence of males peaked in the years 2014 (8.74), 2015 (8.48) and 2016 (7.68), and was lowest in 2019 (4.60). In contrast, snakebite incidence in females peaked in 2015 (5.28) and was still above average in 2016 (3.42), before in 2017 dropping to its lowest value within 2013-2019 (1.58). The age of snakebite patients ranged from very young (0-4 years) to very old (85-89 years). Between 2013 and 2019, the youngest (0-9 years) and oldest age group (80-89 years) were each represented with 10 recorded cases, and 1.43 cases of snakebite envenoming per year on average. Middle and older age groups were most heavily affected, with 47 cases each in the age groups of 30-39 and 50-59 years (6.71 cases of snakebite envenoming on annual average), and

58 cases in the age group of 60-69 years (8.29 on annual average). Hence, by far the highest mean snakebite incidence for the period of 2013-2019 occurred in the age group of 60-69 years (9.19 per 100,000 population), followed by the age groups of 50-59 (6.24) and 70-79 (5.44). The age group of 80-89 years (4.98 per 100,000 population) was almost equally affected as the age group 30-39 years (5.06), while incidence was lower in the age groups of 40-49 years (4.35) and 20-29 years (4.33). By far the lowest incidence was observed among 0-9-year-olds (1.50) and 10-19-year-olds (2.53) (**Figure 5.6**).

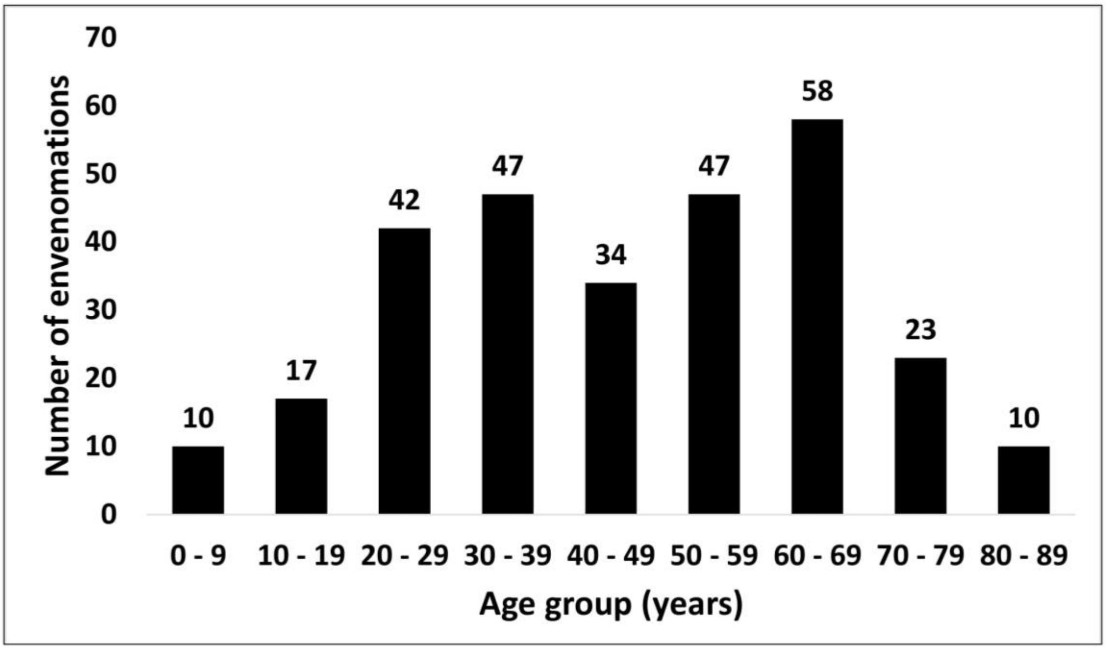

Figure 5.6. Snakebite envenoming in the Republic of Cyprus by age group (2013-2019).

5.3.6 Distribution of snakebite-related hospital admissions among major hospitals in Cyprus

Of all 288 hospital admissions related to snakebite envenoming between 2013 and 2019, 148 cases (51.39%) were recorded at Paphos General Hospital, 86 cases (29.86%) at Limassol General Hospital and 32 cases (11.11%) at Nicosia General Hospital. Another eight (2.78%) and six (2.08%) cases were treated at Kyperounta and Polis Rural Hospital. Ammochostos (Famagusta) and Larnaca General Hospital each reported four cases of snakebite (1.39%) (**Figure 5.7**). While approximately only one case of envenoming per year was recorded on average at the hospitals in Kyperounta (1.14), Polis (0.86), Ammochostos (Famagusta) and Larnaca (each 0.57), the number of envenoming cases per year was greater for the hospitals in Nicosia (4.57) and Limassol (12.29), and by far the highest at Paphos General Hospital, with an annual average of 21.14 snakebite envenomings for the period from 2013-2019. The cumulative number of snakebite envenomings per month and hospital was highest for Paphos Hospital too, where a total of 47 envenoming cases were registered in September (2013-2019). In addition, more than half of all envenomings affecting females (51 cases or 58.62%) as well as 48.26% of all envenomed males (97 cases) were recorded at Paphos Hospital.

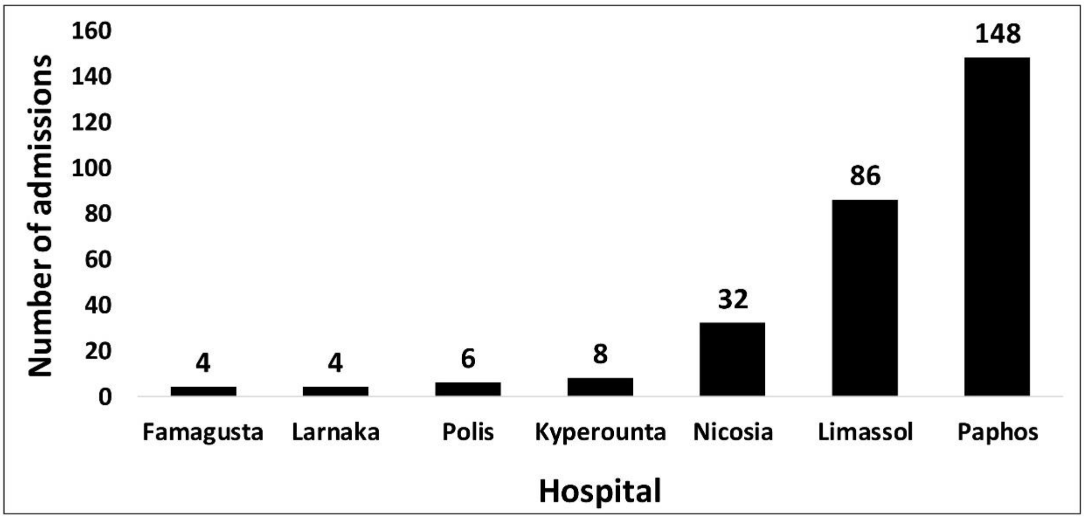

Figure 5.7. Number of snakebite admissions in the Republic of Cyprus by hospital (2013-2019).

5.3.7 Factors increasing the risk of snakebite envenoming in Cyprus

In Cyprus, local people are usually afraid of *M. l. lebetina,* and often try to kill it on sight. Rear-fanged venomous snake species (e.g., *M. insignitus*), if mistaken for vipers, are persecuted as well (V. Schrempf, H.-J. Wiedl, pers. comm., 29 March 2014; E. Erotokritou, H. Nicolaou, pers. comm., 28 May 2014; H. Demetriades, pers. comm., 30 May 2014; H. Hadjistyllis, pers. comm., 6 June 2014). For many people, the adult, black stage of the large whip snake (*Dolichophis jugularis*) is the only snake they do not kill, since they can clearly distinguish it from *M. l. lebetina* (37; H. Hadjistyllis, pers. comm., 6 June 2014).

People working outdoors (e.g., farmers, forestry employees, game wardens, hunters, mosquito control workers and shepherds) encounter vipers more often than those from other occupational groups, thus being constantly exposed to the risk of snakebite envenoming [10]. In spring, blunt-nosed vipers commonly ambush birds and rodents from underneath shrubs, including capers[29] (*Capparis spinosa*). Local people harvesting the popular fruits (see **Figure 5.2c**) thus face an increased risk of blunt-nosed viper bites (38; H.-J. Wiedl, pers. comm., 29 March 2014).

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[29] May deviate from the published version (Jestrzemski et al. 2022).

Shepherds persecute vipers to protect themselves as well as their livestock and dogs from snakebite, thus getting into contact with vipers as well (H.-J. Wiedl, pers. comm., 29 March 2014; V. Schrempf, pers. comm., 10 April 2014; H. Hadjistyllis, pers. comm., 6 June 2014). The killing of vipers by farmers and shepherds is also economically motivated, since the veterinary costs of treating a bitten, envenomed domestic animal often exceed the animal's market value (H. Hadjistyllis, pers. comm., 6 June 2014).

In Cyprus, hunting is a very popular sports activity in which about 25% of the male population are engaged [2], and hunters commonly get into contact with vipers. They are not only afraid of being bitten themselves, but also want to protect their hunting dogs from snakebite (V. Schrempf, H.-J. Wiedl, pers. comm., 29 March 2014; K. Kailis, pers. comm., 15 April 2014; H. Demetriades, pers. comm., 30 May 2014; H. Hadjistyllis, pers. comm., 6 June 2014). As a hunting dog in Cyprus may cost about 2,000 € on average (about 6-10 times as much as a goat or sheep), hunters have a particular economic interest in keeping vipers away from their dogs (H. Hadjistyllis, pers. comm., 6 June 2014).

Farmers often kill blunt-nosed vipers which they find on their lands, in contrast to the large whip snake, which they usually accept (K. Kailis, pers. comm., 15 April 2014; A. Pavlou, pers. comm., 23 May 2014). Sometimes farmers use nets to catch and kill blunt-nosed vipers. In one case, 17 vipers were captured in a single net within few months (H. Hadjistyllis, pers. comm., 6 June 2014).

Mosquito control workers employed by the Health Department enter streams all over Cyprus on a daily basis, thus permanently encroaching on habitats of *M. l. lebetina* [10]. Each mosquito control worker may encounter dozens, and even more than 100 blunt-nosed vipers over the course of a year. They regard vipers as a hazard and commonly kill them (V. Schrempf, pers. comm., 29 March 2014; H.-J. Wiedl, pers. comm., 4 April 2014; H. Demetriades, pers. comm., 30 May 2014; H. Hadjistyllis, pers. comm., 6 June 2014). So far, there are no educational programs on snakebite management for mosquito control workers in Cyprus (H. Demetriades, pers. comm., 30 May 2014), which exacerbates the risk of snakebite faced by this occupational group.

Forestry employees frequently encounter and kill vipers, too, although they are instructed to stay away from snakes (K. Kailis, H.-J. Wiedl, pers. comm., 15 April 2014; H. Nicolaou, pers. comm., 28 May 2014; V. Schrempf, pers. comm., 17 August 2014). Workers of the Cyprus Game and Fauna Service regard vipers as a threat to birds and kill them, particularly when they see them next to bird cages or water pools established for wildlife (H. Hadjistyllis, pers. comm., 6 June 2014).

The ongoing transformation of wild viper habitat into real estate due to property development contributes to higher encounter rates between people and blunt-nosed vipers in Cyprus, thereby increasing the risk of snakebite on the island. Wildfires of anthropogenic origin take place every year and contribute to the loss of wild snake habitat, thus driving snakes closer to people [10]. Another major risk factor are gardens, agricultural areas and other man-made landscapes with ideal conditions for rodent populations (e.g., organic waste), which in turn attract blunt-nosed vipers (38; H.-J. Wiedl, pers. comm., 29 March 2014). The additional presence of wooden debris in gardens and agricultural landscapes provides for suitable snake hiding places. These structures enable vipers to live close to human settlements, which strongly increases the risk of snakebite in Cyprus [10]. Finally, due to the widespread public aversion to *M. l. lebetina,* it is the only snake species that is not protected in the Republic of Cyprus [2], which further increases the risk of persecution by humans, and thus, the snakebite envenoming risk in Cyprus.

5.4 Discussion

5.4.1 Snakebite incidence in the years 2013-2019

Like the absolute number of snakebites, snakebite incidence peaked in the years 2014 (5.55 per 100,000 population), 2015 (6.84) and 2016 (5.50). This temporary increase took place during a slight reduction in the population of the Republic of Cyprus, which was lower between 2014 and 2016 (850,033 on average) compared to the 2013-2019 average of 862,314. At the same time, the mean annual precipitation (2014-2016: 435.6 mm) was lower than the mean precipitation for the 2013-2019 period (475.8 mm). This appears surprising, as rainfall is usually positively correlated with the reproductive activity of venomous snakes, and is of critical importance for agriculture [30]. Consequently, wetter years might have been expected to result in increased farming activities combined with prolonged movements of snakes, resulting in higher encounter rates between people and venomous snakes. The data of the study period suggest that this was not the case in Cyprus: Although the precipitation in 2018 (606.5 mm) and 2019 (796.8 mm) was 27% (2018) and even 67% (2019) above the annual average from 2013-2019, snakebite incidence was only 4.22 in 2018 and 3.49 in 2019, which was 12% (27%) below the annual average from 2013-2019 (4.79).

It is also noteworthy that the wettest year of the 7-year period (2019) had the second lowest snakebite incidence. However, most of the rainfall of 2019 occurred in December (208.3 mm), a month with generally low levels of snakebite. Still, precipitation during the dry season from June to September was much higher in 2018 (41 mm) and 2019 (43.4 mm) than in 2014 (34.5

mm), 2015 (13.3 mm) and 2016 (12.7 mm), although less snakebites were recorded during the dry season in 2018 (26) and 2019 (21) than during the same period in 2014 (33), 2015 (35) and 2016 (30). Collectively, this suggests the possibility that the more widely distributed availability of water in the summers of those years that received unusually high precipitation might have resulted in a reduced need for snakes (and perhaps also humans) to assemble near the usually few remaining bodies of water, which in turn might have resulted in less frequent encounters between snakes and humans.

5.4.2 Snakebite occurrence over the year

The culmination of snakebite cases in Cyprus during the dry summer season is in line with the frequent observation of *M. l. lebetina* close to water during this period. As the Cypriot summer proceeds and landscapes dry out, water bodies provide significant advantages for blunt-nosed vipers such as shelter, ambush sites, ease of water uptake, and thermoregulation. Consequently, there is a constant risk for people to encounter *M. l. lebetina* at water bodies (including swimming pools) during summer [10]. Although blunt-nosed vipers usually hibernate from November to March in Cyprus, they may still be active in winter under favorable weather conditions [2]. This can explain the 22 cases of envenoming recorded between November and March (2013-2019).

5.4.3 Sex ratio, age distribution, length of stay in hospital and snakebite mortality

The highly unequal distribution of snakebite envenoming incidence among sexes (6.85 per 100,000 population in Cypriot males compared to 2.82 per 100,000 population in Cypriot females) underlines the increased risk of bites by venomous snakes that males face compared to females in Cyprus. An explanation might be that in Cyprus, occupational outdoor activities are possibly more commonly pursued by men, who thus may be more likely to encounter *M. l. lebetina*. This sex-specific bias is also found in tropical developing countries, where snakebite is considered an occupational disease most commonly affecting young agricultural workers, particularly men [39, 40].

The presence of very young and old age groups as well as women in the snakebite statistics indicates that not only middle-aged people and men are at risk of venomous snakebite on the island. In fact, snakebites in Cyprus also take place in private gardens, where they are more likely to affect people of both sexes and all age groups alike [10]. People of 50 years and older made up 48% of all registered snakebite victims, although accounting for only 32% (2013), 33% (2014, 2015) and 34% (2016-2019) of the Cypriot population, which hints at an increased risk of snakebite faced by these parts of the population.

One reason might be the higher representation of older age groups (50+ years) in rural areas with nearby blunt-nosed viper populations. However, on the one hand, the proportion of these age groups did not differ strongly between the rural (32.5%) and urban (30.3%) population of Cyprus in 2011 [41]. On the other hand, there were remarkable differences between the districts of Paphos, Limassol, Nicosia and Larnaca in the proportion of older age groups (50+ years) among the rural and urban populations. While the proportion of 50+ year-old people was almost equally represented in rural and urban areas of Nicosia (30.1% vs 31.0%) and Larnaca district (30.4% vs 29.5%), 50+ year-old people made up a higher proportion in the rural population of Limassol district (35.1% vs 30.3% urban), and were especially dominant among the rural population in Paphos district (45.7% vs 28.8% urban) [41]. Thus, older-aged people in Paphos district are more likely to encounter venomous snakes than in other districts.

Interestingly, Paphos is also the district with the highest observed abundance of *M. l. lebetina* in Cyprus [2], which may contribute to generally higher snakebite envenoming rates in this district. Furthermore, it can be assumed that traditional outdoor activities (e.g., shepherding, farmwork) are more commonly pursued by older-aged people (own observations), which additionally increases their risk of encountering blunt-nosed vipers. This assumption is supported by the strong representation of the 50-69-year-old, who account for 76% of all snakebite victims over 50, and who are younger and thus more likely to conduct physically demanding outdoor activities than people of 70-89 years of age.

With approximately 20% of all envenomings, the age group of 60-69 years is most strongly affected, which could be due to less effective prophylactic measures undertaken against snakebite by people within this age group. An explanation may be that older people in rural areas apply modern outdoor safety measures (e.g., wearing safety gloves and protective boots for farm work) less commonly than younger people, as older persons may show less risk aversion than their younger counterparts [42].

The fact that 84% of all patients spent a relatively short time in hospital (up to four days) suggests clinical courses of snakebite envenoming in Cyprus that are mostly mild and/or uncomplicated. This is further highlighted by the low average number of days spent by snakebite patients in hospital (2.65). However, the median (two days) would be a more appropriate measure, as most patients (62.5%) stayed in hospital for a maximum of two days only, while the relatively small share of patients with a stay of five days or more (16%) is responsible for the strong skewness of the distribution to the left.

With one directly and another indirectly related death in 288 hospital admitted snakebite cases between 2013 and 2019, the snakebite mortality in the Republic of Cyprus was very low.

5.4.4 Distribution over hospitals

The numbers of snakebite-related public hospital admissions in four major Cypriot district hospitals (2013-2019) do not correlate with the population sizes (2019) of the corresponding districts. While the population size of Paphos district is 95,400 (only 11% of the population of all five districts), 51% of all hospital-admitted snakebite cases in the Republic of Cyprus were registered at Paphos General Hospital alone. On the other hand, Larnaca and Famagusta, with a combined total of 197,900 inhabitants (22% of the population) only had eight cases (3%) of snakebite envenoming at their two major district hospitals. With 346,400 inhabitants, Nicosia district has a share of 39% of the population, but only 32 people (11% of all cases) were admitted with snakebite envenoming to the district's general hospital from 2013 to 2019. In comparison, the recording of 30% of all snakebites (86 cases) at Limassol General Hospital is approximately proportional to the number of Limassol district residents (248,300), which amounts to 28% of the population of all five districts.

The registration of slightly more than half of all hospital-admitted snakebite cases only at Paphos General Hospital is notable, as Paphos is also the district with the highest density of *M. l. lebetina* in Cyprus (see **Figure 5.8**). While the common occurrence of *M. l. lebetina* in Paphos may hint at an increased risk of snakebite envenoming in this district, it does not necessarily have to correlate with the number of admissions to Paphos General Hospital, as snakebite victims may be transferred from other districts as well. Connectivity may be an important factor, as Paphos General Hospital can be easily reached from Limassol via the A6 highway, and from Polis via the B7 country road, and is situated less than 500 m away from the main junction of both roads. Also, as the five district general hospitals are better equipped to manage cases of snakebite envenoming, snakebite victims may be referred to them from rural hospitals, or directly seek care at secondary or tertiary referral centers. For example, people bitten in the Polis Chrysochous area may prefer to visit Paphos General Hospital, instead of presenting to the nearby hospital in Polis. In fact, the snakebite-related admission numbers in the rural hospitals of Polis and Kyperounta (5% of all cases) are surprisingly low, given that both are situated in areas with common occurrence of *M. l. lebetina* (see **Figure 5.8**), which hints at the above-mentioned trend.

Finally, a possible explanation for the outstandingly high number of snakebite-related admissions to the general hospitals in Paphos and Limassol (together accounting for 81% of all admissions) may be the double-counting of patients, due to the transfer of inpatients from other hospitals (e.g., in Polis). In such a case, a snakebite patient being transferred between two different hospitals will be registered as two different envenoming cases. As the available dataset does not provide the patients' personal information, there is no mechanism to avoid double-counting, and hence the patient can be easily confused with two different persons (each at another hospital). This may lead to particularly high numbers of registered patients in larger hospitals.

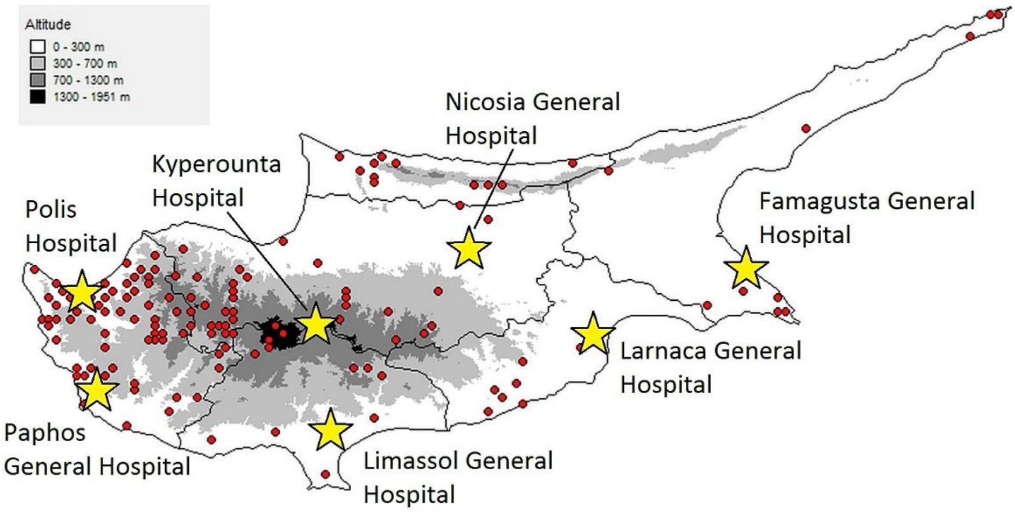

Figure 5.8. Distribution of *Macrovipera lebetina* in Cyprus, shown by red dots (map from Baier et al. 2013 [2]). The location of the seven hospitals in the Republic of Cyprus is shown by yellow stars.

5.4.5 Care-seeking behavior of snakebite victims

Since the available dataset of this retrospective study does not include information on the care-seeking behavior of snakebite victims, there is no empirical basis for identifying and discussing the factors that determine their hospital selection preferences (e.g., accessibility, reputation, cost, specialization, superior equipment, number of available beds). However, public hospitals in Cyprus are free of charge for all patients presenting to the emergency department and are also much less costly for in-patients than private hospitals. Hence, these circumstances suggest that at least snakebite victims with a low-to-average income would preferably visit public hospitals. However, no data were available from private hospitals, for inpatients.

5.4.6 Antivenom administration in Cyprus

Since at least 1971, antivenom is given to documented victims of snakebite envenoming in Cyprus**,** who present to the emergency department of public hospitals. “Polyvalent Snake Venom Antiserum” produced by the Egyptian Organization for Biological Products & Vaccines (VACSERA, Cairo, Egypt) has been used since at least 2009 to treat snakebite envenoming in Cyprus (M. Antoniou [Pharmacist at Nicosia General Hospital], pers. comm., 5 November 2020). The polyvalent antivenom from VACSERA is raised against the venoms of several venomous snake species, specifically from *Naja haje, N. nigricollis* and *Cerastes cerastes* [43], although its efficiency has not yet been tested by the WHO [44]. According to the manufacturer, it also has paraspecific neutralizing activity against the venoms of several oriental viper species, including *M. lebetina* [45].

As several antivenoms exist that are raised against the venoms of palaearctic vipers [44, 46], in some cases even specifically against the venoms of *M. lebetina* ssp.[30] (i.e., monospecific antivenom against *M. l. turanica* by UzbioPharm (Tashkent, Uzbekistan) [44, 47]), it may be expected that the latter have better neutralizing capacities against *M. l. lebetina* venoms. Besides the monovalent antivenom from UzbioPharm, polyvalent antivenoms with stated (specific or paraspecific) neutralizing activity against *M. lebetina* venoms are produced by different manufacturers in addition to VACSERA, including the Institut Pasteur de Tunis (Tunis, Tunisia), the Institut Pasteur d’Algerie (Algiers, Algeria), the Imunološki Zavod (Institute of Immunology, Zagreb, Croatia), the Institute of Virology, Vaccine and Sera TORLAK (Belgrade, Serbia), Inosan Biopharma S.A. (Madrid, Spain), the Razi Vaccine & Serum Research Institute and the Padra Serum Alborz (both in Karaj, Iran), as well as the Vetal Serum ve Biyolojik Ürünler Üretim Sanayi Tic. A.Ş (Adıyaman, Turkey) [44]. The WHO (2016) furthermore lists the National Institute of Health in Islamabad (Pakistan) among the producers of polyvalent antivenom against *M. lebetina* venom [48].

5.4.7 Medical importance of *M. lebetina*

Although *M. lebetina* has been rated as one of the snake species of highest medical importance (WHO category 1) in Algeria, Iraq, Iran, Lebanon, Syria, Turkey, Armenia, Azerbaijan, Georgia, Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan, Turkmenistan and Afghanistan, and as a snake species of secondary medical importance (WHO category 2) in Cyprus, Jordan, Tunisia and Pakistan [49], there clearly is a lack of clinical and epidemiological research on envenoming caused by these widely distributed vipers. While a few published case reports

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[30] May deviate from the published version (Jestrzemski et al. 2022).

illustrate the range of symptoms and signs that may follow envenoming by this species, most are anecdotal or offer little clinical detail [2, 10, 29, 50].

Fraser [12], Hopkins [51] and Göçmen [52] described bites caused by *M. l. lebetina* from Cyprus. In the first case, occurring 1929, a shepherd was bitten on the scalp while kneeling down to drink from a forest pool in Cyprus. He was admitted to the hospital in Famagusta (eight hours after the bite), and discharged 11 days later. This patient initially suffered from a loss of consciousness, massive edema and partial loss of speech, and his bite and incision wounds healed slowly [12]. Two other bites described by Hopkins (1974) occurred when army personnel from the United Nations Peacekeeping Force in Cyprus (UNFICYP) were bitten on the back of the right hand (first case) and on the calf (second case). In the first case, there was initially swelling and vomiting, followed on the third day by high fever and signs of cellulitis. On the 15th day, the patient reported anesthesia and paralysis of his right arm almost up to his elbow. Paralysis resolved within five days and anesthesia within 26 days. In the other case, the patient initially suffered from severe anxiousness and cramps in the injured limb, but was almost free of symptoms on the following day [51].

A particularly severe case was reported by Schweiger (1983) who was bitten by a large (150-160 cm) *M. l. obtusa* in his right forearm near İskenderun (Turkey) and subsequently suffered massive painful edema of the bitten arm extending to the trunk and other arm, blistering, myonecrosis requiring the amputation of the bitten limb, thrombocytopenia, coagulopathy, haemorrhage, hypovolaemic shock, acute kidney injury, necrosis of the lungs, paraesthesia, complete loss of motor function of the legs, and contractures of the knee joints. Ten months after the bite, the patient had largely regained his ability to walk, albeit with highly reduced leg strength [53].

Göçmen (2006) reported a bite on a 40-year-old male researcher who was bitten into a right-hand finger by a 75-cm-long male Cypriot *M. l. lebetina*. Five minutes after the bite, edema started to develop and within three hours advanced to the center of the right arm, accompanied by severe pain. Further symptoms included hypotension, shock, tissue necrosis, hemorrhage, melanoderma, and considerable quantitative differences in the fractions of albumin, globulin and albumin/globulin ratios. After 24 hours, the patient's condition had normalized, and he was discharged from hospital. Complete recovery took place after the sixth week [52].

Sharma et al. (2008) described a case from Jammu and Kashmir, in which a 33-year-old male soldier resting in his bed was bitten in his left hand by a 94.5 cm long *M. l. cernovi*. Twelve days after the bite, the bitten finger had become necrotic and was amputated [54]. In another case in Kashmir, a 46-year-old farmer suffered a *M. lebetina* bite to his penis while urinating on an open field [55]. In both cases, the main symptoms of envenoming included edema, bruising and necrosis around the bite site. The second case in addition had mild coagulopathy but overall a milder clinical course and was discharged 36 hours after the start of treatment. Kazemi et al. (2019) presented five cases of snakebite envenoming by *M. l. obtusa* in Northwestern Iran, which occurred in spring 2019 and resulted in severe clinical courses and long-term musculoskeletal disabilities in some of the bite survivors. In three cases, a fasciotomy had to be performed, affecting the arm of a 15-year-old boy, the hand of a 36-year-old man, and the leg of another, 30-year-old man. In the latter case, extensive necrosis led to the amputation of the leg at the knee. All five patients received polyvalent anti-snake venom from the Razi Vaccine and Serum Research Institute, Karaj, Iran [56].

Cattaneo (2020) provides the first literature record of a bite incident by *M. l. schweizeri*. The victim, a young woman bitten on the right hand, was transferred to the hospital, where a sodium bicarbonate therapy was applied, coupled with the admission of drugs helping to replenish electrolytes and body fluids. The bitten limb swelled to the armpit, accompanied by pain, increased blood sugar and platelets, a reduction in red blood cells, minor hypotension and a decreased heart rate. The patient was discharged after two days, although the swelling and pain in the affected limb needed some additional days to dissolve [57].

Finally, Abukamar et al. (2022) reported of an envenoming by *M. l. obtusa* in Jordan. A 36-year-old male farmer bitten on his left foot presented to his local hospital, with initial symptoms such as dizziness and burning pain in the left leg. His bitten foot and ankle were swollen and hot, with extensive ecchymosis at the bite site. Despite treatment with antivenom, intravenous fluids and antibodies, his condition worsened. Even after transfer to a tertiary hospital in Amman, his platelet count continued to drop. Although neutrophilic leukocytosis and normochromic normocytic anemia were diagnosed, the patient finally recovered, and was discharged after five weeks [58].

5.4.8 Recommendations for snakebite envenoming prevention

Since the risk of snakebite envenoming is a primary concern in Cyprus, educational workshops for occupational outdoor groups such as mosquito control workers, hunters, shepherds, farmers, forestry employees and game wardens are critical for raising awareness of the risks and prevention of snakebite envenoming. Workshops should also be offered to rural communities and schools and include the promotion of non-lethal methods for preventing human-viper conflict such as snake deterrence or translocation. The risk of encounters between people and *M. l. lebetina* in Cyprus can be further reduced by legally protecting valuable snake habitats such as wild riparian areas and rocky slopes with confirmed *M. l. lebetina* populations, with prohibition of land transformation and strict regulations concerning further anthropogenic interventions (e.g., grazing). This could be achieved by designating new areas for the Natura 2000 network of the European Union. In all conservation areas, hunting should be prohibited.

Finally, a major contribution towards reducing the risk of snakebite envenoming in agricultural landscapes and gardens is the removal of organic waste and other structures attracting rats, and of wooden debris and other elements serving as potential snake shelters (H.-J. Wiedl, pers. comm., 29 March 2014).

5.4.9 Limitations of the study and suggestions for research protocol improvement

While the collection of data on annual hospital admissions due to snakebite envenoming in the Republic of Cyprus is a novel step towards a better understanding of the island's snakebite epidemiology, the research protocol for further studies should include additional information, which will allow for a more precise snakebite data analysis. Since all records of hospital-admitted patients were based on the ICD-10 code T63.0, other ICD-10 codes related to snakebite envenoming (i.e., X20, contact with venomous snakes and lizards) were generally not listed by the hospitals, with exception of the two fatal cases, where the underlying causes of death were coded X20. However, X20 would be highly suitable for the non-fatal cases as well, as it explicitly includes vipers. Additionally, the applied ICD-10 code T63.0 can be split into four subcategories: accidental (001), intentional self-harm (002), assault (003) and undetermined (004) [59]. Although this sub-division was not provided for the dataset of this study, it can be assumed that all of the registered envenomings on Cyprus occurred accidentally (T63.001), as no case of assault or intentional self-harm (i.e., attempted suicide) involving snakes has – to the best of our knowledge – ever been published for Cyprus.

Although this article provides for the first time systematically collected data on snakebite epidemiology in the Republic of Cyprus, it is doubtful that all cases of snakebite envenoming between 2013 and 2019 were included. This point can be illustrated by one incident in which an author of this article (DJ) was bitten on the back of his hand while handling a large male *M. l. lebetina* in Polis (Paphos district) on 29 May 2014. Although the bite was delivered into a safety glove, one fang penetrated the glove and punctured the skin. The bitten herpetologist was subsequently transferred to Polis Rural Hospital where he was given injections of cortisone (Solu-Cortef) and antihistamines (Phenergan). As no signs of envenoming could be detected, he was discharged after treatment. This bite accident, consequently, is not included in the 2014 record of ICD-10-coded diagnosis for Polis Rural Hospital. In fact, no case of snakebite envenoming was recorded in Polis Hospital during that year. Hence, future surveys on snakebite-related hospital admissions in Cyprus should also include incidents which might be considered as minor due to the absence of envenoming signs. Our dataset also does not show whether the 288 snakebite admissions refer to 288 or less persons. Accordingly, we cannot exclude the possibility that a person was bitten and admitted repeatedly to public hospitals, although the likelihood of such an occurrence can be considered as rather low. Yet, knowing the exact number of snakebite patients is critical for the calculation of incidence rates.

As the recorded data do not include information on the distribution of age group and sex over month of admission and length of stay, it is not possible to investigate differences between seasonal peaks of snakebite envenoming in males and females in Cyprus, nor between the affected age groups over the year. It is further not possible to examine the data for a correlation between age group (and sex) and length of stay in hospital. In the same way, the missing information of the monthly distribution of length of stay prevents an analysis of possible seasonal trends in this regard. Protocols of future retrospective reviews could help to fill this gap by including this information.

Additionally, the missing information on the localities where the bite accidents occurred currently prevents a statistic of snakebite envenoming in each district. As snakebites often take place in remote areas, people may travel large distances and cross borders to reach the nearest hospitals. Hence, to conclude from the frequency of snakebite-related admissions in a certain hospital to the presence of nearby areas with a possibly increased risk of snakebite envenoming, or with higher abundance of *M. l. lebetina*, is fraught with uncertainty. Therefore, recording the location of the accidents will help to identify snakebite envenoming hotspots and enable a snakebite statistic for each district. Furthermore, the snake species responsible for each bite should be recorded, if reliable identification is possible, in order to distinguish between bites by front-fanged and non-front-fanged snakes in Cyprus. This is necessary to determine whether a potential life-threatening envenoming (*M. lebetina*), a mild envenoming at most (*M. insignitus, T. fallax*), or no envenoming (any other snake species) has occurred. A correct snake species identification is also very helpful for a better assignment of clinical courses to the corresponding species. In the same way, the documentation of the major envenoming symptoms will facilitate distinguishing between potentially life-threatening and mild clinical courses, which is not currently possible with the data at hand.

These points should be addressed by creating a more comprehensive, systematic reporting system for snakebite envenoming on Cyprus, which should include data of admissions from public as well as private hospitals. Finally, the establishment of a poison information center in

Cyprus would be highly useful for a more comprehensive collection of data related to snakebite envenoming on the island.

5.5 Conclusions

Although *M. l. lebetina* is a large viper with a large venom yield, and widely distributed in Cyprus, deaths from snakebite are very rare in the Republic of Cyprus. Over the year, the hazard of snakebite is highest in late summer, particularly during the peak of the dry season in August and September. Occupational outdoor groups (farmers, forestry employees, game wardens, hunters, mosquito control workers and shepherds) are probably at highest exposure of encountering *M. l. lebetina* and experiencing snakebite envenoming. There is further an increased risk in areas supporting rat populations, which in turn attract blunt-nosed vipers. Males are at higher risk of snakebite than females, as are middle- and older age groups in their 60s. Public hospital admission data (years 2013-2019) suggest that the risk of snakebite in the Republic of Cyprus is highest in Paphos district. Although the short average length of hospital stay suggests that the clinical course of most snakebite envenoming cases may not be severe or complicated, further research is warranted to clarify questions regarding the clinical management of such patients. In order to raise awareness of the snakebite risk and how to prevent it, educational programs on snakebite prevention and first aid should be provided especially to outdoor occupational groups, rural communities and schools. Other prevention measures include an improved protection of wild snake habitats, and the removal of structures attracting rats and providing snake shelter in anthropogenic landscapes. Critical information, which is missing in the current dataset (e.g., the localities where the bite accidents occurred, the snake species responsible for the bites, major envenoming symptoms), should be documented in prospective studies in the future. The procurement of antivenoms to treat envenoming by *M. l. lebetina* in Cyprus should be guided by comparative pre-clinical safety and efficacy studies using Cypriot *M. l. lebetina* venoms and different commercially available candidate products, and clinical studies.

5.6 Abbreviations

°E: Eastern longitude; °N: Northern latitude.

5.7 Acknowledgements

The authors would like to thank Stephanie Zeitouni and Michalis Antoniou (Pharmaceutical Services, Ministry of Health), Koulia Onisiforou (Statistical Service), Haris Nicolaou

(Department of Forests, Ministry of Agriculture, Rural Development and Environment), Elena Erotokritou and Elena Stylianopoulou (Department of Environment, Ministry of Agriculture, Rural Development and Environment), Volker Schrempf and Hans-Jörg Wiedl (Cyprus Herpetological Society), as well as Eftychia Demetriade and all interview partners from Cyprus (2014).

5.8 Funding

We acknowledge support by the Faculty of Forest Sciences and Forest Ecology and the Open Access Publication Funds of Göttingen University. Open Access funding was enabled and organized by the Projekt DEAL. The fieldwork in Cyprus was generously supported by the Mohamed bin Zayed Species Conservation Fund, the German Society for Herpetology and Herpetoculture (DGHT, via an award from its Hans-Schiemenz-Fonds), the Department of Forest Zoology and Forest Conservation of the University of Göttingen, and the Nature and Biodiversity Conservation Union (NABU, Germany).

5.9 Authors’ contributions

MA and VS provided the statistical information on snakebite epidemiology in Cyprus and corrected the manuscript. DJ analyzed the data and wrote the manuscript draft, with the participation of UK, FG, BS and PG, who also provided critical feedback for the completion of the final version. All authors read and approved the final manuscript.

5.10 Availability of data and materials

The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

5.11 Ethics approval

The study on hospital admissions due to snakebite envenoming in the Republic of Cyprus (2013-2019) was approved by the Cyprus National Bioethics Committee by decree EEBK ΕΠ 2021.01.193 on 7 October 2021.

5.12 Competing interests

The authors declare that they have no competing interests.

5.13 References Chapter 5

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Chapter 6

General discussion

6.1 Water proximity and habitat use of the Cypriot blunt-nosed viper

In semi-arid regions with annual drought periods, water bodies are of ecological importance for snakes, as they attract their prey, while riparian vegetation and rocky structures inside and around streambeds provide for thermoregulation and protection from natural enemies. Water bodies further facilitate contact between snakes and people, which has contributed to the global problem of snakebite envenoming. In the Levant and Middle East, blunt-nosed vipers are frequently observed close to and even inside water bodies during the dry summer season (e.g., Jestrzemski & Kuzyakova 2018, Al-Sheikhly et al. 2021), which led to the investigation of the relationship between water occurrence and blunt-nosed viper presence in western Cyprus by Jestrzemski & Kuzyakova (2018).

Analyzing the water proximity of wild blunt-nosed vipers in spring 2014 and late summer 2015, we did not find significant differences between these seasons with respect to the distances of *M. l. lebetina* to the nearest water body in general ($P = 0.537$), nor to the nearest natural ($P = 0.497$) or artificial ($P = 0.635$) water body. This contradicted our previous assumptions that the vipers would be closer to water bodies (especially artificial ones) during the dry summer season than in spring, and closer to natural water bodies in spring than in late summer. Likewise, the distances of *M. l. lebetina* to the nearest artificial and natural water body neither differed significantly during spring ($P = 0.295$), nor during late summer ($P = 0.717$), which conflicted with our expectation that the vipers would be closer to natural water bodies during the rainier spring, and closer to artificial water bodies during the dry summer season.

However, it is possible that operational limitations of the field research also limited our ability to properly test these hypotheses. First, our dataset was based on the sampling of a single spring and summer season each, thus containing less data variability than if sampling had been carried out during several consecutive years. In addition, the sampling could not take place in the same year (which would have been ideal), and the data collection periods were not equal (10 weeks in 2014, but only five weeks in 2015). Thus, factors such as climatic differences between 2014 and 2015 might have influenced the spatio-temporal presence and activities of vipers in the field, and a smaller time frame in 2015 allowed for less viper observations in that year. While the sampling in 2014 and 2015 enabled us to measure the water proximity of all recorded vipers, no individual was observed twice. With or without repeated observations of the same vipers, the measured distances to the nearest water body could only reflect the temporary position of the snakes, without providing information about a viper's movement before and after the encounter. For example, a viper observed near an artificial reservoir on one day might have moved to a natural water body (e.g., a stream pool) a few days later, and vice versa. Obtaining data on such short-term movements is only possible via a radiotelemetry survey, which is resource-intensive in terms of equipment and personnel, but can enable researchers to focus on the movements of selected viper individuals throughout the year, and thus cover their whole range of activity, including distances to the nearest water bodies. Finally, the method applied for measuring the distances in the field within 30 m (via a tape and/or Google Maps) and farer away (via Google Maps) did not allow for a fully reliable coverage of all water bodies. First, this was due to physical limitations in the field, as the researcher (DJ) could not access several private properties near the viper encounter spots and was physically unable to survey larger areas for small water bodies during the field trips, especially in difficult terrain. Second, the detectability of water bodies (particularly small ones) on Google Maps was sometimes doubtful due to limited resolution.

If these limitations could be addressed and the data collection methodology improved (i.e., via conducting a radio-telemetry survey including water distance measurements), the research questions on the water proximity of *M. l. lebetina* could be formulated as hypotheses and tested again to obtain more robust conclusions on possible differences in the water attraction of *M. l. lebetina* during spring and summer.

Nonetheless, based on our current knowledge, we can assume that habitat structures which attract blunt-nosed viper prey and conspecifics (i.e., potential mating partners) and allow for thermoregulation, protection from natural enemies, and hibernation in winter, are the most important elements for determining the presence or absence of *M. l. lebetina*, since they are directly related to the survival of the snakes (Jestrzemski & Kuzyakova 2018). Thus, whether a water body attracts a blunt-nosed viper or not certainly depends on its importance for the snake's survival (by providing the above-mentioned critical functions) and on the absence of non-water structures providing those functions as well. For example, Cypriot *M. l. lebetina* have been regularly encountered nearby or inside bird cages (own observations), with the smell of birds possibly being a stronger attractant than the presence of nearby water bodies. Surely, the seasonal needs of a blunt-nosed viper determine the structures that it is most attracted to (see Ščerbak & Böhme 2005). For example, during the mating season, males might ignore water bodies (and thus potentially better opportunities to encounter prey) while following the scent trails of sexually mature females.

While it is not possible to comprehensively investigate the spatial ecology of *M. l. lebetina* based on short-term visual encounter surveys only (see Jestrzemski & Kuzyakova 2018), a radio-telemetry survey would be highly useful for examining the ecological importance of the habitat structures frequented by blunt-nosed vipers over the course of one or more years, especially regarding their relevance for fulfilling the seasonal needs of these snakes. A radio-telemetry survey would further help to investigate how the annual movements and habitat use of the studied viper individuals overlap with areas used by humans, and thus, how the spatial and temporal variability of *M. l. lebetina* movements affects the risk of snakebite in the study area (see Glaudas 2021).

6.2 What do the body mass data of the Cypriot blunt-nosed viper tell us?

Presenting first-time systematically collected of data on the body weight, body length and body condition of Europe's largest and heaviest viper (*M. l. lebetina*), Chapter 2 (Jestrzemski & Kuzyakova 2018) has filled a knowledge gap, as formerly only anecdotical statements on the body weight of blunt-nosed vipers (continental specimens) had been available in the literature. These included Sochurek (1979) mentioning a large male blunt-nosed viper from the USSR exceeding 3 kg in body weight, and Ščerbak & Böhme (2005) reporting a maximum weight of 2,700 (2,000) g for males (females) of *M. lebetina*. Furthermore, different claims on the maximum total length of blunt-nosed vipers have circulated on the internet, several of which are non-scientific, unproven and possibly sensation-seeking (e.g., a Cyprus tourism promotion website claimed that Cypriot blunt-nosed vipers can reach 2 m length and 5 kg weight, accessible at https://www.cyprusisland.net/cyprus-snakes/viper-macrovipera-lebetina). Countering these statements, the present study has established in Chapter 2 the function $Y = \exp(-6.33)*X^{2.86}$ for describing the allometric relationship between SVL and body weight of Cypriot *M. l. lebetina*, thus limiting the maximum weight of the Cypriot blunt-nosed viper to about 2,240 g.

6.3 Fluctuation of body condition and weight in female Cypriot blunt-nosed vipers

The determination coefficient R^2, which describes the relationship between weight and SVL, was markedly lower in *M. l. lebetina* females as compared to males, which hints at factors other than only SVL influencing female body weight in *M. l. lebetina* (see Jestrzemski & Kuzyakova 2018; Chapter 2)

Most likely, these factors are related to the different reproductive role and thus different body composition of female vipers. In the animal kingdom, male selection favors a well-developed skeletal musculature (facilitating mate searching and male-male combat), whereas females need to obtain, process and store nutrients needed for reproduction (i.e., the production of energy-rich gametes). Thus, the alimentary tract, fat bodies and liver can be expected to be disproportionately larger in female organisms. This was confirmed by Bonnet et al. (1998) who found females of *Vipera aspis* to have significantly larger fat stores, liver and viscera mass than males. This sexual dimorphism reflects the substantial sex-specific differences in snake body composition. The larger sizes of organ systems necessary for energy processing and storage underline the relevance of energy stores for reproduction in female snakes.

As energy storage varies during the annual cycle, the time of sampling determines which sex will have the largest fat stores (Bonnet et al. 1998). Hence, female body mass in snakes can be expected to shift over the year. This was also observed in the significant differences in BCI ($P = 0.001$) between female *M. l. lebetina* collected during the breeding season (spring 2014) and after it (late summer 2015). Not surprisingly, females caught during the breeding season were generally heavier, likely reflecting a higher need for energy storage for reproduction, as compared to late summer, when energy storage was partially depleted, and body masses had dropped. Another reason for body mass fluctuation in female *M. l. lebetina* may be the additional mass of ovarian follicles in spring, followed by postpartum weight loss in summer, given that the females had reproduced in that year (Jestrzemski & Kuzyakova 2018). In fact, postparturient female vipers can be extremely emaciated and often do not survive the year of parturition, with mortality rates as high as 75% in French *V. aspis* (Aubret et al. 2002).

Against this background, it may be assumed that the sampling season (in this study: spring or late summer) is a critical determinant of female *M. l. lebetina* mean body mass and thus, mean BCI. Although the mean BCI was overall significantly higher in females than in males ($P = 0.048$, Chapter 2), the results probably would have been different if more females of *M. l. lebetina* had been sampled outside the breeding season. For example, females collected exclusively in late summer would certainly have a lower mean BCI than those collected in spring (see Jestrzemski & Kuzyakova 2018).

Unlike in males, the amassment of considerable energy reserves is a requirement for female vipers to initiate reproductive activities (e.g., migration to nesting sites). In line with this assumption, *V. aspis* females with below-average body condition had low levels of the major sex hormone estradiol, and did not respond to male courtship behavior, while those with markedly higher reserves had high estradiol levels and were sexually responsive (Aubret et al.

2002). In contrast, males of all body condition indices had significant testosterone levels and actively engaged in courtship (Aubret et al. 2002). Thus, one can assume that the maintenance of an above-average body condition is a critical requirement for the reproductive success of *M. l. lebetina* females in spring.

6.4 Variation of body condition in male blunt-nosed vipers from Cyprus

While numerous authors mention cases of snake infestation with diseases and parasites (e.g., Nasiri et al. 2014), published literature on the correlation between snake health and morphometric parameters is scarce. Therefore, to our best knowledge, Chapter 2 also represents the first study linking a body condition index, as an indicator of general health, to the mean SVL of vipers (and possibly snakes in general). In this regard, the significant differences between the mean BCI values of small to medium-sized and large-sized males of *M. l. lebetina* ($P = 0.0258$) indicate a trend towards deteriorating health condition in older vipers, which is supported by the dataset of Israeli *D. palaestinae* (Chapter 2). Here, even stronger differences were found between the mean BCIs of both subgroups of male Palestine vipers ($P = 0.0004$), which hints at decreasing body condition with increasing size (and thus, age) as a general trend in male vipers in the genera *Macrovipera* and *Daboia*. With 186 male Palestine vipers, the Israeli dataset is more than 13 times larger than the Cypriot dataset (14 male blunt-nosed vipers), and thus, the BCI differences in male *D. palaestinae* are even more meaningful.

We can assume that snakes with a higher BCI have an advantage over those with a lower BCI, as they are more likely to survive periods of food or water scarcity, withstand parasite infestation over longer periods of time, and have more fat reserves as buffer for balancing energy-depleting periods of the year (see Sperry & Weatherhead 2008). However, while a higher BCI certainly also benefits male blunt-nosed vipers in combat dancing, the generally larger sizes and weights of older, longer individuals may be a more important factor for male mating success in snakes. Thus, BCI might not be the decisive factor for this aspect of the reproductive ecology of male blunt-nosed vipers. A study on male mating success in red-sided garter snakes (*Thamnophis sirtalis*) confirmed both the advantages of large body size and above-average body condition, as larger and heavier males obtained more matings while larger males also mated with larger females and possessed larger hemipenes and testes (Shine et al. 2000).

Albeit it would be interesting to investigate possible differences in BCI also within females of *M. l. lebetina*, any trend (e.g., towards a lower or higher BCI with increasing SVL) would be likely harder to detect than in male vipers, due to the more distinct annual body mass fluctuation in female *M. l. lebetina* (Bonnet et al. 1998). Further research on possible correlations between body size parameters (e.g., SVL, TaL) and BCI should be conducted with additional species (and subspecies) of the *Macrovipera* and *Daboia* complexes to investigate possible trends with ecological implications.

6.5 Sex-specific variation and relative tail length

The significantly higher SVL ($P = 0.039$), TaL ($P = 0.026$), ToL ($P = 0.02$) and HL ($P = 0.011$) observed in adult males (Chapter 2) reflect the overall larger body dimensions in males of *M. l. lebetina*. Although males exceeded females in HL, there were neither significant sex-specific differences for HW ($P = 0.135$), nor for the ratio HL/HW ($P = 0.134$). This is surprising since longer heads would hint at a higher HL/HW ratio in males. However, if the trend of greater head length in males and equal head width in both sexes persisted with increasing sample size, the HL/HW ratio would likely also become significantly larger in males. Despite males having longer tails, sex-specific differences were insignificant for relative tail length (TaL/SVL, exact $P = 0.195$), which implicates that TaL/SVL is an unsuitable indicator for sex determination in *M. l. lebetina.*

In general, relative tail length is among the most widespread examples of sexual dimorphism in snakes (Shine et al. 1999), with several possible explanations for its evolution. While sexual dimorphism in SVL may be biased towards males or females, TaL/SVL is typically considered higher in male snakes (King 1989). Three hypotheses have been created to explain this dimorphism: While the “Morphological Constraint Hypothesis” suggests that TaL is relatively longer in males for the accommodation of hemipenes and retractor muscles, the “Female Reproductive Output Hypothesis” proposes a relatively shorter TaL in females, originating from natural selection for higher reproductive capacity. Finally, the “Male Mating Ability Hypothesis” suggests that relatively longer tails in males are advantageous during courtship and thus favored by sexual selection (King 1989). Both the first and third hypothesis were tested and confirmed with *T. sirtalis* by Shine et al. (1999), who found relative TaL longer in males, and a biologically relevant trait affecting male mating success. In fact, longer tails in males can be of combined advantage by providing space for larger hemipenes and enhancing male mating ability, which could have been critical in the evolution of sex-specific tail-length dimorphism (Shine et al. 1999). Hence, longer relative TaL in males is considered a sexual selected trait (Sivan et al. 2020).

In this regard, it is possible that the differences in relative tail length between male and female Cypriot blunt-nosed vipers will become significant with increasing sample size, thus confirming the assumption of sexual selection favoring males with relatively longer tails. As snakes have a strongly simplified morphology, sexual tail dimorphism in snakes is also highly suitable for studying evolutionary hypotheses (Shine et al. 1999).

6.6 Occurrence of reduced tails in female blunt-nosed vipers

Interestingly, the correlation coefficients between SVL and TaL (R) show a significant correlation between both characters for adult males (N = 16, R = 0.64, *P* = 0.008), but not for adult females (N = 21, R = 0.37, *P* = 0.095). Thus, we can assume that *M. l. lebetina* tail length varies more strongly in adult females than in adult males, which prevents tail length predictions in females based on SVL. Furthermore, the natural expectation that TaL grows proportionally with SVL in vertebrates was not met by the studied females of *M. l. lebetina*.

A possible reason for this remarkable deviation could be that some of the measured females had been born with abnormally short tails or had lost their tail tips due to accidents or attacks by natural enemies. In the latter case, female *M. lebetina* might be more prone to attacks because of their more sedentary lifestyle (see Ščerbak & Böhme 2005). Staying for longer time periods in structures shared with other animals might render them more exposed to potential predators sharing the same habitat. These could include small carnivores (e.g., red foxes), but also smaller mammals which might prey on juvenile *M. l. lebetina*, such as insectivores (e.g. hedgehogs) and rats, the latter of which are extremely common in Cyprus (Jestrzemski & Kuzyakova 2018). Furthermore, snake tails may be confused with earthworms or insect larvae by other insectivorous animals such as birds (see Da Fonseca et al. 2019). Additionally, snakes in Cyprus are directly preyed upon by humans and human-introduced predators such as feral cats (Baier et al. 2013), while potential avian predators of blunt-nosed vipers include snake-eating raptor species such as the short-toed snake eagle (*Circaetus gallicus*) (Roth & Corso 2007).

6.7 Venom proteome variability among blunt-nosed viper subspecies

During the last two decades, increased research has been conducted on blunt-nosed viper venoms, which resulted in the unraveling of the venom proteomes of *M. l. transmediterranea* from Tunisia (Bazaa et al. 2005), *M. l. obtusa* from Armenia (Sanz et al. 2008), Southeastern Anatolia (İğci & Demiralp 2012) and Dagestan (Pla et al. 2020), and finally of Cypriot *M. l. lebetina* and Iranian *M. l. cernovi* (Ghezellou et al. 2022). Still, the venom proteomes of *M. l. schweizeri* and *M. l. turanica* have not yet been analyzed. Although the presently known venom compositions are fundamental to our understanding of blunt-nosed viper venomics, the analyzed samples represent only a negligible percentage of the actual venomic diversity within the species complex *M. lebetina*, also given that ontogenetic and sex-specific comparative venomics have not yet been performed for blunt-nosed vipers.

Among the identified toxin protein families of *M. l. lebetina*, SVMPs clearly dominated the venom proteomes of the subspecies *M. l. transmediterranea* (67%), *M. l. lebetina* (28%), Armenian *M. l. obtusa* (32%), Southeast Anatolian and Dagestani *M. l. obtusa* (each 24%) and Iranian *M. l. cernovi* (35%) (İğci & Demiralp 2012, Ghezellou et al. 2022). While PIII class SVMPs prevailed especially in *M. l. transmediterranea* (67%) and in *M. l. lebetina* (25%) and were also quite prominent in *M. l. cernovi* (16%), the PI class made up the largest portion in Armenian (28%) and Dagestani (15%) *M. l. obtusa,* and in *M. l. cernovi* (20%) (Ghezellou et al. 2022).

Playing a critical role in viper envenoming pathologies, SVMPs are common toxins in blunt-nosed viper venoms and major factors for local and systemic hemorrhage. As they also disrupt the hemostatic system and weaken the components of basement membranes, their lethal hemorrhagic activity causes typical symptoms of viper envenoming such as internal bleeding, intravascular clotting, edema, inflammation, and necrosis (Takeda et al. 2012).

Interestingly, enormous differences in the hemorrhagic potential of SVMPs can be observed, which are related to the presence or absence of additional domains. PIII SVMPs are stronger hemorrhagic toxins than PI SVMPs, as they comprise Dis-like and Cys-rich domains in addition to the metalloproteinase domain, while PI SVMPs only have the catalytic domain (Gutiérrez et al. 2016). Thus, P-III SVMPs are the most potent and diverse SVMP class, as they can induce both local and systemic bleeding, while P-I SVMPs mainly only induce local hemorrhage (Takeda et al. 2012). Furthermore, PII and PIII SVMPs are more effective in causing capillary damage, and able to inhibit platelet aggregation (thus enhancing the hemorrhagic state), although these effects on hemostasis have been observed in vitro, but not yet in vivo (Takeda et al. 2012, Gutiérrez et al. 2016). In this regard, the stronger expression of the PIII SVMPs in the Mediterranean subspecies *M. l. transmediterranea* and *M. l. lebetina* may indicate that their venoms have higher hemorrhagic activities than those of continental subspecies (i.e., *M. l. obtusa* and *M. l. cernovi*), where PI SVMPs prevail. However, as PI SVMPs are known to disrupt muscle tissue, besides causing consumption coagulopathy, the venoms of *M. l. obtusa* and *M. l. cernovi* may show higher myotoxic activity (Ghezellou et al. 2022).

Besides SVMPs, another major group of enzymes found in snake venoms, SVSPs, was most prominent in *M. l. lebetina* (17%), Dagestani (23%) and Armenian *M. l. obtusa* (15%) venom, and in the venom of *M. l. cernovi* (16%) but constituted only 9% in *M. l. transmediterranea* venom and 6% in the venom of Southeast Anatolian *M. l. obtusa* (Ghezellou et al. 2022). Despite the different expression of this enzyme group, the latter subspecies is geographically relatively close to Armenian *M. l. obtusa*. The overall rather strong presence of the SVSP group is not surprising, as it is commonly found in blunt-nosed viper venom, impacting several physiological functions (e.g., blood coagulation, fibrino(geno)lysis and platelet aggregation) of the target organism (Siigur et al. 2019).

Disintegrins constituted the third major toxin group found in the *M. lebetina* venom proteome and were most abundant in *M. l. lebetina* venom (16%), followed by *M. l. cernovi* venom (14%), and by Dagestani (13-14%) and Armenian as well as Anatolian *M. l. obtusa* venom (each 11%) (İğci & Demiralp 2012, Ghezellou et al. 2022). The disintegrin content was lowest in *M. l. transmediterranea* with 6-7% (Ghezellou et al. 2022). Dimeric disintegrins constituted the vast majority of disintegrins in *M. l. transmediterranea* (6%), *M. l. lebetina* (13%), Armenian *M. l. obtusa* (9%) and *M. l. cernovi* (14%) venom, while the remaining portions belonged mostly to the short disintegrin subgroup (Ghezellou et al. 2022). No information on the division of integrins was provided for Southeast Anatolian *M. l. obtusa* venom (İğci & Demiralp 2012). The strong presence of disintegrins furthermore adds to the lethal effect of *M. l. lebetina* venom on prey organisms, by selectively blocking the function of integrin receptors (Calvete et al. 2005) and inhibiting platelet aggregation (McLane et al. 1998). It should be noted that PIII-SVMPs, the major fraction of SVMPs in the analyzed *M. l. transmediterranea* and *M. l. lebetina* venom proteomes, include disintegrin-like domains (Fox and Serrano 2005). Dimeric disintegrins belong to the snake venom-based integrins with anti-neoplasic properties, while obtustatin is the shortest disintegrin yet described (Marcinkiewicz et al. 2003).

Interestingly, PLA2 was by far the most common protein family found in the venom of Southeast Anatolian *M. l. obtusa* (34%), whereas its proteome share was much lower in Armenian (15%) and Dagestani (14%) *M. l. obtusa* (İğci & Demiralp 2012, Ghezellou et al. 2022). Yet, it still constituted 9% each in *M. l. lebetina* and *M. l. cernovi* venom, while its share was lowest in the venom of *M. l. transmediterranea* (4%) (Ghezellou et al. 2022). The prominence of PLA2 in *M. l. obtusa* venom is not surprising, as it is one of the most widespread toxin protein families in vipers and elapids, being responsible for neurotoxicity, hemolytic activity, myotoxicity, anticoagulant, antiplatelet and antibacterial effects, as well as antitumor and antiangiogenic activities (Zouari-Kessentini et al. 2013).

The fifth group of relatively common toxins, C-type lectin-like proteins, was most abundant in *M. l. transmediterranea* (10%) and Armenian *M. l. obtusa* venom (15%), but less abundant in the venom of Southeast Anatolian (8%) and Dagestani (9%) *M. l. obtusa*, and in the venom of *M. l. cernovi* (8%). It was least represented in the *M. l. lebetina* venom proteome (4%) (İğci & Demiralp 2012, Ghezellou et al. 2022). C-type lectin-like proteins form a subgroup within the biologically active C-type lectins (CLEC), which strongly affect platelet aggregation (Clemetson et al. 2009, Siigur et al. 2019).

6.8 Phylogenetic relations between Eastern Mediterranean blunt-nosed vipers, island immigration theories and venom proteome variability

Molecular evidence by Stümpel (2012) suggests that the insular blunt-nosed viper populations (*M. l. schweizeri*, *M. l. lebetina*) and those from the southern Anatolian mainland originate from a common recent ancestor, which likely inhabited a closed area along the southern Turkish coastline and probably colonized Cyprus during the Middle Pleistocene (Stümpel 2012). Thus, the nominate subspecies *M. l. lebetina* might not be restricted to Cyprus but could also occur between the Turkish coastal cities of Iskenderun and İncekum, where morphologically similar blunt-nosed vipers have been recorded (e.g., Entzeroth 1989). Stümpel (2012) classifies the Eastern Mediterranean blunt-nosed vipers (*M. l. schweizeri*, *M. l. lebetina*) as monophyletic, posing a sister clade to the other taxa of *M. lebetina*. Thus, within the genus *Macrovipera*, both taxa can be considered as the most closely related subspecies (Stümpel 2012). In fact, Freitas et al. (2020) recovered *M. schweizeri* and *M. lebetina* as a single unit and conclude that the *Macrovipera* subclade "*lebetina* + *schweizeri*" diverged about 2.62 million years ago (MYA).

Yet, the establishment of the insular populations of *M. lebetina* (Cyprus, Cyclades) remains elusive, with conflicting theories of how the immigration of *M. lebetina* from the Levant mainland to Cyprus could have happened. One theory includes the possibility of immigration via a terrestrial corridor during the Messinian salinity crisis (MSC, 5.96 to 5.33 MYA), when the Mediterranean was disconnected from the Atlantic circulation and large-scale desiccation drastically lowered the sea level (Hsü et al. 1973, Baier et al. 2013). Although a Messinian terrestrial overpass between Cyprus and the mainland has not yet been geologically proven, the high congruence between the herpetofauna of Cyprus and the nearest mainland hints in fact at a colonization via a terrestrial corridor (Schmidtler et al. 2009).

Another theory is that the colonization of the east Mediterranean islands by *M. lebetina* occurred by crossing the sea during eustatic lows (Stümpel 2012). During Pleistocene glacial maxima, the sea level dropped by more than 120 m (compared with today), which reduced the distance between Cyprus and the Turkish mainland to a minimum of 30 km, thus enabling active maritime migration (Baier et al. 2013). The low share of endemic Cypriot reptile and amphibian species further points at a relatively recent colonization during the Pleistocene (Böhme & Wiedl 1994, Corti et al. 1999).

The third theory hints at a possible (accidental or intentional) human transportation of *M. lebetina* to Cyprus (Böhme & Wiedl 1994, Corti et al. 1999). Consequently, Cypriot *M. l. lebetina* could not be older than the Neolithic, when humans first colonized Cyprus (Böhme & Wiedl 1994). This view is also shared by Torres-Roig et al. (2020), who refer to burnt vertebrae remains of *M. l. lebetina* dating back to the 10th millennium before Christ (BC), which were discovered on Cyprus' oldest archaeological site Aetokremnos, together with bones from the Cyprus grass snake (*Natrix natrix cypriaca*), the large whip snake (*D. jugularis*) and other vertebrate taxa (Simmons 2002). However, while humans have hunted blunt-nosed vipers on Cyprus already more than 10,000 years ago, there are major arguments against the theory of a human introduction. Given the arduous undertaking of colonizing a new island under Neolithic living conditions, it appears highly unreasonable that the earliest settlers of Cyprus would have deliberately translocated an extremely dangerous snake species like *M. lebetina*. Even if individual vipers (e.g., juveniles) would have been accidentally transferred to Cyprus, it is doubtful that they would have survived, successfully reproduced, and colonized the island (C. Andrijczuk, pers. comm.). However, if the Cypriot *M. l. lebetina* and/or the Cycladic *M. l. schweizeri* population would indeed originate from anthropogenic translocation, their venom compositions might show much more congruence (also with the venom compositions of southern Anatolian populations) than under the scenario of a natural, much earlier colonization of Cyprus.

A first venomic study addressing the unresolved taxonomic relationship between Cypriot and Anatolian blunt-nosed vipers was carried out by Göçmen et al. (2006), who compared the electrophoresis patterns of their venom proteins and found discernible differences, concluding that the Anatolian specimens do not belong to the nominate subspecies *M. l. lebetina*.

In this regard, a future in-depth comparative investigation of the venom proteomes of *M. l. schweizeri* (Cyclades), *M. l. lebetina* (Cyprus) and *M. lebetina* ssp. from the Anatolian coast would be useful for obtaining new insights into the evolutionary history and phylogenetic relationships of these three enigmatic taxa.

6.9 Venom lethality and proteome composition of insular and continental blunt-nosed vipers in the context of dietary differentiation

Macrovipera lebetina ssp. are large viperids that depend on vertebrate prey, especially on small mammals and birds. Despite the intraspecific venomic diversity within *M. lebetina*, blunt-nosed viper venoms generally are very potent and able to quickly kill mammalian and avian prey (Ščerbak & Böhme 2005). However, significant intraspecific differences between the proteomic compositions of blunt-nosed viper venoms exist, which may be the outcome of "evolutionary arms races" between the vipers and their prey, and thus related to the dietary differentiation of *M. lebetina* ssp. (see Murphy 2010).

In this regard, a particular focus should be on the comparison between insular and mainland *M. lebetina* ssp., since the latter reach greater body sizes, have access to a larger variety of prey taxa, and are of greater medical importance. The most striking contrast is probably between the large Eurasian blunt-nosed vipers (e.g., *M. l. obtusa*, *M. l. cernovi*, *M. l. turanica*) and the much smaller *M. l. schweizeri*, a (sub)species restricted to four Cycladic islands. In contrast to the continental subspecies, stomach contents from insular blunt-nosed viper populations have provided a more comprehensive overview of their prey taxa, as the viper prey fauna on islands is geographically more restricted.

For *M. l. schweizeri* from Milos, Adamopoulou et al. (1997) identified rodents (*Rattus* sp., 43%), lizards (21%) and invertebrates (28%, mainly Coleoptera) as the dominant prey items, whereas birds made up only 7%. In contrast, Nilson et al. (1999) pointed out that *M. l. schweizeri* had evolved preying on small birds, particularly during the avian migration in spring and autumn, while the rodent species found on Milos (*Mus musculus*, *Rattus rattus*) are not autochthonous and were possibly introduced by paleolithic seafarers (Nilson et al. 1999).

The dietary preferences of Cypriot blunt-nosed vipers are similar to those of their Cycladic relatives, with rodents (*Rattus* sp., *Mus* sp.) and birds (mostly Passeridae) as main prey items. Juveniles feed on insects, small mice, and lizards including *Ablepharus budaki* and *Ophisops elegans* (see Osenegg 1989, Baier et al. 2013, Jestrzemski & Kuzyakova 2018).

Continental blunt-nosed vipers feed on a similar prey range. In Central Asia, mammals constitute up to 70% of their diet, followed by birds (up to 20%) and reptiles (up to 10%, with lizards as the main diet of juveniles). Regional differences exist between the Kopet Dag mountains in the border region of Iran and Turkmenistan, where blunt-nosed vipers mainly prey on mammals, and the Nuratau mountains (Uzbekistan), where they primarily target birds (Bogdanov 1962). Caucasian blunt-nosed vipers (*M. l. obtusa* in Georgia and Azerbaijan) prey on various mammals, including relatively large (juvenile *Lepus europaeus*) and potentially dangerous species (*Mustela nivalis*), as well as birds (e.g., *Sturnus vulgaris*, *P. domesticus*), and lizards (e.g., *O. elegans*, *Lacerta strigata*) (Alekperov 1978). Similarly, the diet of Iranian *M. l. cernovi* comprises small birds (including *Alectoris chukar* and *Galerida cristata*) and rodent species (e.g., *Rhombomys opimus*) (Ghezellou et al. 2022).

Despite partially contradicting literature statements on the preferred diet of *M. l. schweizeri*, and the dietary similarities between blunt-nosed viper subspecies, it can be assumed that Cycladic blunt-nosed vipers prey on smaller species than their much larger mainland relatives. The intravenously measured LD50 in laboratory mice (23.5 mg/kg) of *M. l. schweizeri* is not much different from that of the mainland subspecies *M. l. obtusa* (30.1 mg/kg), *M. l. cernovi* (17.6 mg/kg) and *M. l. turanica* (20.5 mg/kg) (Archundia et al. 2011). This was confirmed by García-Arredondo et al. (2019), who found the LD50 of *M. l. schweizeri* (17.32 µg/mouse) to be between *M. l. obtusa* (16.32 µg/mouse), *M. l. turanica* (18.36 µg/mouse) and *M. l. cernovi* (19.71 µg/mouse). These findings suggest a relatively high toxicity of *M. l. schweizeri,* which is impressive, since no fatal envenomings of people by Cycladic blunt-nosed vipers have been recorded so far, contrary to the mainland subspecies. The high toxicity could indicate that these relatively small blunt-nosed vipers need a fast-acting, potent venom to shut down their major prey organisms, migratory birds, and then proceed to swallow them (see Nilson et al. 1999).

The intraperitoneally measured LD50 of Cypriot *M. l. lebetina* (7.58 mg/kg) (Nalbantsoy et al. 2012) is higher than that of *M. l. obtusa* (1.057 mg/kg, Demiroz et al. 2018; 1.74-1.85 mg/kg in mice and 1.86-2.0 mg/kg in rats, Darbinyan et al. 2018; 1.8-1.9 mg/kg in mice, Voskanyan et al. 2010) and Algerian *M. l. transmediterranea* (2.5 mg/kg, Bennacef-Heffar & Laraba-Djebari 2003). This is surprising, since Cypriot blunt-nosed vipers prey on animals of similar size and defensibility (e.g., *Rattus* sp.) as their mainland counterparts. Thus, the higher LD50 of *M. l. lebetina* cannot be explained by weaker prey species. However, since there is no comparative LD50 study for the genus *Macrovipera*, methodological differences between the authors prevent an exact comparison and validation of the results. Considering that Cypriot blunt-nosed vipers commonly prey on large-sized and defensive rodents (see Jestrzemski & Kuzyakova 2018), their venom must contain powerful toxins to quickly shut down the prey organism and prevent injury by struggling rats, or the escape of the prey.

Hence, it is not surprising that the first detailed venom proteome analysis for *M. l. lebetina* (Ghezellou et al. 2022) shows a prevalence of potent toxin families such as SVMPs (28%, causing internal bleeding, intravascular clotting, edema, inflammation and necrosis), SVSPs (17%, impacting physiological functions such as blood coagulation, fibrino(geno)lysis and platelet aggregation), disintegrins (16%, selectively blocking the integrin receptor function and inhibiting platelet aggregation), PLA2 (9%, with neurotoxic, hemolytic, myotoxic, anticoagulant, antiplatelet and antibacterial effects) and LAAO (8%, inhibiting ADP or collagen-induced platelet aggregation), which is also responsible for the yellow color of blunt-nosed viper venom (**Figure 6.1a**).

On the North African and Eurasian mainland however, the number of potential prey taxa and likely also the variation in the venom composition of *M. lebetina* is much higher than in Cyprus and the Cycladic islands. Yet, only five in-depth venomic studies have been published so far for continental *M. lebetina* ssp., covering *M. l. transmediterranea* (Bazaa et al. 2005), *M. l. obtusa* (Sanz et al. 2008, İğci & Demiralp 2012, Pla et al. 2020) and *M. l. cernovi* (Ghezellou et al. 2022), while no in-depth investigation of the venom proteome has yet been conducted for *M. l. schweizeri*, *M. l. turanica* and *M. razii*. In the absence of this information and considering that even the published studies cover only a small fraction of the potential venomic variability within *M. lebetina*, a comprehensive comparison of the venomic variability and dietary preferences of *M. lebetina* ssp. is not yet possible.

Additionally, fundamental methodological differences exist in the procurement of venom samples, which further hinders a meaningful comparison. While venom from captive-bred individuals was used for the studies of *M. l. transmediterranea* (Bazaa et al. 2005) and *M. l. lebetina* (Ghezellou et al. 2022), the venom samples of *M. l. obtusa* (Sanz et al. 2008, İğci & Demiralp 2012, Pla et al. 2020) and *M. l. cernovi* (Ghezellou et al. 2022) were harvested from wild specimens with a completely different diet. Finally, the taxonomic status of some continental subspecies (e.g., *M. l. transmediterranea*) is controversial and unresolved.

In this regard, advances in the taxonomic resolution of *M. lebetina* ssp., the provision of more detailed information on the diet of sampled *M. lebetina* populations, and the implementation of more venomic studies (involving unresearched subspecies and populations) would be very helpful for drawing first conclusions on the impact of dietary preferences on the venom proteome variability of insular and continental blunt-nosed vipers.

6.10 Natural enemies and their potential impact on the blunt-nosed viper venom proteome and toxicity

Snakes are an integral part of the diet of various animals, particularly birds of prey, small carnivores and ophiophagous reptiles. As predators and their prey species are interlocked in a “co-evolutionary arms race” (Murphy 2010), selection pressure exerted by ophiophagous predators has contributed to the high toxicity of several snake taxa (see Phelps 2010), and possibly also played a major role in shaping the venom proteome expression of *M. lebetina* ssp.

The Cypriot blunt-nosed viper lives in sympatry with the large whip snake *D. jugularis* (non-venomous) and Eastern Montpellier snake *M. insignitus* (mildly venomous, **Figure 6.1b**), whose diet includes snakes and which occur in the same habitat as *M. l. lebetina* (Baier et al. 2013). Thus, both colubrid species are possibly significant potential natural enemies of *M. l. lebetina* in Cyprus. Furthermore, blunt-nosed vipers in Cyprus have been sharing their island with small carnivores since at least the Pleistocene.

In pre-Neolithic times, the Cypriot fauna included mammals such as the pygmy hippopotamus (*Phanourios minutus*), the pygmy elephant (*Elephas cypriotes*) and genetts (*Genetta plesictoides*) (Hadjisterkotis & Masala 1996). The latter are known to feed on a variety on small wildlife, including snakes (Janzen 1976), and could have posed a threat to blunt-nosed vipers in prehistoric times. With the arrival of the first humans, new mammal species were introduced from the Levant mainland, including carnivores (e.g., *M. nivalis, Felis silvestris, Vulpes vulpes*), which are known to attack and eat snakes, and compete with them for prey resources (Klauber 1972, Stobo-Wilson et al. 2021). While most mammalian species from the Pleistocene were extirpated from Cyprus by man (including the genetts), newly introduced predators such as feral cats and red foxes have persisted until today (Hadjisterkotis & Masala 1996), together with the introduced long-eared hedgehog (*Hemiechinus auratus*) (Boye 1991). Although house cats are very fast predators (see Baier et al. 2013), blunt-nosed vipers succeed in envenoming them (own observation, **Figure 6.1c**). Red foxes encroach on blunt-nosed viper habitats as well (own observation, **Figure 6.1d**). Besides mammals, Cypriot blunt-nosed vipers have been co-existing with various species of raptors, which act both as predators of snakes and competitors for food (Roth & Corso 2007, Ion et al. 2011).

Considering a scenario of constant predation and food competition by small carnivores, raptors and other snake species in Cyprus, one may deduct that *M. l. lebetina* developed a relatively potent venom, with a low LD50 value. However, given the presumably higher predation pressure experienced by continental blunt-nosed vipers, it is unsurprising that the intraperitoneally measured LD50 of *M. l. obtusa* (1.057 – 1.85 mg/kg in mice) and *M. l. transmediterranea* (2.5 mg/kg) were lower than that of Cypriot *M. l. lebetina* (7.58 mg/kg) (Bennacef-Heffar & Laraba-Djebari 2003, Nalbantsoy et al. 2012, Darbinyan et al. 2018, Demiroz et al. 2018). The venom proteome composition of *M. l. lebetina* shows some minor differences to the mainland subspecies as well, for example, a relatively high share of SVSPs (17%) and disintegrins (16%) as well as the lowest share of C-type lectin-like proteins (only 4%).

In contrast to *M. l. lebetina*, no ophiophagous carnivores co-evolved with *M. l. schweizeri* on the four Aegean islands (Milos, Kimolos, Polyaigos and Sifnos) inhabited by this geographically highly restricted subspecies (Nilson et al. 1999). It is also not known that any potential predators of *M. l. schweizeri* were transferred to Milos by early seafarers, except for recently introduced feral cats (Nilson et al. 1999). Even small rodents (i.e., *Rattus* sp. and *Mus* sp.) were unknown to blunt-nosed vipers before the first humans arrived on the Cyclades. Although birds of prey commonly feed on snakes (Janzen 1976), and bird migrations take place via the Cyclades, no raptor predation on *M. l. schweizeri* is mentioned by Nilson et al. (1999) and Nilson (2005). Equally, no potential ophidian predators of *M. l. schweizeri* occur on its native islands (Cattaneo (2020).

Hence, as *M. l. schweizeri* probably did not evolve under high predation pressure nor had to struggle with potentially dangerous prey (*Rattus* sp.), they presumably did not need to develop particularly powerful toxins. Although no fatal human envenomings by *M. l. schweizeri* have yet been recorded (Nilson et al. 1999, Nilson 2005, Cattaneo 2020), its surprisingly potent venom (see Archundia et al. 2011, García-Arredondo et al. 2019) is an effective weapon for anti-predator defense. This hints at a limited influence of predation pressure on the toxicity of blunt-nosed vipers, with other factors being possibly stronger drivers of venom proteome evolution. Since no LD50 comparison between the insular taxa *M. l. lebetina* and *M. l. schweizeri* has yet been published, it is not possible to directly compare their venom toxicity.

On the Eurasian mainland, however, a much larger variety of ophiophagous predators are found. Key predators of snakes include several species of raptors (e.g., *Circaetus ferox* and *Buteo rufinus*) and mammals (e.g., *V. vulpes*), as well as reptiles (e.g., *Varanus griseus* and *Naja oxiana*) (Ščerbak & Böhme 2005). Among the snake fauna of continental Eurasia, the colubrids *D. jugularis* and *M. insignitus*, whose distribution overlaps in large parts with that of the blunt-nosed viper (including Cyprus), are likely the most important potential predators of *M. lebetina* (see Latifi 1991, Baier et al. 2013). Of several small carnivore species in North Africa and Western Asia, major snake predators such as mongooses and honey badgers (Janzen 1976) can be regarded as natural enemies of blunt-nosed vipers, with the Javan mongoose (*Herpestes javanicus*) being a key predator of *M. lebetina* in the Mesopotamian marshes (Al-Sheikhly et al. 2021). Wild boar (*Sus scrofa*) can be a predator of blunt-nosed vipers as well, as it includes snakes in its diet (Graitson et al. 2019), and its distribution fully overlaps with that of *M. lebetina* ssp. (IUCN 2021).

Since young *M. lebetina* are exposed to a larger variety of natural predators than adults, it can be assumed that the venom of juvenile blunt-nosed vipers displays a higher toxicity to certain predators than during the adult life stage, when the diversity of natural enemies has decreased, and former potential predators may become blunt-nosed viper prey (see Mushelišvili 1970), while also a much greater amount of venom can be delivered in defense. Hence, an ontogenetic study of *M. lebetina* ssp. venom lethality and proteome composition would be very helpful to investigate a possible "evolutionary arms race" between blunt-nosed vipers and their predators.

Despite recent advances in venomic research, the small number of in-depth venomic studies available for *M. lebetina* ssp. (i.e., for the taxa *transmediterranea, lebetina, obtusa* and *cernovi*) is outweighed by the numerous unsampled populations in North Africa and particularly the vast Eurasian mainland, where the distribution of *M. lebetina* spans over 3,700 km. Hence, more venomic studies are required, also covering the taxa *M. l. turanica* and *M. razii,* and generally more localities within all taxa of *Macrovipera*. Besides evolutionary influences on the venom composition of *M. lebetina* ssp. due to ontogenetic development, diet, natural enemies and competitors, other factors such as genetic variability and environmental pollution should be considered as well. Regarding the evolutionary relationships of insular populations of *M. lebetina*, a future investigation of the *M. l. schweizeri* venom proteome and a comparative LD50 study including both island subspecies would contribute to a better understanding of possible correlations between the evolutionary pressure exerted on insular blunt-nosed viper populations and their venom proteome composition and toxin expression.

Figure 6.1. a The flavoprotein LAAO is responsible for the yellow color of blunt-nosed viper venom (*M. l. lebetina* held by H.-J. Wiedl, 14 April 2014). **b** The Eastern Montpellier snake (*M. insignitus*) is a large, rear-fanged venomous colubrid native to the Mediterranean region and sympatric to *M. lebetina* in several countries, including Cyprus (northern Paphos district, 1 June 2014). This generalist predator also feeds on snakes, thus being both a competitor and potential natural enemy of *M. lebetina*. **c** One-year old male feral cat with a 2-day-old, necrotic wound occurring from snakebite (probably by *M. l. lebetina*), in veterinary treatment (Paphos city, 3 September 2015). As feral cats commonly hunt snakes and other reptiles, the bite was possibly delivered in defense. **d** Sharing their habitat with *M. l. lebetina*, young red foxes (*V. vulpes*) climb on a rock pile established for reptiles (Polis Chrysochous, 9 May 2014). Wild canids are natural predators of small reptiles and include snakes in their diet. Photos: D. Jestrzemski.

6.11 A new antivenom for blunt-nosed viper envenoming

The development, manufacture, and distribution of effective antivenoms at affordable prices remains a key strategy against snakebite envenoming (Williams et al. 2011). Yet, the serious side effects of some antivenoms pose a threat to snakebite patients. For example, the polyvalent VACSERA antivenom, which is raised against *Naja haje, Cerastes cerastes, Echis coloratus* and *Bitis arietans* and applied for snakebite treatment in several African countries, caused the highest rate of adverse reactions in Ethiopian snakebite patients (18.3%, compared to 1.3% in patients treated with Fav-Afrique antivenom, and 7.1% in patients treated with EchiTAb-PLUS-ICP antivenom), while the rate for uncomplicated outcomes was lower for VACSERA (74.4%) than for Fav-Afrique (93.2%) and EchiTAb-PLUS-ICP (90.4%) (Steegemans et al. 2022).

From 2021 to 2022, the coverage of the EchiTAb-PLUS-ICP antivenom was expanded to incorporate Middle Eastern venomous snakes, with *M. l. obtusa* from Azerbaijan (venom sample from Latoxan) included in the new immunization mixture. The new product shows a neutralization level of about 2.4 mg of *M. l. obtusa* venom per ml of antivenom (mg/mL), which is much higher than the already existing antivenoms that achieve around 0.3 mg/mL neutralization against *M. lebetina* venom (Philip Price, pers. comm., 3rd February 2023). Thus, estimating a mean venom yield of 50-60 mg for *M. lebetina* (based on the data from Latifi 1984), only 2.5 EchiTAb-PLUS-ICP vials (each 10 ml) can potentially neutralize the full venom amount delivered from a typical blunt-nosed viper bite, as compared to more than 20 vials of the antivenoms that are currently on the market (P. Price, pers. comm., 3rd, 28th February 2023). This expanded formulation of the EchiTAb-PLUS-ICP antivenom could soon be registered locally in Costa Rica. However, the situation is more complicated in Cyprus, where extensive clinical studies would be required for a registration of the product, which is not yet registered in any EU member country. Such clinical studies are not easy to perform because the annual number of patients with proven *M. lebetina* envenoming serious enough to require antivenom treatment is low in Cyprus. Thus, this new antivenom is “likely to get stuck at the regulatory stage for a long time” (P. Price, pers. comm., 3rd February 2023).

6.12 Development of a chemo-ecological snake repellent to deter blunt-nosed vipers from human settlements

Despite the enhanced availability of antivenoms and an improved hospital infrastructure in several Middle Eastern countries (see Jestrzemski et al. 2022), snakebite envenoming by *M. lebetina* remains a public health hazard in the region (e.g., Kazemi et al. 2019). Unsurprisingly, a tremendous public interest exists to keep this medically important snake species away from human settlements and reduce the incidence of snakebite envenoming by *M. lebetina* ssp. (see Jestrzemski & Kuzyakova 2018, Jestrzemski et al. 2022). Yet, conventional snake repellents have proven ineffective so far, failing to deter snakes from selected areas (San Julian & Woodward 1985).

Despite these difficulties, a promising approach lies in developing a deterrent for in- and outdoor application, based on chemical communication in reptiles. Chemical communication plays a crucial role in snake foraging, in the detection of and defense against natural enemies, in the recognition of conspecifics and for partner searching, selection and courtship (Mason & Parker 2010). Thus, chemical substances like snake scent compounds not only influence the interactions between snakes, but also the distributions of species and populations (Scott et al. 2013). Snake scent volatiles are distributed via faeces, urine, skin lipids and other secretions (Mason & Parker 2010), and elicit different behavioral traits in snakes (e.g., changes in their tongue flick rate, defensive postures), which can be recorded and quantified by behavioral experiments involving olfactometers. Originally designed by entomologists, olfactometers can be applied to test the impact of scent compounds on the choice behavior of snakes (see Vet et al. 1983). In this regard, a fundamental question is to what extent chemical compounds are transmitting information at spatial and temporal scales (Van Buskirk et al. 2014).

Of particular interest regarding venomous snakes are kairomones. These substances are emitted by an organism and provide advantages to individuals from another species, for example a predator sensing a nearby prey species, or a potential prey species being warned about the presence of its natural enemy (Sabelis & Van de Baan 1983, Schoeppner & Relyea 2009). These interactions are illustrated by the prey-predator relationships between ophiophagous snakes and their ophidian prey, which often includes venomous species. For instance, pit vipers (Crotalinae) show a strong defensive reaction (“ophiophage defense response”) when exposed to the kairomones of kingsnakes (*Lampropeltis getula*), which are ecologically important predators of rattlesnakes and other North American crotalines (Bogert 1941, Weldon & Burghardt 1979, Gutzke et al. 1993). In South America, Mussuranas (*Clelia* spp.) are well-known predators of pit vipers of the genus *Bothrops*, which includes highly venomous species (e.g., *B. asper*) responsible for most snakebite incidents in the Americas (Delia 2009, Sasa et al. 2009).

Although blunt-nosed vipers do not have strictly ophiophagous natural enemies among the sympatric snake fauna of Western and Central Asia, the large whip snake and Eastern Montpellier snake, whose distributions range from Cyprus to Iran, also include snakes in their diet, and thus are potential predators of (juvenile) *M. lebetina* ssp. In Cyprus, the large whip snake is among the most common snake species (Baier et al. 2013) and regarded as a natural enemy of blunt-nosed vipers by rural people. Cypriot farmers even believe that the presence of the large whip snake keeps blunt-nosed vipers away, and thus tolerate and appreciate its presence in their fields (K. Kailis, 15 April 2014, pers. comm.). Consequently, it can be hypothesized that the smell of the large whip snake has a deterring effect on the blunt-nosed viper, and that its scent compounds could be used for developing a biological deterrent to keep blunt-nosed vipers away from human settlements.

Thus, future research should investigate the role of snake odor in the predator-prey relationship between the blunt-nosed viper and the large whip snake. In particular, the hypothesis should be tested that the odor of the large whip snake has an impact on the movements of the blunt-nosed viper and can keep the latter away from selected areas. The tests could be performed in an olfactometer with four wings (and thus, four possible directions of movement), for obtaining results with higher statistical power (**Figure 6.2**). To examine a potential aversion of the blunt-nosed viper particularly against the large whip snake, the trials could be repeated with the non-venomous coin snake (*Hemorrhois nummifer*), which is not a predator of the blunt-nosed viper and thus could be used as a control species.

Accordingly, the first two research objectives would be to investigate how the presence of the large whip snake, its scent and the corresponding chemical compounds affect the behavior of the blunt-nosed viper, and vice versa. The third objective would be to examine the traditional beliefs of Cypriot farmers, who assume that the presence (and thus, potentially the smell) of large whip snakes keeps blunt-nosed vipers away from human settlements and agricultural areas.

The results of this research project could be of great value for a better understanding of the mechanisms underlying the spatial and temporal occurrence of *M. lebetina* across its distribution range. The overall goal of the project would be to create the scientific and technical fundaments for a new approach of repelling blunt-nosed vipers from human settlements, thus contributing to prevent snakebite and reduce the human-snake conflict in the distribution range of the blunt-nosed viper.

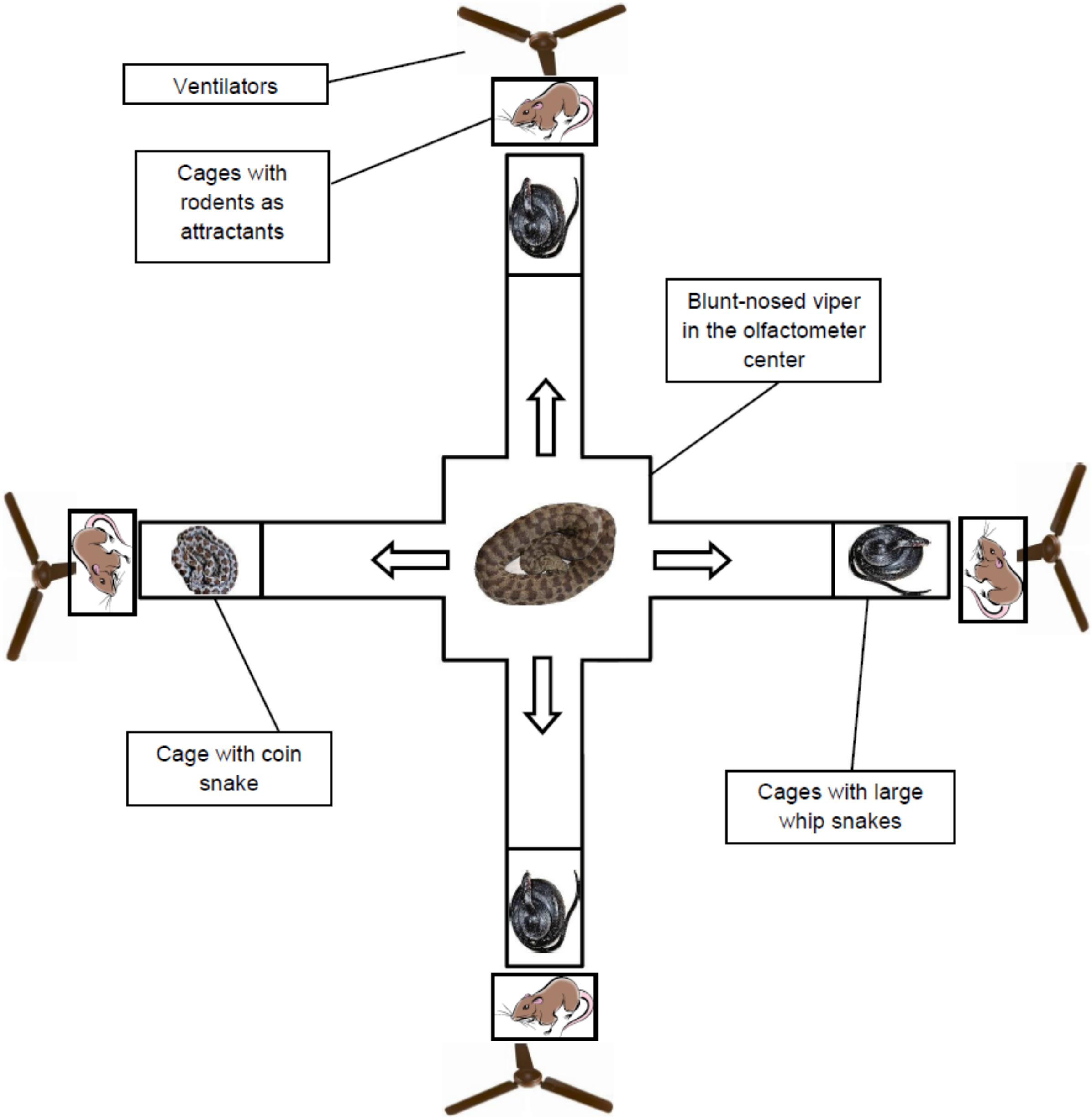

Figure 6.2. Four-wing olfactometer for testing the reaction of *M. l. lebetina* on a potential ophidian predator (*D. jugularis*), with *H. nummifer* as control species. Photo of *M. lebetina*: D. Jestrzemski. Photos of *D. jugularis* and *H. nummifer*: George Constantinou.

6.13 Blunt-nosed viper conservation and venom diversity in the light of anthropogenic interference and climate change

The current geological epoch is defined as the anthropocene (Crutzen 2006, Lewis & Maslin 2015) and characterized by a combination of accelerating global changes of anthropogenic origin, which negatively affect the wellbeing of the global ecosystems, thus endangering the survival of millions of fauna and flora species (Sauerborn & Matthies 2006). Numerous snake species are threatened by the degradation, fragmentation, pollution and destruction of their habitats, the spread of new diseases, the introduction of alien organisms, wildlife trade, and by other forms of human persecution (Gibbons 2000, Murphy 2010, Böhm et al. 2013). These factors interfere with the natural processes that drive the evolution of snake species and their venoms (see Vonk et al. 2011).

Blunt-nosed vipers are affected in several ways by anthropogenic changes. Their habitats (including Mediterranean macchia and garrigue shrublands), are being destroyed and fragmented on a large scale for different reasons, including agricultural expansion, real estate development, urban expansion, road development and ungulate overgrazing (Ščerbak & Böhme 2005, Jestrzemski & Kuzyakova 2018). As snake habitats fulfill critically important ecological functions (e.g., the provision of cover, prey attraction and conspecific aggregation), their loss is probably a major cause of population declines in *M. lebetina* ssp. (see Jestrzemski & Kuzyakova 2018). Blunt-nosed viper habitats are also threatened by wildfires of anthropogenic origin, which can wipe out whole snake populations (Böhm et al. 2013). The expansion of road networks further increases the number of blunt-nosed vipers killed by traffic. At risk are populations whose annual migration routes are intersected by roads, for example *M. l. schweizeri* on Milos (Nilson et al. 1999). These processes contribute to a reduced gene pool within *M. lebetina* populations, as they decrease the gene flow between them (see Dubey et al. 2011), interfering with the natural forces that shape the evolution of *M. lebetina* ssp. Hence, this trend may also lead to a reduced venom diversity within blunt-nosed viper populations (see Vonk et a. 2011).

However, if the transformation of Cypriot macchia and garrigue shrublands resulted in an extension of agricultural plantations and croplands, the habitat range of *M. lebetina* populations might be sustained since blunt-nosed vipers are adapted to preying on rodents in agrarian landscapes with sufficient habitat structures (Jestrzemski & Kuzyakova 2018). An agrarian expansion might also establish new potential corridors for the genetic exchange between *M. lebetina* populations, while curbing the importance of rodents (i.e., *Rattus* sp.) as their major prey species. Over a very long time period, this could even promote a co-evolutionary arms race between blunt-nosed vipers and their rodent prey: as rats may become more resistant to the venom, selective pressure may favor those vipers with the most potent toxins (see Murphy 2010).

Human predation, another threat to blunt-nosed vipers, most commonly targets the more visible (i.e., larger and mature) life stages in snakes, while young and small individuals are more often overseen. Consequently, the removal of the fittest individuals selects against traits that are advantageous under natural conditions, inducing “changes in a suite of traits, including physiology, development, morphology, behavior, and life history” (Sasaki et al. 2008). Hence, the systematic persecution of blunt-nosed vipers could induce irreversible genetic changes and result in populations exhibiting truncated size distributions, with smaller adult sizes, higher neonate mortality rates and other disadvantageous traits that could endanger local populations. These consequences have been observed in hunted populations of the Japanese pit viper *Gloydius blomhoffii*, where neonates were smaller and with fewer vertebrae compared to neonates from unhunted populations, indicating that they would grow to smaller adult body sizes, which in turn results in lower population fecundity. Such detrimental evolutionary changes generate reduced population growth rates and fitness, which decreases population sizes and their recovery potential. Additionally, an increased fleeing activity of hunted snakes will cause higher energetic costs to the populations, resulting in a loss of opportunities for other activities such as foraging and basking (Sasaki et al. 2008). Although no venomic studies of *M. lebetina* have yet been undertaken in the context of human persecution, the ongoing hunting of this large viper species could bring about adverse evolutionary changes, which could lead to a decline in intraspecific and ontogenetic venom proteome variability of *M. lebetina* ssp.

Globalized trade has brought about the spread of alien organisms, starting in the Neolithic, with rodents and small carnivores (including feral cats) following human expansion. For example, the colonization of Cyprus established the rodent populations that today are a fundamental food resource for blunt-nosed vipers across the island (Watson 1951, Hadjisterkotis & Masala 1996) and possibly enabled the common occurrence of *M. l. lebetina* in Cyprus. The increased global mobility of people and goods also dramatically facilitated the circulation of previously unknown diseases including the zoonotic coronaviruses (Zheng 2020), as well as the introduction of new natural enemies of blunt-nosed vipers. For instance, the uncontrolled spread of non-native snake predators such as *Herpestes javanicus* and *H. edwardsii* in Iraq (Al-Sheikhly & Mallow 2013) may pose a new threat to Iraqi *M. lebetina* populations, with *H. javanicus* now being its prime predator in the Mesopotamian marshes (Al-Sheikhly et al. 2021). Thus, the establishment of alien predatory species could trigger a co-evolutionary arms race between blunt-nosed vipers and their new enemies, with evolutionary pressure towards the development of more potent toxins in the venom proteome of *M. lebetina* ssp.

Finally, climate change is a key driver of global ecosystem deterioration, pushing countless species to the edges of their biogeographic zones and beyond, while promoting the migration of exotic organisms and the spread of new, dangerous diseases (Murphy 2010, Bickford et al. 2010). These include previously unknown pathogens affecting snakes, such as the novel snake fungal disease (SFD), which is regarded as a major potential threat to snake populations worldwide (Lorch et al. 2016), analogous to the spread of the devastating amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) (Olson et al. 2013). It is yet unknown which impact new diseases such as SFD have on the venom proteome of venomous snakes. However, as SFD may cause considerable damage to the venom apparatus of pit viper species, accompanied by several other pathological clinical signs directly affecting the health condition and metabolism of the infected snakes (Allender et al. 2011, 2015), it likely also influences the venom proteome composition of its hosts.

Besides the threat of diseases, climate change-induced heat stress could severely deteriorate the living conditions of numerous vertebrate taxa in Southwestern Asia, including reptiles, mammals and birds (Murali et al. 2023). Thus, a projected increased exposure to future heat extremes could threaten blunt-nosed vipers directly by heat stress, and indirectly by extirpating their mammalian and avian prey populations (Murali et al. 2023). Droughts could also reduce the availability of water needed by snakes for drinking and shedding, and facilitate the outbreak and rapid spread of wildfires, which are already a hazard in Cyprus during the summer season (see Jestrzemski & Kuzyakova 2018).

With new species setting foot in Western Asia due to climatic changes (e.g., other snakes, small carnivores, raptors), blunt-nosed viper populations could experience additional stress through increased competition for food resources (see Losos et al. 1993).

A combination of all these factors could result in the loss of snake habitats and habitat connectivity, cause increased snake mortality, and thus further isolate, weaken, and even extirpate *M. lebetina* populations, and threaten the gene flow between the remaining ones. Consequentially, with a growing pressure on *M. lebetina* ssp. to adapt to the rapidly occurring, human-induced environmental changes, their evolution is put under increasing risk. If blunt-nosed viper habitats cannot be preserved and their population mortality rates cannot be kept below critical threshold levels, genetic variability and thus venomic diversity are likely to decline within *M. lebetina* over longer periods of time. This would threaten the existence of an ecologically and medically highly important, enigmatic viper species, while the loss of its venom resources would deprive modern medicine of "a rich source of bioactive molecules with known or potential therapeutic applications" (see Puschhof et al. 2021).

Thus, to establish improved species and habitat conservation programs benefiting blunt-nosed vipers (and other sympatric reptile species), it is imperative to enhance our knowledge on the conservation biology of *M. lebetina* by in-depth ecological studies of their insular and continental populations, ideally by also applying radiotelemetry.

6.14 References Chapter 6

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Figure 4.2 Venomic analysis of *M. l. lebetina* from Cyprus and *M. l. cernovi* from Iran. (A) Reverse-phase chromatographic separation of the venom proteome of *M. l. lebetina*, and (B) *M. l. cernovi*. The chromatographic fractions of both venoms were collected manually and analyzed by SDS-PAGE in both, reduced (upper section) and non-reduced (lower section), condition. (C, E) The pie charts indicate the identified protein families and their relative abundances (as percentage of total detected proteins) within the venom of *M. l. lebetina*, and (D, F) *M. l. cernovi* as determined either from the peak areas of reverse-phase chromatography with the help of SDS-PAGE protein bands (C, D) or based on the normalized spectral abundance factor from single-shot whole venom bottom-up proteomics (E, F). Acronyms: SVMP, snake venom Zn2+-metalloproteinase; PLA2, phospholipase A2; SVSP, snake venom serine protease; CTL-like, Snaclec, C-type lectin-like protein; LAAO, l-amino acid oxidases; DIS, disintegrin; CRISP, cysteine-rich secretory protein; NGF, nerve growth factor; VEGF, vascular endothelial growth factor; PDE, phosphodiesterase; AP, aminopeptidase; ACE, angiotensin-converting enzyme; BPP, bradykinin-potentiating peptides; PLB, phospholipase B; PLA2i, phospholipase A2 inhibitor; HYAL, hyaluronidase; NP, natriuretic peptide; 5′NTD, 5′-nucleotidase; DC, disintegrin-like/cysteine-rich fragment of PIII-SVMP; SVMPi, snake venom metalloproteinase inhibitor; NGF, nerve growth factor.

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Table 4 Comparison of toxin families detected in the venom proteomes of *M. lebetina* subspecies, investigated by almost the same proteomic technique. Overview of the relative abundances of toxin families in the venom of Cypriot *M. l. lebetina* and Iranian *M. l. cernovi* along with published data on Russian *M. l. obtusa* [13], Armenian *M. l. obtusa* [12] and Tunisian *M. l. transmediterranea* [11].

Table 5 Representatives of Cypriot institutions interviewed on the conflict between people and blunt-nosed vipers in Cyprus.

Acknowledgements

The successful implementation of this project was only made possible by the support of numerous people and institutions, which I appreciate beyond words.

First, I am highly indebted to my second supervisor Dr. Ulrich Kuch for his constant, great support and his expert advice throughout all years of my PhD project, and for continuously motivating me to pursue my research in an unconventional yet fascinating topic: venomous snakes. I am also extremely grateful to my first supervisor, PD Dr. Frank Gessler, and to my third supervisor, Prof. Bernhard Spengler, for their kind support and expert guidance during my PhD project. Furthermore, I would like to express my deep appreciation to Prof. Bertram Brenig and Prof. Ralph Mitlöhner for kindly agreeing to referee my dissertation. Also, thanks go to my former first supervisor Prof. Stefan Schütz for supporting me during the first years of my PhD studies.

This endeavor would not have been possible without the friendly support of Dr. Parviz Ghezellou with the proteomic part of my research project, and without the kind help of Dr. Irina Kuzyakova with the statistical data analysis – I learned a lot during these years. Further, I thank Maria Athanasiadou and Dr. Vasos Scoutellas for kindly providing the data on snakebite-related hospital admissions in Cyprus and for supporting me throughout the data analysis, manuscript writing, submission, and revision procedures. I am also thankful to Peter van Issem for the friendly provision of blunt-nosed viper venom samples.

Great thanks go to the Volkswagen Foundation for the financial support of my research. My work was further generously funded by the Mohamed bin Zayed Species Conservation Fund and by the Institute of Occupational, Social and Environmental Medicine (Goethe University Frankfurt), as well as by the German Society for Herpetology and Herpetoculture (via the Hans-Schiemenz Fonds), by the Department of Forest Zoology and Forest Conservation (Faculty of Forest Sciences and Forest Ecology, University of Göttingen) and by the Nature and Biodiversity Conservation Union (NABU, Germany). In this regard, I would like to mention Tom Kirschey and Prof. David Groneberg, as well as Gabriele Volante and Dr. Axel Kwet.

Moreover, I want to acknowledge the support for open access publishing of the thesis chapters 2 and 5 by the German Research Foundation, the Open Access Publication Fund of Göttingen

University and the Faculty of Forest Sciences and Forest Ecology. In this regard, I would like to recognize Sabine Witt (SUB) for her administrative support with open access publishing-related issues.

Likewise, my thanks go to Elena Erotokritou and the Department of Environment of Cyprus (Ministry of Agriculture, Rural Development and Environment), which approved my field work in Cyprus (Reference numbers 02.15.001.003, 04.05.2002.005.006, 02.15.007.003.001), and to the Cyprus National Bioethics Committee for approving my study on hospital admissions due to snakebite envenoming in the Republic of Cyprus (2013–2019) (decree ΕΕΒΚ ΕΠ 2021.01.193).
Farther, I thank everyone who supported my fieldwork locally in Cyprus, in particular Volker Schrempf, Hans-Jörg "Snake George" Wiedl, Antreas Kourides and Eftichia Demetriade, as well as Marios Theodorou, Savvas Zotos, Haris Nicolaou, Spyros Sfenthourakis, Elias Christodoulou and his family, Dannys Kourides, and several more people. I am also indebted to Oliver Becker for his outstanding engagement and support.
George, your love and relentless passion for snakes and their conservation have been an inspiration for life. Volker, it has been a big pleasure to meet alternately in Cyprus and in Göttingen, and have lively discussions over a cup of tea. Antreas, your engagement for snakes, research and environmental education in Cyprus is wonderful. Looking back, I am very grateful for having been able to conduct five fascinating field excursions to Cyprus!

Furthermore, I would like to acknowledge Christoph Andrijczuk for inspiring discussions and advice. Also, I thank Prof. Ulrich Joger and Dr. Nikolaus Stümpel for thematic discussions at the beginning of my PhD project. My PhD research was further supported by the staff from the Department of Forest Zoology and Forest Conservation (University of Göttingen), and by the staff from the Institute of Inorganic and Analytical Chemistry (Justus Liebig University Giessen). Likewise, I am grateful for the help by the GWDG (Göttingen). Additionally, I would like to recognize the GFA Göttingen for advice and support regarding the coordination of my PhD studies – I had numerous talks, especially with Dr. Inga Mölder.

Finally, I am highly indebted to my wonderful family for their continuous support throughout my PhD studies. Likewise, I thank my lovely wife Thi Phuong Hoang for selflessly tolerating my absence while I was abroad in Cyprus.
Lastly, I thank **God** for the successful realization of my PhD research.

Publications related to this PhD project

Jestrzemski, D. (2022). „Wen rieche ich, und wenn ja, wie viele?“ – Grundlagenforschung zur chemischen Ökologie der zypriotischen Levanteotter (*Macrovipera lebetina lebetina*). TERRARIA/elaphe 1/2023: 72-75.

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Presentations related to this PhD project

9/2020	Venoms and Toxins 2020 (LibPubMedia, Oxford): „A 7-year review of snakebite envenoming in the Republic of Cyprus (2013-2019)” (Poster).
9/2020	Venoms and Toxins 2020 (LibPubMedia, Oxford): „Ecology and conservation of Europe's largest viper (*Macrovipera lebetina*) on Cyprus” (Poster).
05/2019	Symposium „First Myanmar-Germany Symposium on Biodiversity, Chemical Ecology and Health“ (East Yangon University, Yangon): „Field survey of the Cypriot blunt-nosed viper *Macrovipera lebetina lebetina* (Linnaeus, 1758), a medically important snake species of Western Asia” (Oral presentation).
05/2015	Jahrestagung der DGHT AG Schlangen (Diepziger Hof, Diepzig): „Die zypriotische Levanteotter (*Macrovipera lebetina lebetina*) und ihre Naturschutzsituation” (Oral).
10/2014	4th Biology of the Vipers Conference (Oasis Hotel, Athens): „A field survey of the Cypriot blunt-nosed viper (*Macrovipera lebetina lebetina*) in northern Paphos province, Cyprus” (Oral presentation).
10/2014	Jahrestagung der Deutschen Gesellschaft für Herpetologie und Terrarienkunde (DGHT, Zoologisches Forschungsmuseum Alexander König, Bonn): „Wildes Zypern – auf den Spuren der Levanteotter (*Macrovipera l. lebetina*)” (Oral presentation).