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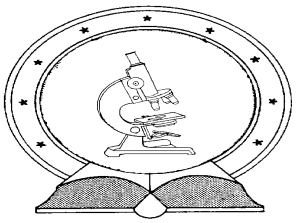

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# VENOM VARIATIONS AND THEIR CLINICAL SIGNIFICANCE IN CASE OF AN ISOLATED POPULATION OF THE COMMON ADDER (*VIPERA BERUS*) IN EASTERN HUNGARY

Egyetemi doktori (Ph.D.) értekezés


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**A doktori értekezés betétlapja**

Ezen értekezést a Debreceni Egyetem Természettudományi Doktori Tanács a **Juhász-Nagy Pál Doktori Iskola Biológia** programja keretében készítettem a Debreceni Egyetem természettudományi doktori (Ph.D.) fokozatának elnyerése céljából.
Debrecen, 2015.

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a jelölt aláírása

Tanúsítom, hogy **Malina Tamás** doktorjelölt 2009-2015 között a fent megnevezett Doktori Iskola **Biológia** programjának keretében irányításommal végezte munkáját. Az értekezésben foglalt eredményekhez a jelölt önálló alkotó tevékenységével meghatározóan hozzájárult. Az értekezés elfogadását javasolom.
Debrecen, 2015.

……
a témavezető aláírása

**A doktori értekezés betétlapja**

# MÉREGVARIÁCÓK ÉS KLINIKAI JELENTŐSÉGÜK EGY IZOLÁLT KELET-MAGYARORSZÁGI KERESZTES VIPERA (*VIPERA BERUS*) ÁLLOMÁNYNÁL

# VENOM VARIATIONS AND THEIR CLINICAL SIGNIFICANCE IN CASE OF AN ISOLATED POPULATION OF THE COMMON ADDER (*VIPERA BERUS*) IN EASTERN HUNGARY

Értekezés a doktori (Ph.D.) fokozat megszerzése érdekében
a Biológia tudományágban

Írta: **Malina Tamás** okleveles Biológus

Készült a Debreceni Egyetem **Juhász-Nagy Pál Doktori Iskolája**
(**Biológia** programja) keretében

Témavezetők:
Dr. Vasas Gábor ……

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# 1. Introduction

The common adder (*Vipera berus*) is the most distributed terrestrial venomous snake; its geographical range covers almost the whole Palaearctic region and this is the sole species, which can be found beyond the Arctic Circle, as well (SAINT GIRONS 1980; CARLSSON 2003).

This old-world viperid species is native to Scotland, England, Wales, Sweden, Finland, Denmark, Netherland, Belgium, Latvia, Estonia, Germany, Poland, central-France, Czech Republic, Slovakia, Hungary, Austria, northern Italy, Slovenia, Romania and most of the territory of Balkan peninsula, reaching eastwards as far east as the Pacific coast of Russia through Korea to the Sakhalin Island (CARLSSON 2003; NILSON et al. 2005). It is absent from Ireland, the whole Iberian Peninsula, the central and southern areas of Italy and Greece (NILSON et al. 2005). Three subspecies are distinguished on the bases of their current taxonomic status: the nominate subspecies, *Vipera berus berus*, the Sakhalin adder (*Vipera berus sachaliensis*), and the Balkan subspecies, *Vipera berus bosniensis* (CARLSSON 2003; NILSON et al. 2005). Although, the taxonomic status for the latter has not yet been clarified within the *V. berus* complex (JOGER et al. 1997; KORSÓS 2007); it is frequently a key element of negotiations among taxonomists and herpetologists.

The native herpetofauna of Hungary includes only two venomous snake species, the common adder (*V. berus*) and the Hungarian meadow viper (*V. ursinii rakosiensis*), both with a restricted distribution in the country. Up to now, *V. berus* had been described from three main, separated regions in Hungary (KORSÓS & KRECSÁK 2005): 1) the species has a relatively coherent geographical distribution range in the north-eastern areas, on the Zemplén Hills and Eperjes - Tokaj Range (JANISCH 1979, 1987), 2) it can be found in the valley of the Upper River Tisza in the East, involving the Bereg and Szatmár Plain (AGÓCSY 1958; MARIÁN 1960; JANISCH 1979, 1987), and 3) also occurs in the south-western regions of the country, Somogy (MARIÁN, 1956; JANISCH 1979, 1987; TÓTH & SÓS 2003) and Zala counties (FEJÉRVÁRY 1923; JANISCH 1979, 1987; TÓTH & SÓS 2003). According to the most recent and authenticated data, a new *V. berus* locality was found in Zala

county, near Zalabaksa (MIZSEI 2014. pers. comm.). *Vipera berus* is strictly protected species in Hungary.

There have been some further *V. berus* localities, of which some of them are still unclear today. One population exists near Monok in the Szerencs Hills (*Magyar Természettudományi Múzeum, Budapest, 1972/2, Monok, leg. Horváth R., 1988*), which most probably can be in connection with the adder populations of Zemplén Hills, about 5-6 km far from the closest *V. berus* locality, near Tállya, although, there is no authentic data about its recent status. An early record is known from Nógrád County (northern Hungary), near Salgótarján from 1943 (FEJÉRVÁRY-LÁNGH 1943). Later, DELY & MARIÁN (1960) re-mentioned this adder locality. However, its real existence is still an open question and according to our recent zoo-geographical knowledge, it is quite absurd. KORSÓS (2007) denoted Barcs (Somogy County) as the southernmost locality of the species in Hungary. While MARIÁN – who mapped the distribution range of the adder in Somogy County during several years – was unable to show the species and emphasized its absence around Barcs (MARIÁN 1981). The species presumably has already been extincted from this area (MARIÁN 1981).

Two subspecies of the common adder occur in Hungary: the nominate subspecies (*V. b. berus*) in the north-eastern mountainous areas and the eastern lowland corner of the country, and the Balkan or Bosnian adder (*V. b. bosniensis*), which reaches its northernmost distribution in the lowlands of south-western Hungary (DELY & MARIÁN 1960, DELY 1978, KORSÓS & KRECSÁK 2005). Native populations of the two subspecies, *V. b. berus* and *V. b. bosniensis*, do not show significant differences in their appearance for laymen. The hill country *V. b. berus* populations of north-eastern Hungary, are isolated from the eastern lowland adder populations. Although, they differ slightly also in morphology (MARIÁN 1960; KORSÓS & KRECSÁK 2005) and habitat preferences (MARIÁN 1960; KORSÓS, 2007), they are both recognized as *V. b. berus* (MARIÁN 1960; DELY & MARIÁN 1960; KORSÓS & KRECSÁK 2005; KORSÓS 2007). Based on their mitochondrial DNA, these two populations belong to two different evolutionary clades (KALAYABINA-HAUF et al. 2004).

Despite that some authors (i.e. FRITZSCHE & OBST 1966; DELY 1972, 1978; JANISCH 1979; TÓTH & SÓS 2003) argued that the south-western Hungarian adders can be referred to the subspecies of *V. b. bosniensis*, their

taxonomic status has still not been completely clarified. In case of the adders of Somogy County, MARIÁN (1956) was the first, who raised the possibility that these adders might not be *V. b. bosniensis* but rather a local form. These adders also differ in their certain morphological characters – which is based on their morphological analysis –, and living in different ecological conditions than the traditionally recognized *V. b. bosniensis* (ÚJVÁRI et al. 2001; KORSÓS & KRECSÁK 2005). Though, on the bases of a mitochondrial DNA study, the adders of Somogy are found to be near to *V. b. bosniensis* (KALYABINA-HAUF et al. 2004). The presumption that the adders of Somogy is not totally *V. b. bosniensis* also suggests that *V. b. bosniensis* was originally described in highlands and its other populations are also known from the mountainous regions of the Balkan (KRECSÁK 2001, 2005). Nevertheless, the adders of Somogy County and populations living across the River Drava – mainly in the valley of the River Sava and lowlands of Croatia – constitute a group with strong resemblance (KRECSÁK 2005). However, SCHREIBER (1912) described the latter-mentioned populations as a local and lowland form of the adder, namely *V. berus var. pseudaspis*, which is the synonym of *V. b. bosniensis*, nowadays (KRECSÁK 2007). According to certain authors (i.e. FEJÉRVÁRY 1923; VÖLKL & THIESMEIER 2002), *V. berus* populations occurring in the valley of the River Sava through the lowlands of Croatia and till the lowlands of south-western Hungary, should be distinguished as a subspecies, namely as *V. b. pseudaspis* from the mountainous *V. b. bosniensis* populations. Today, *V. berus* populations native to northern Croatia along the Sava and Drava rivers are currently recognized as *V. b. bosniensis* (KREINER 2007) similarly as the south-western Hungarian adder populations.

In Hungary, the species microhabitat preferences extend in the regions mentioned above that is appropriate for its survival, mainly on woodland edges and wood-cuts, bushy and damp meadows, brambly escarps, blueberry-hedgerows but also on boggy fields, edges of alder marshes and willows (MARIÁN 1956, 1960; DELY 1978; JANISCH 1987). Closed forests and timber forests, rocky grasslands, cultivated fields are non-preferred habitats of *V. berus* (JANISCH 1987). However, urban environments are basically also avoided by the species but it can appear directly next to the villages; thus adder envenoming had already occurred within the border of village (VIRÁGH & TASS 1986). Habitats combined with hot and dry microclimate are also not

preferred by the adder, while it shows passive activity and/or active only during the early mornings and evenings on the heatwaved-days. *Vipera berus* hibernates at the end of October/early November and becomes active again usually in March (MARIÁN 1956; ÚJVÁRI et al. 2001), when the maximum air temperature is steadily between +10 and 12°C (MARIÁN 1956). Since *V. berus* is a cold-adapted species (WÜSTER 1998; NILSON et al. 2005), it becomes also active during the winter in case of significant mild weather. Only a few published data is available in the literature about the diet preferences of *V. berus* within its Hungarian territory. The natural diet of adult *V. berus* specimens mainly consists of small rodents (*Apodemus sp*., *Microtus sp*., *Arvicola sp*.) and insectivorous mammals (*Sorex sp*.) but also certain frogs (i.e. *Rana dalmatina*, *R. arvalis*), while juveniles feed on the young specimens of *Zootoca vivipara*, *Lacerta agilis*, and *Triturus vulgaris* (MARIÁN 1956, 1957, 1960).

# 2. Literature background

Intraspecies venom variation of snakes can have scientific interest from ecological, chemotaxonomical, clinico-epidemiological, but also antivenom production point of view (WÜSTER et al. 1999; BARLOW et al. 2009). Such venom variations can be found at several levels, e.g. between different populations regionally, including the inter-subspecies venom variations and intra-populations, which covers gender specific, diet/habitat, seasonal and ontogenetic differences (CHIPPAUX et al. 1991; WILLIAMS et al. 1988; SASA et al. 1999; WÜSTER et al. 1999). This venom variability is notably influenced by adaptation to available prey species and can lead to regionally distinct clinical patterns observed in envenomed humans (CHIPPAUX et al. 1991; DALTRY et al. 1997; BELT et al. 1997; FERQUEL et al. 2007; ALAPE-GIRÓN et al. 2008; BARLOW et al. 2009; BABOCSAY 2010; ÖHLER et al. 2010).

The venom of the European viperids has an arsenal of a complex mixture of enzymes. Of the phospholipases $A_2$ ($PLA_2$), the post-and presynaptically acting neurotoxins, e.g. different isoforms of ammodytoxin and vaspin, are the most significant, which can cause peripheral neurotoxic effects on humans (FERQUEL et al. 2007; JAN et al. 2007). Significant

differences have been detected in the $PLA_2$ content of venom of the asp vipers (*V. aspis*) in different regions of France (FERQUEL et al. 2007). Venom composition differences were also verified among the different populations of the long-nosed viper (*V. ammodytes*) in Croatia (BALIJA et al. 2005). The individual venom variations are known in the following members of the genus *Vipera*: in *V. aspis* (DETRAIT & DUGUY 1966), *V. latastei* (AREZ et al. 1994) and *V. ammodytes* (MASTER & KORNALIK 1965, HALASSY et al. 2011).

*Vipera berus* may have the highest clinical significance among the monophyletic genus of the European *Vipera* due to having the broadest distribution range and, therefore, the highest frequency of snakebite-incidents in Europe (PERSSON 1995). With regard to *V. berus* bites that occurred in Hungary, KÓSA (1989) emphasized that he treated about 70-80 bitten patients by *V. berus* during 30 years – in the formerly Korányi-Frigyes Sándor Hospital, Budapest – and highlighted, he had more patients in critically and severely envenomed condition following *V. berus* bite (KÓSA 1989).

The species has three phylogenetically separated main clades: the *V. b. bosniensis*-clade, the Southern clade, and the Northern-clade. This latter involves plus four sub-clades (Carpathian, North-western, Western, and Eastern (URSENBACHER et al. 2006)). Taking into account the above facts and the significance of regional variations of a given species that is already emphasized by several authors (SAINT GIRONS & DETRAIT 1992; DALTRY et al. 1997; STÜMPEL & JOGER 2009; BARLOW et al. 2009), the possible implication of venom variations among the phylogenetically distinct clades and sub-clades of *V. berus* very probably have high implication from taxonomical, toxicological and clinical perspective, as well.

However, the venom of *V. b. berus* has been extensively studied in the past 20 years, only the venoms of certain populations from the Eastern sub-clade (from Russia) (SIIGUR et al. 1979; NEDOSPASOV & RODINA 1992; KRIŽAJ et al. 1993; CALDERÓN et al. 1994; MALENEV et al. 2007; RAMAZANOVA et al. 2008), and the venoms of some populations from the western sub-clade (from France) (SAINT GIRONS & DETRAIT 1992; GUILLEMIN et al. 2003) have been investigated. A study (MEBS & LANGELÜDDEKE 1992) used venoms from a population in the area of former Czechoslovakia, which belongs to the Central European sub-calde (URSENBACHER et al. 2006). The venom characteristics of other sub-clades of *V. berus* have not yet been studied. However, KRIZAJ et al. (1993) mentioned

that DELORI used *V. berus* venoms to his research in 1973, originated from Hungary. Neither any data, nor any information with indication was found for the exact geographical origin of venoms in DELORI's works (1971, 1973); samples were obtained from the Berne Institute (DELORI 1971, 1973).

The venoms, derived from adder populations mentioned above, contain a mixture of the following activities: oedema-forming, anti-haemostatic, anticoagulant, fibrinolytic, proteolytic, haemorrhagic and myotoxic activities (SAINT GIRONS & DETRAIT 1978; MEBS & LANGELÜDDEKE 1992; CALDERÓN et al. 1993). According to several authors (KRIŽAJ et al. 1993; JAN et al. 2002; GUILLEMIN et al. 2003; RAMAZANOVA et al. 2008; DE HARO et al. 2009; MAGDALAN et al. 2010), *V. b. berus* venom is devoid of neurotoxic properties. However, neurological deficits after envenoming by some populations of *V. berus* are occasionally reported, as it was emphasized by WARRELL (2011). On the other hand, it has been known since the 1930s that the venom of the Balkan subspecies, *V. b. bosniensis* is capable of inducing neurological disturbances dominated by cranial nerve dysfunctions (REUSS 1930, 1937; SCHÖTTLER 1938). While case reports from the 1920s, raised the possibility that the venom of certain *V. b. berus* populations occurring in parts of north and central Germany, is also neurotoxic (OTTO 1929). More authors mentioned, detailing the symptoms and signs on humans envenomed by *V. b. berus*, may develop neurological disturbances and leading to cranial nerve dysfunctions such as ptosis (REUSS 1930; FRANCKE 1937), dysphagia (FRANCKE 1937), impaired vision and speaking difficulties (SCHIEMENZ & BIELLA 1978).

New data were published in the last years about unambiguous neurotoxic manifestations following *V. b. berus* bites. In 2004, an envenomed patient developed bilateral ptosis and blurred vision following *V. b. berus* bite in Poland (CISZOWSKI & MODLA 2004). Ptosis is the classical early sign of snakebite neurotoxicity, while blurred vision resulting from impaired visual accommodation is also a familiar feature of neurotoxic envenoming (WARRELL 2003). WEINELT et al. (2002) have also reported a case from northern Germany, when the bitten patient had permanent partial left ptosis and persistent left facial nerve palsy following a bite in the left fronto-temporal area by *V. b. berus*; but it was explained by direct local effects of the venom and associated gross swelling of the face, orbit and eyelid rather than by systemic neurotoxicity. Also a neurotoxic *V. berus* envenoming

occurred in south-eastern Romania in 2012 (GAFENCU et al. 2012). Although, in this Romanian case, the taxon was erroneously recorded as *V. b. bosniensis* by the authors because only *V. b. berus* occurs in Romania (ZINENKO et al. 2010). The above reports unambiguously suggest that the venom of certain *V. b. berus* populations may contain one or more neurotoxins.

Until now, no study has addressed the nature of venom of *V. b. berus* within its Hungarian distribution range. This is an important omission because the Hungarian *V. b. berus* populations belong to the Carpathian sub-clade (KORSÓS 2007) – which is an ancestral lineage of this taxon (URSENBACHER et al. 2006) – and venom composition differences have a potential implication in understanding the link between phylogeny and intraspecific venom variations (CHIPPAUX et al. 1991), as well as the regionally distinct clinical picture of envenomed patients by the different populations of the same taxon.

# 3. Aims of the study

The present Ph.D. dissertation contains three chapters altogether. Each chapter is based on results published as an impacted paper of the author and/or manuscripts which are under preparation by the author at the time of the preparation of the dissertation.

**Chapter 1.** In Europe, *V. berus* is extensively distributed and causes more bites than any other species within the genus *Vipera*. In Hungary, envenoming by the native *V. berus* is relatively rare compared to other European countries, although, certain cases may have more serious consequences than usual and leading unique and challenging medical emergency situation mainly due to the possible geographical venom variations. In this chapter, we provide here a review and discussed the main epidemiological aspects and significance of incidents inflicted by *V. berus* in Hungary: 1) *Epidemiological aspects of Vipera berus envenomings in Hungary, and 2) Envenomings that published in the Hungarian literature – Brief review*.

**Chapter 2.** Envenomings by *V. berus* result in characteristic systemic symptoms including early ‘anaphylactic’ features (i.e. tachycardia, gastrointestinal symptoms), dizziness, hypotension, shock, coagulopathy and

neutrophil leucocytosis. These symptoms resemble those caused by *V. aspis*, reflecting the similarities in the composition of their venoms. Systemic neurotoxicity has been described in patients envenomed by certain populations of some subspecies of *V. aspis* and *V. ammodytes* in Europe. It has been attributed to pre-synaptic (ammodytoxins) or post-synaptic neurotoxic phospholipases $A_2$ in their venoms. However, neurotoxicity is the most unusual and unexpected clinical feature of *V. berus* envenoming. This chapter demonstrates two clinical reports from eastern Hungary with other interesting features and review the scanty and somewhat obscure literature on this phenomenon: entitled as *Case reports from eastern Hungary; Case report 1, Case report 2.*

**Chapter 3.** Since intraspecific venom variability of the taxon may also have important clinical implications, the third aim of the dissertation was to demonstrate experimentally and biochemically the venom individualities of a given population of *V. b. berus* in eastern Hungary. We studied the individual venom variability in this Hungarian adder population by: 1) *Comparison of the electrophoretic protein pattern of venom samples, 2) Analysis of the phospholipase $A_2$ ($PLA_2$) content of venoms by MALDI-TOF-MS, 3) Phospholipase $A_2$ ($PLA_2$) activity assay, 4) Protease assay, 5) Determination of murine $LD_{50}$ of the crude venoms and effects on chicks in vivo, 6) Neuromuscular effects of venom on frog nerve-muscle (FNM) preparation, 7) Neuromuscular effects of venom on chick biventer cervicis (CBC) preparation, and 8) Inhibitory venom effect on a glutamergic synapse of the rat brainstem slice preparation.* This chapter may help to better understand the nature of their venom complexity based on our results and the symptoms-manifestation on envenomed humans, and eventually their evolutionary and taxonomic aspects, as well.

# 4. Materials and methods

### *4.1. Data collection of envenomings*

Hungarian literature was reviewed; searches performed in medical libraries and the private libraries of amateur and professional Hungarian snake experts as well as the online available articles on Medline, PubMed and

Google. One case report has based on my personal clinical experience on adder bite published it previously (i.e. MALINA et al. 2008a). The other case report, which has also been published (i.e. MALINA et al. 2013), obtained from the Paediatric Ward of Szatmár-Bereg Hospital (Fehérgyarmat, eastern Hungary) beside the permission of usage of the original medical case record and patient's discharge letter from the Medical Director.

*4.2. Collection and storage of venom*

All venom samples of the eastern Hungarian *V. b. berus* were collected in the field (Upper Tisza River valley, eastern Hungary) during mid-spring in order to avoid differences that may result from seasonal changes of the venom composition from 2010 to 2012. The Ministry of Water and Environmental Protection of Hungary issued the permit for venom collection (No.: 14/1690-4/2010). Only healthy specimens were milked from both sexes (n=25: 14 from females and 11 from males, plus 2 control samples were used. The details of controls are below in the last paragraph in this section) by glass capillaries and/or Pasteur pipettes, or by manual gland massage following the bite on Eppendorf tubes very soon after capture, their total length were measured and recorded and then, the snakes were released. After milking, the venoms were flash frozen in liquid nitrogen. Prior to the usage of venoms, each sample was lyophilized, measured their dry weight and stored in the dark at -80°C until used. Only individual venoms – and not pooled – have been used in each experimental and biochemical study of this research in order to investigate the individual venom variability, based on CHIPPAUX et al.' (1991) recommendation.

Two control venom samples that originated from areas where neurotoxic symptoms have not been reported in human envenomings, were used: a non-neurotoxic *V. b. berus* venom (n=1, with unknown sex) collected in the eastern Austrian Alps (Ybbs Mountain) in late May 2006, and a *V. nikolskii* venom (n=1, with unknown sex) collected in the central region of Ukraine (Pidlisne) in early May 2009.

*4.3. Limitations*

The species, *V. berus* has much lower venom yield compared to the tropical and subtropical viperid species. Taking into account that venom yield and the successful milking are influenced by several factors (WILLEMSE et al. 1979; CHIPPAUX et al. 1991; CHIPPAUX 2006; MIRTSCHIN et al. 2006), venom samples used in the various experimental and biochemical studies in this research are not derived from the same specimen in every case. In addition, only venom sample from an Austrian *V. b. berus* and a Ukrainian *V. nikolskii* specimen was available for us and used as a control in this research.

*4.4. Protein content determination*

Protein concentration was determined by the method of BRADFORD (1976) using bovine serum albumin (BSA) as the standard. Absorbance was measured at 595 nm.

*4.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

In order to estimate the molecular mass of the proteins, the individual crude venoms were initially separated by SDS-PAGE, in a discontinuous gel and buffer. The resultant pellets were dissolved in the sample buffer and the electrophoresis was performed on 10 to 18% linear sodium dodecyl sulfate (SDS)-polyacrylamide gradient gels as described previously (LAEMMLI 1970). Marker proteins (used for quantification of molecular weight of the venom) were included in the runs and the gels were stained with coomassie blue and subsequently analysed using UVIDoc software (UVI, Cambridge, UK). For statistical analysis of SDS-PAGE the band obtained for the individual samples (n=25+2 controls) have been grouped into eight groups, as follows: juvenile female (n=2), juvenile male (n=3), sub-adult female (n=4), sub-adult male (n=5), adult female (n=8), adult male (n=3), and controls of the Austrian *V. b. berus* (n=1) and the Ukrainian *V. nikolskii* (n=1). Bands presence (1) and absence (0) were scored. Hierarchical clustering, Unweighted Pair-Group Method on group average (UPGMA) using Jaccard`s similarity coefficient, was run using the SYN-TAX 2000

program package (PODANI 2001). In order to determine the congruence between the dendrogram and the underlying resemblance matrix, the cophenetic correlation coefficient ($r_{cs}$) was also calculated.

*4.6. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)*

MALDI-TOF-MS is a suitable analytical tool in the detection and direct measurement of molecular weights of components in crude snake venoms (FAVREAU et al. 2006). MALDI-TOF-MS measurements of the individual crude venoms were carried out in linear mode using a Bruker Biflex III mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). External calibration was applied using the $[M+H]^+$ m/z: 12361.1971 and $[M+2H]^{2+}$ m/z: 6181.1023 peaks of Cytochrome c as calibrants. Sinapinic acid (3,5-dimethoxy-4-hydroxacinnamic acid) was the matrix solution at final concentration of 20 mg/ml using 0.1 % trifluoroacetic acid (TFA) solution in water: acetonitrile 2:1 as solvent. The venom was dissolved in 200 µl of 0.1 % TFA and a 10 µl sample plus 15 µl of matrix solution were mixed and 0.5 µl was applied to the target plate and allowed to dry at room temperature. Spectra from multiple (at least 100) laser shots ($N_2$ laser, 337 nm) were averaged prior to analysis. One sample was chosen and analysed four times to check the accuracy and reproducibility of the results; the average variance is maximally 5 Da in each molecular mass range.

*4.7. Phospholipase* $A_2$ *activity assay*

Phospholipase $A_2$ activity of the venoms was determined by the method of SANTORO et al. (1999). Briefly, venoms were diluted in 1 mL PBS pH 7.4, and 1 µg protein was added to 1.5 mL of reaction solution (100 mM NaCl, 10 mM $CaCl_2$, 7 mM Triton X-100, 0.265% soybean lecithin, 98.8 mM phenol red, pH7.6) in a spectrophotometer cuvette. The solution was immediately homogenized and read at 558 nm. The definition of 1 U of $PLA_2$ activity was taken as the amount of venom (mg of protein/assay) producing a decrease of 0.001 absorbance units per minute under the conditions described. Phospholipase activity was expressed as U/mg of two independent

experiments. Statistical analysis was performed on MATLAB Version 7.7.0471 (R2008b) and descriptive statistics were computed with the aid of built-in-routines and one-way ANOVA to test the probability of equal means.

*4.8. Protease activity/gelatin-zymography*

Gelatin-zymography can be applied to evaluate protease activities of venoms (HASSON et al. 2004; MALTA et al. 2008). For the determination of proteolytic activity of venoms, samples were homogenized with 100 mM Tris–HCl buffer pH 8.0 (Sigma-Aldrich) containing 150 mM NaCl (Reanal, Budapest, Hungary). The homogenates were sonicated and shaken to disintegrate remaining organelles as described by SCHLERETH et al. (2000). After centrifugation (13.000×g, 2x5 min, Biofuge), the supernatants were used as crude protein extracts. Protease activity analysis of samples (10-20 µl of 22 µg protein) was carried out using gelatin-containing SDS slab gels according to HASSON et al. (2004) and MALTA et al. (2008). Samples were mixed with equal volumes of protein loading buffer lacking β-mercaptoethanol and loaded on 10% SDS-polyacrylamide gels containing 0.04% gelatin. The gels were run at 20 mA/gel at 4$^{o}$C in the dark. To renature venom proteolytic enzymes, SDS was removed from gels by three 10 min washes in 2.5% (v/v) Triton X-100 in reactivating buffer. The gels were then incubated overnight at 37 $^{o}$C in reactivating buffer (50 mM Tris-HCl pH 8.0, 5mM $CaCl_2$, 10 ng $NaN_3$) in dark (HASSON et al. 2004). Local gelatin degradations were visible after Coomassie Blue staining (Coomassie Brilliant Blue R250) and revealed the sites of proteases with gelatinolytic activities.

*4.9. Determination of venom toxicity ($LD_{50}$) on mice and in vivo experiments on chicks*

All experiments were authorised by the Committee of Animal Research of the University of Debrecen. Toxicity was assessed as described previously by THEAKSTON & REID (1983) on Swiss-Webster male mice (n=6/group). Four individual venoms were chosen randomly from the Hungarian adder population and tested together with the control samples (n=2). The dose range of venoms was the following: 1.0; 0.8; 0.7; 0.6; 0.5; 0.4; 0.3; and 0.25 µg/g i.v. Control mice (n=6) were injected with normal

(venom free) saline solution. $LD_{50}$ calculation was performed by probit analysis using Minitab 16 software. Distribution fitting was tested using Person's goodness-of-fit test. Additionally, pairwise relative potency tests have been performed to compare the $LD_{50}$ fiducial estimated toxicity between each pair of samples. Chicks (6-8-day-old males, weighing 82.6 and 98.2 g; n=2) were injected with fresh-milked and undiluted venom (20 µl, unknown dry weight) subcutaneously (s.c.) and observed for developing symptoms.

*4.10. Frog nerve-muscle preparation*

Adult, wild caught frogs (*Pelophylax* kl. *esculentus*) weighing 21.5-29.2 g were used in the experiments. Frog nerve-muscle (FNM) preparations were set-up using *nervus ischiadicus* and *musculus gastrocnemius* as described previously by SCHÖTTLER (1938). All experiments were carried out at room temperature (20-22°C). Preparations were allowed to stabilize for approximately 20 minutes in venom-free frog ringer prior to the start of the experiments. Fresh frozen venom (5.5 mg in dry weight) was dissolved in 1000 µl frog ringer solution of the following composition (mM): NaCl 113.0; KCl 2.5; $CaCl_2$ 1.8; $NaHCO_3$ 3.0; and pH: 7.2-7.4. Preparations were stimulated with supra-maximal stimuli (1 V; 0.5 Hz; 1 msec pulses) via the nerve with bipolar electrodes and responses to sub-maximal concentrations of acetylcholine (Ach) (0.01 µM) and KCl (67 mM) before and after venom exposure. Two experimental techniques were employed: i) venom (550 µg) was injected into an area of the *m. gastrocnemius* that was remote from the *n. ischiadicus* (n=6), and ii) venom was injected (3300 µg) into the lymph sac of live frogs (n=4). Frogs were observed to develop symptoms of neuromuscular failure and, before death, were decapitated and the preparation was set up for twitch tension experiments. Mechanograms were recorded on a thermo-mechanical Harvard kymograph. Thermopaper tapes with the graphs were then scanned, aligned, marked and background was subtracted to improve the clarity of the trace.

*4.11. Neuromuscular studies on chick biventer cervicis*

The chick *biventer cervicis* (CBC) preparation is sensitive to snake toxins and allows prejunctional effects to be distinguished from postjunctional effects (HARVEY et al. 1994). The CBCs were removed from chicks which had been killed by exposure to $CO_2$, and the preparations were set up as described previously by HARVEY et al. (1994). Preparations were mounted under 1 g resting tension in 10 ml glass organ baths containing Krebs-Henseleit solution with the following composition (mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; $KH_2PO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 11.1. The solution was maintained at 37°C and continuously bubbled with 95% $O_2$/ 5% $CO_2$. Preparations were stimulated through the motor nerve via silver ring electrodes (0.2 msec pulses, voltage greater than required for maximal twitches at 0.1 Hz), and responses were also obtained to sub-maximal concentrations of ACh (1 mM), carbachol (20 μM) and KCl (40 mM) before and after exposure to venom.

In some experiments, preparations were set up as described and then the Krebs-Henseleit solution was changed to one in which the $CaCl_2$ was replaced by an equimolar amount of $SrCl_2$ (HARVEY & KARLSSON 1982). Responses to ACh, carbachol, and KCl were re-established and the responses to indirect stimulation allowed to stabilise before the addition of venom.

*4.12. Electrophysiology on identified neurons of rat brainstem*

To study the origin of possible neurotoxic effects of the venom on non-cholinergic synaptic transmission, the end-bulbs of Held were investigated. These transitions are formed between the glutamatergic nerve terminals of the acoustic nerve and the cell bodies of the bushy neurones of the cochlear nucleus in the central auditory pathways of the rat (WANG & MANIS 2008). Experiments were performed on 200 μm thick parasagittal brainstem slices prepared from 10-14-day-old Wistar rats (n= 8). The tissue sections were maintained in an artificial cerebrospinal fluid (aCSF) with the following composition (mM): NaCl, 125; KCl, 2.5; $NaHCO_3$, 26; glucose, 10; $NaH_2PO_4$, 1.25; $CaCl_2$, 2; $MgCl_2$, 1; myo-inositol, 3; ascorbic acid, 0.5; sodium-pyruvate, 2. For the preparation of the brainstem slices, low-sodium

aCSF was applied, in which NaCl was replaced by equimolar sucrose (250 mM). Brain slices were visualized using an Axioskop FS microscope (Carl Zeiss, MicroImaging GmbH, Jena, DE) equipped with differential interference contrast optics and a 63×water immersion objective. The slices were continuously perfused with aCSF bubbled with a mixture of 95% $O_2$ and 5% $CO_2$ at 34°C. Patch pipettes were filled with a solution containing (mM): K-gluconate, 114; KCl, 4; HEPES, 10; $K_2$-ATP, 4; $Na_3$-GTP, 0.3; $Na_2$-phosphocreatinine, 10; $K_2$-Lucifer Yellow, 2; biocytin, 8. After filling with the pipette solution, the resistance of the microelectrodes varied between 1.8 and 2.2 MΩ. Whole-cell patch-clamp recordings were made using the voltage-clamp configuration and employing an Axopatch 200A amplifier (Molecular Devices, Union City, CA, USA). During the electrophysiology experiments in the voltage-clamp configuration, the holding potential was -60 mV. The evoked postsynaptic currents (PSCs) were induced by using a monopolar-stimulating electrode that was connected to a BioStim STC-7a stimulator device (Supertech Ltd., Pécs, HU). The stimulatory electrode was placed on acoustic nerve fibres within the anteroventral cochlear nucleus (aVCN). Excitatory postsynaptic currents were elicited by 50 Hz stimulation with the lowest effective stimulatory amplitude. The inhibitory synaptic transmission was inhibited by 1µM strychnine and 10µM bicuculline. Data acquisition was achieved by using the Clampex and Fetchex 6.0 software (Molecular Devices Inc., Union City, CA, USA). Data analysis was performed using the Clampfit 9.0 (Molecular Devices Inc., Union City, CA, USA) program. During the analysis, paired-pulse-ratio (PPR) was calculated as the ratio of the amplitudes of the second and first postsynaptic currents, respectively (ZUCKER & REGEHR 2002).

Data are presented as mean ± SEM. Statistical significance was determined using Student's two-sample t-test (with one-tailed distribution and two-sample equal variance (i.e. homoscedastic)). The level of significance was set to 0.05. All animal experiments were authorised by the Committee of Animal Research of the University of Debrecen, Hungary.

# 5. Results
# Chapter 1

## 5.1. Epidemiological data

### *5.1.1. Epidemiological aspects of Vipera berus envenomings in Hungary*

Only *V. berus* can be significant from medical and toxicological points of view from the two native viper species (MALINA et al. 2011a), although, *V. ursinii* is also noted as a medically important species in Hungary (WHO 2010). There is no doubt that envenomings by *V. berus* and *V. ursinii* are relatively rare in the country (MALINA et al. 2012) with a mean annual morbidity rate of 0.46 or 0.0046/100,000 inhabitants, estimated for both species (MALINA et al. 2008b). Compared with the consequences and frequency of native *Vipera* cases, bites by exotic species, especially the tropical and subtropical viperids often result snakebite incidents (MALINA et al. 2008b; MALINA & KRECSÁK 2008), which is a major challenge for the Hungarian physicians (MALINA et al. 2008b).

Howbeit, there has been preliminary nationwide study about *V. berus* envenomings in Hungary (i.e. MALINA 2011), demonstrating the clinical picture of bitten patients within the Hungarian distribution range of species (**Fig. 1**), currently we have no exact epidemiological data about the incidence rate of *V. berus* envenomings.

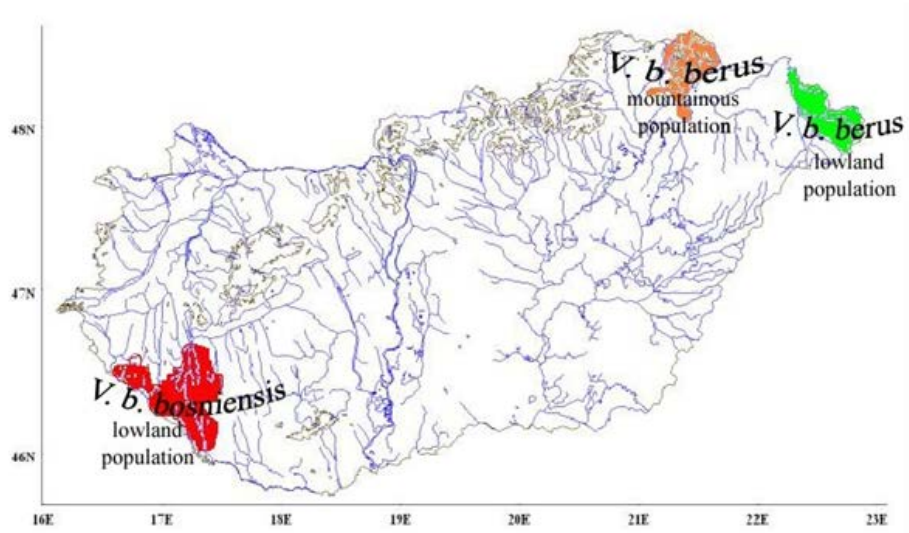



**Figure 1.** Restricted distribution range of *Vipera berus* in the three main separated regions of Hungary.

Nevertheless, *V. ursinii* envenomings are negligible compared to *V. berus* bites (KRECSÁK et al. 2011). On the other hand, it seems that the adder related incidents are much less frequent in Hungary than in any other European countries where *V. berus* occurs.

In spite of the fact that the species has quite potent and toxic venom (CALDERÓN et al. 1993; WÜSTER 1998; MALINA et al. 2012), fatal incidents are very rare, not only in Hungary (MALINA et al. 2008b, 2012) but also throughout Europe (REID 1976; WÜSTER 1998; WARRELL 2005; KARLSON-STIBER et al. 2006). Amount of the injected venom for a single bite is quite small in case of *V. berus*, which significantly contributes to a favourable outcome of envenoming, increasing the chance for survival (MALINA et al. 2012). In Europe, the last *V. berus* envenoming that sought human life occurred in the neighbouring Transylvania (Maros County) on 16 August 2010 (ANONYMOUS 2010a). Envenoming by *V. berus* had been ended in death only three occasions in Hungary since the 1940s. Adult males were the

victims in all the cases. Though, only two cases are authentic while the third one is quite dubious. These fatal cases are briefly summarized, below.

Firstly, MARIÁN (1956) recorded a fatal case from Somogy County, while the last fatal envenoming occurred in the valley of the Upper River Tisza in 2001 (TÓTH 2003). Although, in this latter case, the death of victim had been attributed to snake venom induced anaphylaxis (a complication specific to snake-handlers as well as professional herpetologists (MALINA et al. 2008b; DE MEDEIROS et al. 2008)) rather than the direct toxic effect of the venom, since this victim had several snakebites prior to the fatal event (MALINA et al. 2008b). The third case was reviewed by VIRÁGH & TASS (1986). In this case, a 55-year-old male died presumably due to *V. berus* bite in the Zemplén Hills on 13 August 1956 (VIRÁGH & TASS 1986). The authors were unable to confirm the authenticity of this fatal incident, but the case has been still existed as a fatal envenoming by *V. berus* (VIRÁGH & TASS 1986); cause of the victim's death was the venom had been injected through a larger vessel on the dorsum of hand (KORSÓS 2008. pers. comm.).

### *5.1.2. Envenomings that published in the Hungarian literature – Brief review*

Only a few case series (e.g. MAJOR 1965; VIRÁGH & TASS 1986, 2002) and/or single case reports (e.g. SZIRAY & KÁROLYI 2011) have been issued in Hungarian about the incidents caused by *V. berus* in the north-eastern mountainous areas, namely the Zemplén Hills during the past 50 years. Although, *V. berus* has the widest distribution range in this region of Hungary and most likely the highest population densities are in the Zemplén Hills (MALINA et al. 2011a). Probably the first authentic Hungarian case history is mentioned by PETRASKÓ (1899), although, without any detail referring to the description of envenoming, when an adult male was bitten near Gönc in the Zemplén Hills. Several cases that derive from the early case series are not well-detailed by the authors and combined with inaccurate medical description and thus, just a few of them is useful clinically and toxicologically. From the period of 1998 till 2005, a very poorly detailed ten cases - occurred in the region of Zemplén -, are mentioned in SIMON's MA thesis (2007). The authenticity of these 10 cases is debatable as all of them are based on only personal communications of rural people and

questionnaired responses of the employees from the local government, and not hospital case records and/or discharge letters of patients.

Case descriptions about human envenomings from the south-western Hungarian distribution range of the species are very poor, while more fatal adder bites affected domestic ungulates and dogs, are noted by MARIÁN in his work that is based on the record of the district veterinarian of Somogyszob (MARIÁN 1956). In addition, only a few sentences refer to one or two cases about envenomed patients but mostly without any detailed description. Such cases are mentioned also by MARIÁN (1952, 1956) from Somogy County. Maybe one of the earliest recorded *V. berus* bite from this county is that one, when the patient was transported to the Hospital of Kaposvár in July of 1951 (MARIÁN 1952). Another two envenomings are known and a single case with fatal outcome that occurred between 1941 and 1956 (MARIÁN, 1956). The fatal case was re-mentioned by DOLECSKÓ (1964). Another incident is known from Homokszentgyörgy (southern Somogy County) in 1964, when the local forester was bitten by *V. berus* and was admitted in highly critical and life-threatening condition to the Hospital of Kaposvár (ANONYMOUS 1964b). KOLLÁR (1979), who was a local medical practitioner in Somogy County, has treated more patients, which were bitten by *V. berus* within that area. Interestingly, there are no cases from Zala County, mentioned in the Hungarian literature. The first clinically oriented research paper, which presented *V. berus* envenomings that occurred in Somogy and Zala counties – and discussed it with those incidents inflicted by the lowland Croatian *V. berus* populations –, issued only in 2011 (MALINA et al. 2011b).

There was no known adder bite occurred in the region of the Upper Tisza River valley in the last 15 years as it was reported by AGÓCSY in 1958 and then, MARIÁN in 1960 (AGÓCSY 1958; MARIÁN 1960) but more fatal envenomings among the local livestock and dogs were recorded (AGÓCSY 1958). There is no any other data and/or indication for adder envenomings from the eastern region of the country in the Hungarian journals, but two clinical case reports about envenoming by *V. berus* derive from the region of the Upper Tisza River valley, were published in foreign medical literature in 2008 (MALINA et al. 2008a) and most recently in 2013 (MALINA et al. 2013).

Summarizing the above, the available epidemiological and clinically oriented papers about *V. berus* envenomings are few, published in Hungarian. Additionally, most of them drive from the early literature and thus, these

articles do not contain the updated information concerning *V. berus* bites for the native emergency physicians and clinical toxicologists. (MALINA et al. 2012). Unfortunately, the lack of information (e.g. about the correlation between clinical presentation of patients and the subsequent symptom-manifestation, the predictable factors that can permit a rapid evaluation and predicting the severity of adder bites) is broadening as a result of the false and/or partly insufficient (e.g. about the beliefs persisting both among laymen and medical professionals regarding to the venom toxicity of the species) information has been occasionally issued in the Hungarian medical journals (MALINA et al. 2012).

In connection with certain clinical features of *V. berus* envenomings, occurring within the Hungarian distribution range of the species, we can state without doubt, that the bite of *V. b. bosniensis* in south-western Hungary (MALINA et al. 2011b) and the lowland *V. b. berus* population in eastern Hungary (MALINA et al. 2008a; MALINA et al. 2013) is capable of inducing neurological disturbances on humans. The basis for these results is those case reports and clinical research studies that have been issued in the past few years. We have no knowledge about any case when neurotoxic symptoms and signs would have manifested on envenomed patients, bitten by *V. b. berus* in the Zemplén Hills and Eperjes - Tokaj Range (MALINA 2011). Reviewing the available literature, other authors did not mention any neurotoxic case from this mountainous region, either.

Despite the extremely low mortality rate and the relatively infrequent human-adder encounters in Hungary, the medical relevance of *V. berus* envenomings is not negligible as it is reflected by more case reports and clinically oriented papers (i.e. MALINA et al. 2008a, 2008b, 2011a, 2011b, 2012, 2013; and MALINA 2011), have been issued in the last couple of years.

# Chapter 2

### 5.2. Case reports from eastern Hungary

These two case reports below had been published previously by MALINA et al. (2008a, 2013). Both snakebite incidents were inflicted by a specimen from the flatland *V. b. berus* populations native to the Upper Tisza

River Valley, eastern Hungary. The most interesting feature of these incidents were that both resulted neurotoxic envenoming, characterizing by unambiguous neurological signs and symptoms on the envenomed patients.

*5.2.1. Case report 1.* (MALINA et al. 2008a)

A previously healthy 27-year-old man was bitten by an adult female *V. berus* 70-72 cm in total length on 23 April 2007 in Szabolcs-Szatmár-Bereg County in eastern Hungary. The snake was captured alive and expertly identified. The victim reported no previous snakebites. Two fangs impaled the left thumb, causing burning pain that became throbbing in quality. The bitten finger swelled immediately and a small haematoma formed at the bite site. Swelling associated with erythema extended to the hand in 45 min. The patient applied a tourniquet around the proximal phalanx of the thumb and the wrist, which was released every 15-20 min. It was still in place when he was admitted. Within ~90 min, he was transported to the nearest hospital.

On admission, the patient was fully conscious. He had two attacks of profuse diarrhoea and one episode of mild nausea. Examination revealed tense, tender swelling involving the whole hand and wrist. Erythema was mild and local haemorrhage was confined to the fang marks, which were clearly visible. He was unable to move his fingers or make a fist. He had been slightly nauseated and dizzy during the journey. On arrival at hospital he felt ‘drugged’ although he had taken no medications except two 500 mg calcium tablets. After 20-30 min, he suddenly noticed double vision; his gaze seemed to shift horizontally and there was a dim ghost image. He became increasingly dizzy and experienced true vertigo when he lay supine. On examination, there was no ptosis but diplopia was confirmed. There was definite strabismus and the second image disappeared when one eye was covered. His gait was found to be unsteady. There was no nystagmus and the pupils were moderately dilated, equal in size and reactive to light and accommodation. There was mild fever (37.6 °C). When he was first examined, 1.5 h after the bite, the heart rate was increased (103/min) and the blood pressure was elevated to 180/120 mmHg. This hypertension was controlled with captopril 12.5 mg. The blood pressure decreased to 150/90mmHg over the next few hours, but was still 139/82 mmHg the next morning. According to the patient, his blood pressure is usually about 118/79

mmHg with a heart rate of 65/min. At this stage, 2 h after the bite, he was given tramadol 50 mg i.v. ~1-2 h later, he began to feel drowsy. On admission to hospital, his differential leucocyte count was mildly abnormal: absolute neutrophil count $9.59x10^9$/l, 84.5% (normal: 42-74%), lymphocytes 9.9% (normal: 17-45%), eosinophils 0.5% (normal: 1-7%). Blood coagulation and urine were normal.

Although antivenom is indicated when neurological and/or other systemic symptoms evolve (WARRELL 2005; KARLSON-STIBER et al. 2006) it was not given in this case because the patient feared a reaction. The neurological signs were not considered life-threatening. Diplopia lasted for 11 h after the bite and then resolved spontaneously, while intense dizziness gradually wore off within 2 days. The dorsum of the hand was slightly bluish in colour until the third day. The moderate local swelling was receding by the fourth day. Tender arthralgia of metacarpal and interphalangeal joints of the bitten hand lasted for 8 days.

*5.2.2. Case report 2.* (MALINA et al. 2013)

A healthy 12-year-old girl (body weight, 50 kg) was bitten by a snake during a school trip in Túristvándi (Szabolcs-Szatmár-Bereg County), eastern Hungary on 02 May 2012 at approximately 4:30 PM. The girl confidently grabbed the snake, as she believed it was only a harmless grass snake (*Natrix natrix*). The 50- to 60-cm long snake immediately bit her. The culprit specimen had been photographed with a mobile phone by one of her schoolmates. Later, the snake was identified as a melanistic *V. b. berus* specimen by a keeper from the Venomous Snake Department of the Budapest Zoo, and the identification was reconfirmed by a ranger of the Directorate of Hortobágy National Park (Hungary).

Throbbing pain and local swelling developed within minutes after the bite, suggesting that venom had been injected. The girl received first aid (500 mg of Calcium-Sandoz [calcium] tablet and cold pack) from her teacher and was taken to the local general physician (GP) office. By then she was drowsy and nauseated, but fully conscious. She had no history of snakebite and declared no allergies. The GP requested the transport of the patient to the nearest hospital (Szatmár-Bereg Hospital, Fehérgyarmat). She was transported by ambulance, and admitted to the paediatric ward.

On admission (7:12 PM), the girl's whole left hand was edematous, tense, and painful on palpation. Mild hyperaemic discoloration was observed on the dorsum of the hand. There were 2 fang marks approximately5 to 8mm apart on the extensor side of the left index finger, and another puncture mark was visible on the finger's radial aspect. The victim was pale, weak, and prostrated and was unable to stand. She repeatedly retched and, on a few occasions, regurgitated. On arrival her blood pressure, heart rate, and respiration rate were 109/70 mmHg, 99 beats/min, and 13 breaths/min, respectively. She complained of intensive dizziness and was still drowsy but able to be aroused. The sensorium remained clear, and she was able to give adequate answers when questioned. According to the patient, double vision first developed about 20 to 30 minutes after the bite. During the neurological examination, her pupils were moderately dilated, and mild abnormality of pupillary accommodation was detected. Although she could partially open her eyes on request, palpebral ptosis was obvious. The patient described photosensitivity in the presence of ambient light and experienced eye movement difficulties. Medical examination confirmed a bilateral impairment characterized by oculomotor paralysis with partial bilateral ptosis and gaze paresis but no other cranial nerve or additional neurological deficits were observed. The patient preferred remaining in a lying position because it mitigated the intensity of dizziness occurring when sitting or standing.

Immediate routine laboratory tests did not show abnormalities in blood coagulation, assessed by prothrombin (73.3%; normal, 70-100%), international normalized ratio (INR, 1.17; normal, 1.00-1.20), activated prothrombin time (APTI, 23.7 seconds; normal, <42.0 seconds), and thrombin time (TT, 18.80 seconds; normal, <22.0 seconds). Only slight elevations in blood glucose (6.50 mmol/L; normal, 3.30–6.00 mmol/L) and plasma calcium (2.64 mmol/L; normal, 2.15-2.60 mmol/L) were observed. Within approximately 4 hours of post-bite supportive therapy, 1 vial (5 mL) of Calcimusc injection (calcium, 100 mg/mL i.m.), isotonic saline (Ringer's lactate solution, 500 mL i.v.), tetanusprophylaxis, corticosteroid (120 mg of methylprednisolone, i.v.), and antibiotic therapy (850 mg of amoxicillin-clavulanic acid i.v. every day) was administered. The latter was maintained longer than 48 hours. Antivenom was not administered because the treating physician considered it unnecessary.

Next morning (03 May 2012) the retching and regurgitation ceased. Laboratory results showed slight deviations from normal: C-reactive protein, 7.2 mg/L (reference range, <5 mg/L); white blood cells, 17.96 $10^3$/L (reference range, 4.5-13.0 $10^3$/L); neutrophils, 87% (reference range, 40-74%); lymphocytes, 7% (reference range, 19-41%); and density of urine, 1015.000 (reference range, 1.005-1.020). Some blood coagulation parameters showed an increasing trend as compared with those on the admission day (i.e., APTI, 25.7 seconds [normal, <42.0 seconds], TT, 20.90 seconds [normal, <22.0 seconds]), but remained within the normal range. The patient could open her eyes easier, but bilateral ptosis was still present, and the hand oedema started to decease. On 04 May 2012, the patient showed no systemic symptoms, and the hand oedema, as well as hyperaemia, was significantly reduced. The cranial nerve palsies resolved, and there were no further peripheral neurotoxic signs and symptoms. She remained stable and was discharged the next day (05 May 2012) after an uneventful recovery.

# Chapter 3

## 5.3. Experimental and biochemical studies

### *5.3.1. Snakes*

Specimens were collected and milked in the field from a lowland *V. b. berus* population within a limited area (**Fig. 2**), in the Upper Tisza River valley, eastern Hungary up to an elevation of 100-150 m above the sea level.

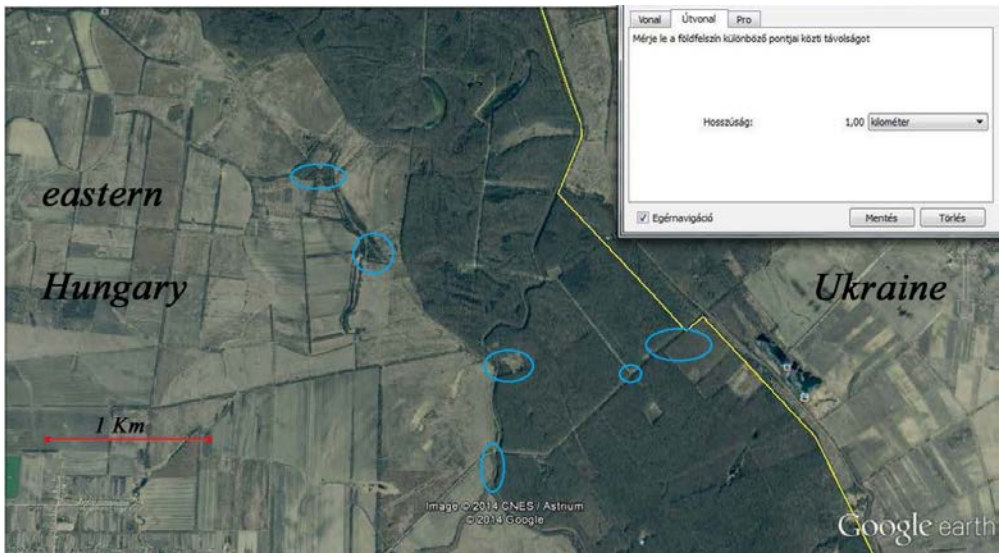



**Figure 2.** Map about the collection of venoms in the habitat of a lowland *V. b. berus* population in eastern Hungary.
**Legend:** yellow line is the border of country, blue circles show the area where adders were captured and milked, and then, released. Red line is the scale.

Overall 37 individuals were milked: 19 males and 18 females, their total length was ranged from 16.2 to 77.4 cm (**Table 1**). The average total length was 52.3 cm (Males: 16.2-68.9 cm, average: 50.3 cm. Females: 26.3-77.4 cm, average: 54.5 cm). According to their total length, snakes were classified into three main approximate age-groups: i) juveniles (n=7; 16.2-38.0 cm), ii) subadults (n=11; 42.5-52.0 cm), iii) and adults (n=19; 58.3-77.4 cm).

Of the 37 venom samples, the dry weight of two venoms was unmeasured. The average dry weight of venom (based on the 35 specimens) was 5.5 mg/specimen in case of the eastern Hungarian adders (average: 4.7 mg in males, 6.2 mg in females), while it was 2.0 mg in case of the specimen of *V. nikolskii*, and 6.3 mg in the individual of the Austrian *V. b. berus*. The average dry weight of venoms was the following in the given age-groups: i) juveniles: 1.9 mg, ii) subadults 4.1 mg, and iii) adults: 7.9 mg. Dry weight of each venom sample is showed by **table 1**.

| No. of specimen | Approximate age-group/total length (cm) | Sex | Dry weight of milked venom (mg) |
|---|---|---|---|
| **1.** | subadult/48.3 | ♀ | 1.2 |
| **2.** | adult/70.0 | ♀ | 10.3 |
| **3.** | adult/62.6 | ♂ | 5.0 |
| **4.** | adult/66.0 | ♀ | 13.2 |
| **5.** | adult/58.3 | ♂ | 6.8 |
| **6.** | subadult/43.3 | ♂ | 3.7 |
| **7.** | juvenile/28.1 | ♀ | 2.6 |
| **8.** | juvenile/22.0 | ♂ | 2.4 |
| **9.** | juvenile/16.2 | ♂ | 0.8 |
| **10.** | juvenile/26.3 | ♀ | 1.7 |
| **11.** | adult/60.1 | ♀ | 8.1 |
| **12.** | subadult/42.5 | ♂ | 1.9 |
| **13.** | adult/61.0 | ♀ | 11.1 |
| **14.** | adult/77.4 | ♀ | 15.7 |
| **15.** | subadult/43.8 | ♂ | 7.0 |
| **16.** | adult/59.8 | ♀ | 4.4 |
| **17.** | subadult/50.7 | ♂ | 9.3 |
| **18.** | subadult/44.9 | ♀ | 2.7 |
| **19.** | subadult//47.6 | ♀ | 3.8 |
| **20.** | adult/73.8 | ♀ | 10.0 |
| **21.** | adult/70.1 | ♀ | 10.3 |
| **22.** | subadult/45.0 | ♂ | 2.3 |
| **23.** | adult/60.2 | ♂ | 5.2 |
| **24.** | subadult/48.1 | ♀ | 2.3 |
| **25.** | adult * | - * | 6.3 |
| **26.** | adult * | - * | 2.0 |
| **27.** | juvenile/36.7 | ♂ | 2.1 |
| **28.** | adult/59.1 | ♂ | 3.4 |
| **29.** | adult/62.7 | ♂ | 4.8 |
| **30.** | adult/66.5 | ♂ | 8.5 |
| **31.** | adult/69.7 | ♀ | 3.1 |
| **32.** | adult/60.9 | ♂ | 6.1 |

| | | | |
|---|---|---|---|
| **33.** | subadult/48.8 | ♀ | 3.9 |
| **34.** | subadult/52.0 | ♀ | 7.0 |
| **35.** | adult/68.9 | ♂ | 7.7 |
| **36.** | juvenile/38.0 | ♂ | 2.5 |
| **37.** | juvenile/30.1 | ♀ | 1.0 |
| **38.** | adult/60.0 | ♂ | ‡ |
| **39.** | adult/59.1 | ♂ | ‡‡ |

**Table 1.** Total length of the milked specimens and the dry weight venoms in the three approximate age-groups of snakes used in this study.
**Legend:** From 1 to 27: venom patterns of the same specimens are presented in figure 2. Number 25 and 26 are the control venoms; 25=Austrian *V. b. berus* and 26=*V. nikolskii*; *=length and sex were not recorded; ‡=unknown dry weight, 20 µl fresh-milked undiluted venom. ‡‡= unknown dry weight, 23 µl fresh-milked undiluted venom.

### *5.3.2. Comparison of the electrophoretic protein pattern of venom samples*

As determined by SDS-PAGE electrophoresis (non-reduced) (**Fig. 3**), there is an unambiguous variation in the number, the location, the abundance, and the intensity of protein bands among the individual venoms. The number of bands varies between 7 and 18 (average: 12.8) in the samples. Control samples presented 11 and 12 bands, respectively that showed the following molecular weights: 82.1, 76.5, 59.1, 55.5, 40.3, 31.3, 24, 18.3, 18.2, 15.5 kDa (Austrian *V. b. berus*) and 77.2, 61.2, 39.3, 34.7, 26.7, 18.8, 17.6, 15.7 kDa (*V. nikolskii*). There is a great predominance of proteins of molecular masses in the range of $PLA_2$s (13-15 kDa), indicated by their intensity and relative quantity. These proteins are the dominant components of the Hungarian adder venoms and of the two control samples, as well. There were notable differences in protein bands in the molecular mass range of 20-24 kDa, 30-40 kDa and 45-70 kDa.

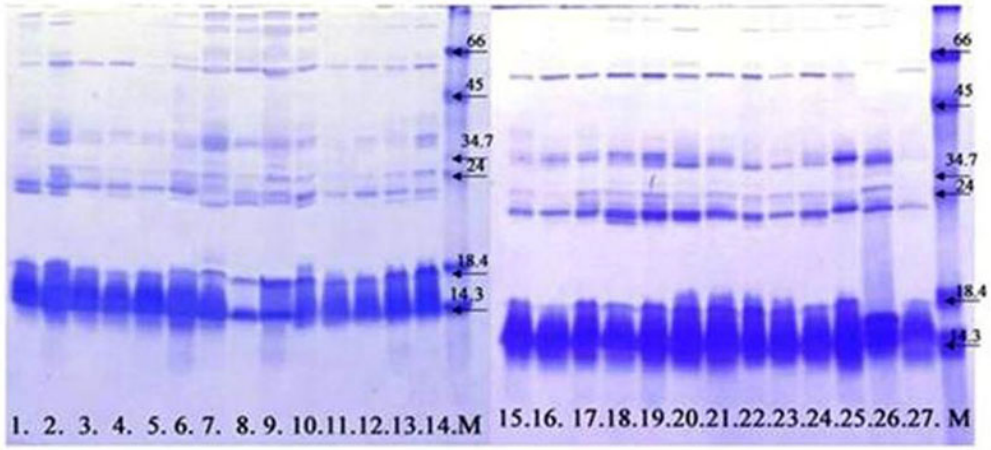



**Figure 3.** SDS-PAGE photograph (non-reduced) of the complete venom pattern of the Hungarian adders and the two control venoms.
**Legend:** M=marker; 25=Austrian *V. b. berus*; 26=*V. nikolskii*

Individual analysis of the venom patterns revealed a few gender-specific differences and similarities. There was no difference in the venom complexity associated with the number of protein bands between females (n=13) and males (n=12): the average number of bands is 12 in males, while it is 11.9 in case of females. Four protein bands (61, 37, 30, and 21 kDa) were found in male venoms that were absent in female ones. On the other hand, there were three protein bands (83.5, 75, 57.5 kDa) found only in the female adder venom (**Fig. 3**).

Differences in venom protein bands of the three taxa (Hungarian *V. b. berus*, Austrian *V. b. berus* and *V. nikolskii*) determined by SDS-PAGE, are shown on **figure 3**. The dendrogram resulting from the UPGMA is showed in **figure 4.** The protein profile of venom of *V. nikolskii* differs from those of *V. b. berus* venoms (Hungarian and Austrian, as well) only in two protein bands (located in 57 and 25.5 kDa range), which are completely absent from *V. berus* venoms. A cross line at the 0.82 dissimilarity level divided the dendrograms into three major groups: one contains the Austrian adder sample and the sample of *V. nikolskii*, while another contains the samples of juveniles, and a third group consists of the samples of subadult together with adult specimens. Our dendrogram had a cophenetic correlation of 0.93,

suggesting that the dendrogram provides an excellent representation of the resemblances.

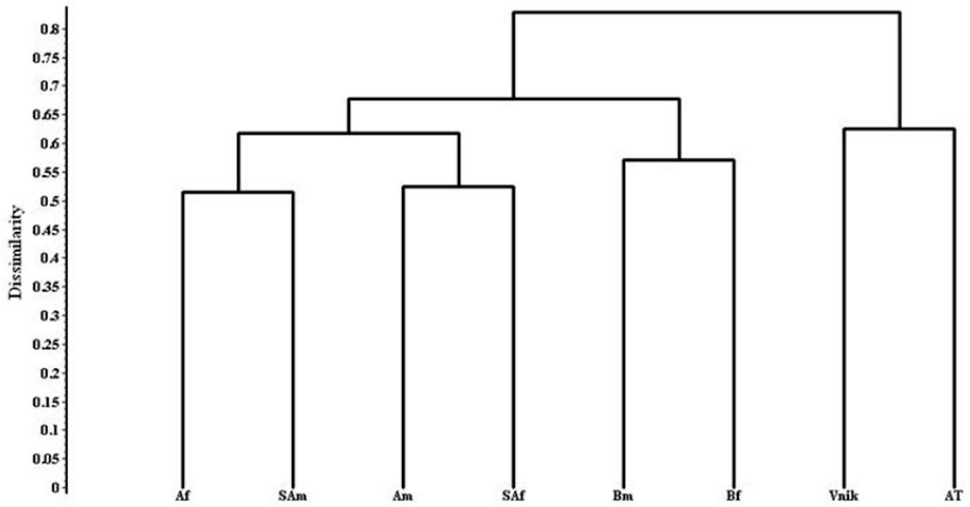



**Figure 4.** Cluster analysis of the grouped electrophoretic results, based on figure 2.
**Legend:** Bf=juvenile female, Bm=juvenile male, SAf=subadult female, Sam=subadult male, Af=adult female, Am=adult male, AT=*V. b. berus* Austria, Vnik=*V. nikolskii*.

### *5.3.3. Analysis of the phospholipase $A_2$ ($PLA_2$) content of venoms by MALDI-TOF-MS*

In order to assess the possible intra-population variations, the MALDI-TOF spectra of $PLA_2$ content of venoms from all but one (n=24) Hungarian adder venom samples are compared (**Appendix 1 and Fig. 3**). The abundance and intensity of bands in the molecular mass range 13-15 KDa on the SDS-PAGE correspond to the expected $PLA_2$s. According to the molecular masses, 9 main groups of these proteins have been detected in the above-mentioned ranges (**Appendix 1**). The samples gave from 4 to 7 peaks - depends on the individuals - in the molecular weights by MALDI-TOF-MS that vary between 13548.35 and 14340.17 Da. The most intense peaks were at range of 13800 Da in 92 % of venoms. Venom of female adders gave an average 5.1 peaks while males gave 5.3 peaks between 13000 and 15000 Da. There was no significant difference among the specimens in the different age groups (their approximate age /age-group was estimated on the bases of the

size of specimens). The average number of peaks in the different age-groups was the following: 4.8 in juveniles, 5.4 in subadults and 5.3 in adults. The control venoms gave 5 (Austrian *V. b. berus*) and 3 peaks (*V. nikolskii*), detected by MALDI-TOF-MS (**Fig. 5**).

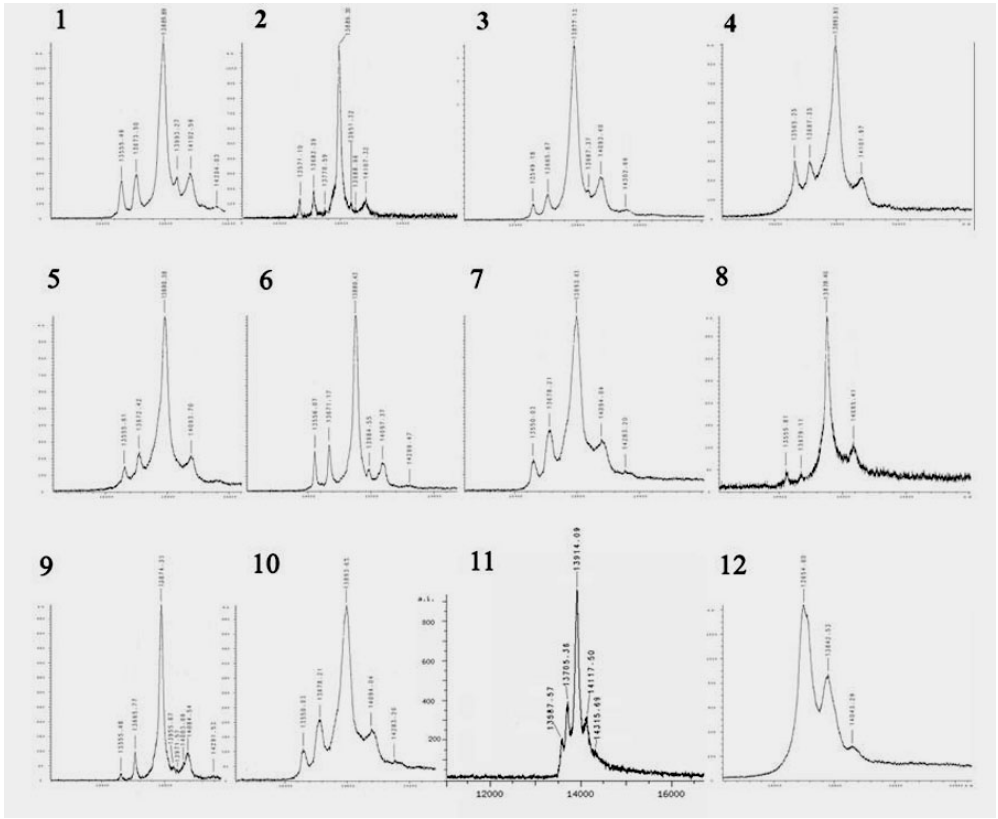



**Figure 5.** Representative MALDI-TOF mass profiles (dimers are not shown) of the $PLA_2$ content of individual venoms in case of the eastern Hungarian *V. b. berus* population and in the two control venoms. Mass (in Da) is given each peak for each component present. **All the detected molecular masses in the tested samples (n=24) are listed in Appendix 1. Legend:** Spectrum represents four experiments. Samples are the same as listed in figure 2 (in numerical order on SDS-PAGE): 1 =sample 1; 2=sample 13; 3=sample 23; 4=sample 9; 5=sample 1; 6=sample 22; 7=sample 25 (control as Austrian *V. b. berus*); 8=sample 5; 9=sample 16; 10=sample 27; 11=sample 11; 12=sample 26 (control as *V. nikolskii*)

### *5.3.4. Phospholipase $A_2$ ($PLA_2$) activity assay*

The total $PLA_2$ activities of the individual venoms (samples) were also compared. Of the Hungarian specimens, the lowest activity was 385 kU/mg, while the highest was 619 kU/mg. Results are presented as mean ±

SEM: i) juveniles: 515 kU/mg (±57), ii) subadults: 506 kU/mg (±61), and iii) adults: 492 kU/mg (±54). The activity of individual samples is differing from each other but this difference was not significant ($p$= 0.745) between the three age-groups. The control samples showed 546 kU/mg (Austrian *V. b. berus*) and 431 kU/mg (*V. nikolskii*), respectively.

*5.3.5. Protease assay*

Clear individual variations of the venoms' proteolytic activity were seen on gelatine-zymograms, shown in **figure 6.** Gelatinolytic activity could be observed in bands around 34-37, 45-50, and 60 kDa. Only 8 of 21 investigated venoms showed unambiguous gelatine-degradations on the gel, which indicates enzymatic activity. Of these 8 venoms, certain venoms (samples: 1=subadult female, 5=adult male, 8=juvenile male, 9=juvenile male) had relatively strong protease activity, while the others (samples: 2, 4, 11, 14= all of them derived from adult females) had lower activity (**Fig. 6**). The control Austrian *V. b. berus* and *V. nikolskii* venom, were devoid of protease activity (**Fig. 6**).

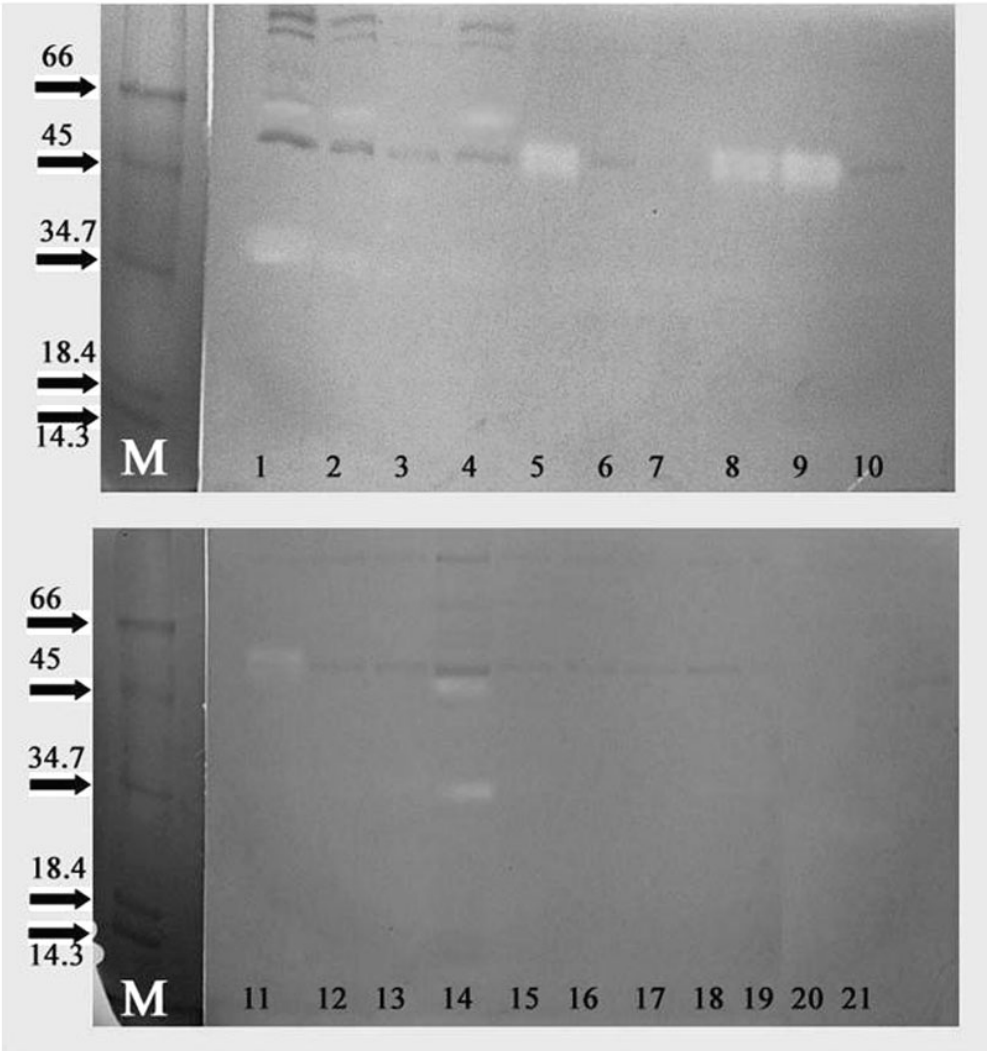



**Figure 6.** Gelatinolytic activity (15μg) of the venoms of the eastern Hungarian adders and the two controls.

**Legend:** M=marker. Arrows with the numbers correspond to the position of molecular mass markers (M). Enzymatic activity of the samples is indicated by the clear degradations (plaques) on the gel. Samples are the same in numerical order that were listed in table 1 and presented on figure 2 (from 1-18 and sample 21, with the exception of 19 that is the Austrian *V. b. berus* and sample 20, which is *V. nikolskii*).

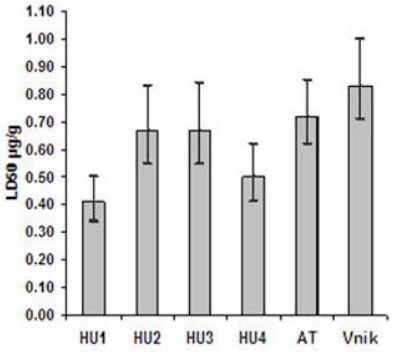
*5.3.6. Determination of murine $LD_{50}$ of the crude venoms and effects on chicks in vivo*

In case of all the tested (n=4) venom samples, envenoming resulted in characteristic symptoms in mice (i.e. head-drop and floppy neck, progressive respiratory paralysis preceded by initial increase in respiration rate and limb-paralysis) prior to death from respiratory paralysis. Following administration of 0.80 µg/g to mice, these symptoms developed within circa 40 min, and death followed 1-1.5 h after venom injection.

Limb-paralysis and floppy-neck on mice – the mice were able to move till the last moment, when the respiration had become irregular and very intensive directly prior to death – could be observed after the injection of the Austrian adder venom in case of the maximum tested dose 1.0 µg/g i.v. (it was lethal in 100%), neither. While these symptoms were also visible after the injection of *V. nikolskii* venom. Similar symptoms were observed, i.e. headshake, balance disorder, marked head-drop, dyspnoea and flaccid paralysis in chicks following the injection (s.c.) of multiple amount of lethal dose of an eastern Hungarian adder venoms (20 µl fresh-milked undiluted). Oedema did not develop in any group of mice or chicks.

$LD_{50}$ values are presented in **figure 7.** The Log-logistic distribution fitted best for our data as revealed by the Person's goodness-of-fit test (Person $\chi^2$=19.77, df=27, p=0.84; Deviance $\chi^2$=23.38, df=27, p=0.66). The regression slopes equality among the venoms was not violated (Test for equal slopes $\chi^2$=9.07731, df=5, p=0.106).

| | HU2 | HU3 | HU4 | AT | Vnik |
|---|---|---|---|---|---|
| HU1 | 1.61* (1.20:2.15) | 1.62* (1.21:2.16) | 1.21 (0.92:1.60) | 1.74* (1.36:2.23) | 2.00* (1.56:2.60) |
| HU2 | | 1.00 (0.73:1.37) | 0.75 (0.56:1.02) | 1.08 (0.82:1.42) | 1.24 (0.94:1.65) |
| HU3 | | | 0.75 (0.56:1.01) | 1.07 (0.82:1.41) | 1.23 (0.94:1.64) |
| HU4 | | | | 1.43* (1.10:1.84) | 1.64* (1.27:2.14) |
| AT | | | | | 1.14 (0.91:1.45) |




| | LD50 (95% confidence limits) |
|---|---|
| HU1 | 0.41 (0.34:0.50) |
| HU2 | 0.67 (0.54:0.83) |
| HU3 | 0.67 (0.54:0.84) |
| HU4 | 0.50 (0.41:0.62) |
| AT | 0.72 (0.62:0.85) |
| Vnik | 0.83 (0.71:1.00) |

**Figure 7.** $LD_{50}$ values of individual venoms and pairwise relative potency comparison and between the venom samples.
**Legend:** *=significant difference, values in parenthesis are represented the 95% confidence limits. The same venoms were used as in table 1. HU1=sample 23; HU2= sample 31; HU3=sample 33; HU4=sample 36; Controls: AT=Austrian *V. b. berus*; Vnik=*V. nikolskii*

### *5.3.7. Neuromuscular effects of venom on frog nerve-muscle (FNM) preparation*

In general, venoms from the Hungarian *V. b. berus* specimens produced a time-dependent, irreversible block of neurotransmission of nerve-muscle preparations.

With *in vivo* injection of 3300 µg venom into live frogs, throat oscillations started to increase after the first minute and became irregular; this was followed by paralysis of the anterior and posterior extremities, complete absence of righting reflex, and maximally dilated pupils with slow accommodation to the light, and, finally, flaccidity. During the progression of symptoms, repeating muscle spasms developed on the dorsal part of hind limbs, which grew weaker as the flaccid paralysis progressed. The average time of the development of total flaccid paralysis was 5 h ± 2 min. The

complete absence of the spinal reflexes was noted during decapitation, and the spinal nerves did not respond to galvanic spinal stimulation.

FNM preparations from envenomed frogs were set-up and stimulated directly and then indirectly. The muscle responded to the direct stimulation but was reduced in amplitude (approx. 20%) when compared to control tissues (**Fig. 8**). The FNM preparation was unresponsive when it was stimulated via the nerve (**Fig. 8**). However, it remained responsive to the exogenous application of either ACh or KCl.

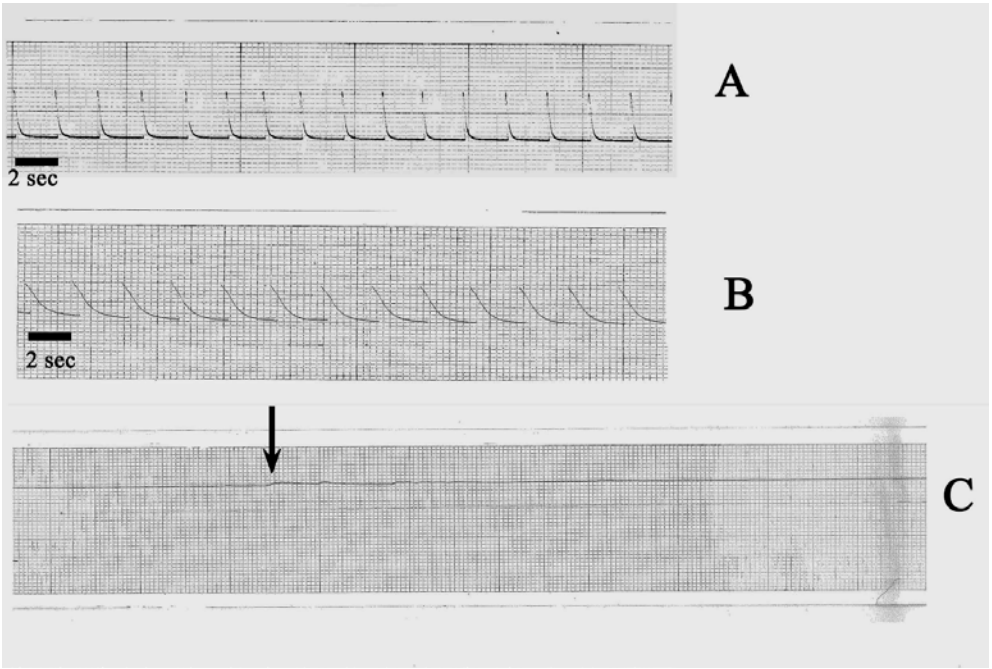



**Figure 8.** Mechanogram of the frog nerve-muscle preparation (*in vivo*) from envenomed specimens, after the development of neuromuscular block.
**Legend: A)** control twitch (made from non-envenomed specimens); **B)** Response of the muscle during direct muscle stimulation; **C)** Responses of the muscle during direct neural excitation. Black arrow shows the washing with physiological solution (venom-free).

Prior to neuromuscular blockade, the venom induced a sustained muscle contracture (*ex vivo* preparations). Single shock stimulations of the motor nerve were associated with tetanic contractions of the muscle. The first tetanic muscle contracture appeared at 5 ± 0.9 min after venom exposure

(**Fig. 9A and B**). These contractures diminished with time. Such tetanic responses did not appear in the absence of nerve stimulation.

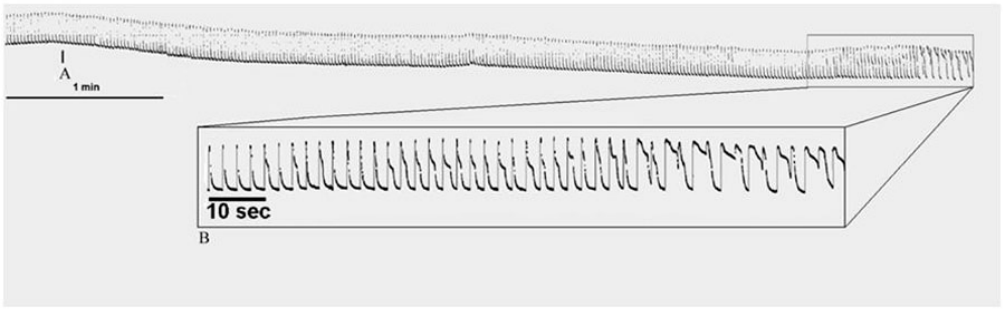



**Figure 9.** Isolated frog neuromuscular preparation (*ex vivo*) prepared from intact animals, exposed to *V. b. berus* venom.
**Legend: A)** administration of venom (120 µg/100 µl). Scale is 1 min. **B)** Rectangle: Expanded view of the repetitive phasic tetanic muscle contractures developed in every 1-2 sec. interval.

Following the development of flaccidity in preparations, the muscle was able to contract during direct stimulation, the sensitivity of muscles remained intact to ACh, while reduced response of the muscle could not be observed in the preparations to the exogenously applied KCl (**Fig. 10**).

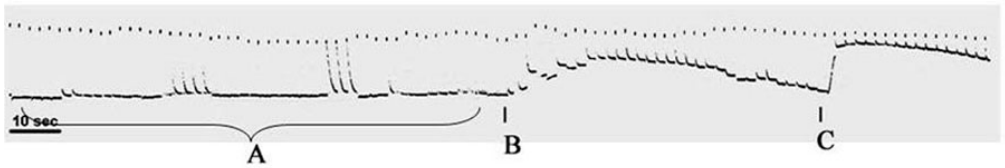



**Figure 10.** Responses of frog nerve-muscle preparation (*ex vivo*) to direct muscle stimulation (A), exogenous ACh (0.01 µM) (B) and to KCl (67 mM) (C) after the development of complete flaccidity. Scale is 10 sec.

### *5.3.8. Neuromuscular effects of venom on chick biventer cervicis (CBC) preparation*

Experiments were started only with three samples for the first time on CBC preparations; two eastern Hungarian adder venoms and the Austrian adder venom at 10 µg/ml concentration. All the three samples caused a time-dependent inhibition of twitches when the preparation was stimulated through

the motor nerve and led to a slow progressive reduction in the height of the twitch response (**Fig. 11A**). There was some initial augmentation in preparations exposed to the Austrian adder venom (**Fig. 11A**). When these venoms applied at higher concentration (100 μg/ml for sample "23" and "37", while the Austrian sample ("25") at 30 μg/ml due to its smaller available amount) the reduction of twitch responses to indirect stimulation was faster and all preparations were completely blocked (**Fig. 11B**). Some preparations showed a small, slowly developing and slowly waning contracture during the onset of twitch block (not shown). Responses to Ach, carbachol and KCl were tested after complete twitch block: there was little change in preparations exposed to samples "23" and "37", but responses to all three stimuli were markedly reduced in preparations exposed to the Austrian *V. b. berus* sample ("25") at 30 μg/ml (**Fig. 11C**). There was no recovery after wash-out of the venoms (not shown).

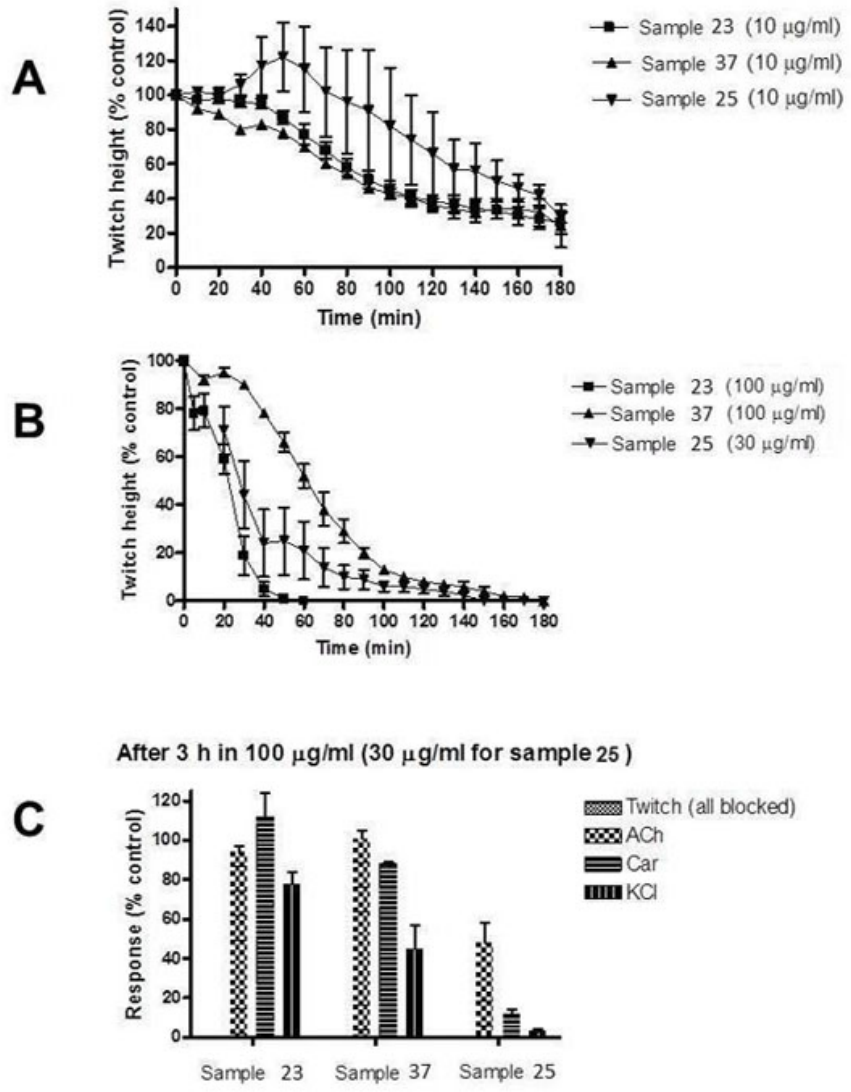



**Figure 11.** Venom effects of eastern Hungarian and the Austrian *V. b. berus* on chick *biventer cervicis* nerve-muscle preparation.

**Legend: A)** Reduced twitch responses to nerve stimulation. Venom concentrations: 10 µg/ml. After 180 min in venom at 10 µg/ml, twitch height was about 20% of control (pre-venom) height. **B)** Reduced twitch responses to nerve stimulation with venom concentration 100 µg/ml for sample 23 and 37, while 30 µg/ml for sample 25. **C)** Responses of chick *biventer cervicis* preparations to acetylcholine (ACh; 1 mM), carbachol (Car; 20 µM) and potassium chloride (KCl; 40 mM) after exposure to venoms. Sample 23 and 37=eastern

Hungarian adder venoms, 25=Austrian *V. b. berus*. The same samples were used that presented in table 1.

In the second experiment, six samples were randomly chosen from the eastern Hungarian *V. b. berus* venoms and the venom of *V. nikolskii*, tested on CBC preparations. All the Hungarian *V. b. berus* venoms caused time-dependent inhibition of twitches induced by stimulation of the motor nerve, except for the sample of one adult female specimen, which had no effect on the CBC preparation (**Fig. 12**).

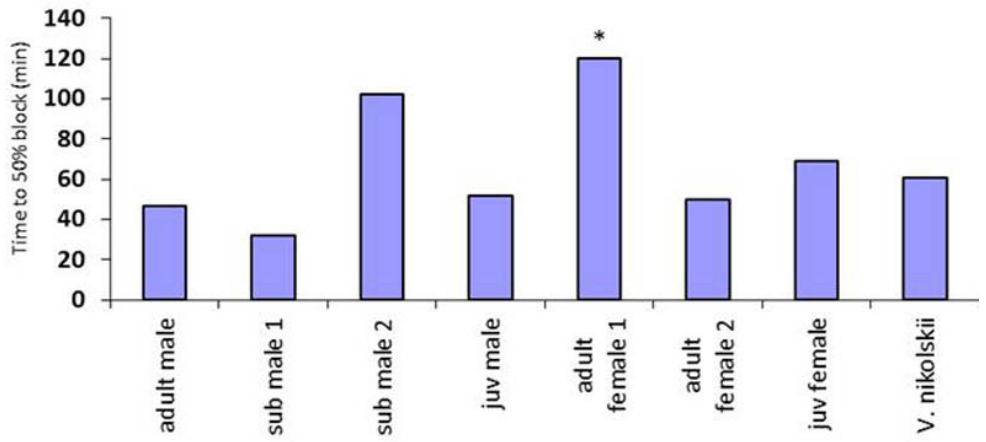



**Figure 12.** Effects of *Vipera* venoms on twitch responses of chick *biventer cervicis* preparations. Columns show the time (min) for the responses to nerve stimulation to be reduced to 50% of control, pre-venom heights (n= 2-4).
**Legend:** Seven samples from the east Hungarian *V. b. berus* specimens are shown: adult male and female, sub-adult males, juvenile male and females, and *V. nikolskii*. * = this sample of venom from an adult female adder did not cause twitch blockade to 50% in <120 min.

The times to a 50% reduction in twitch height in the presence of venom at 100 μg/ml varied from 50 to 75 min. There were no obvious differences in effects of venoms from snakes of different maturity or sex (**Fig. 12**). *V. nikolskii* also blocked responses to nerve stimulation. (**Fig. 12**). The progressive neuromuscular blockade of CBC preparations caused by these venoms was also concentration-dependent (10-100 μg/ml). The resting baseline tension was unchanged during the onset of twitch block.

As shown in **figure 13**, for one of the samples from a Hungarian specimen (adult male, numbered as "23" in table 1 and the same as used in Fig. 11), after 180 min incubation with venom at 10 µg/ml, twitch height was about 20% of control height; at a higher concentration (100 µg/ml), the reduction in twitch responses to indirect stimulation was much faster than with 10 µg/ml, with total failure of neuromuscular transmission within 60 min of exposure; at 30 µg/ml, the venom produced a block of twitches at a rate between 10 and 100 µg/ml (**Fig. 13**). There was no recovery after wash-out of the venom with venom-free physiological salt solution (data not shown). When venoms from the Hungarian snakes had blocked twitch responses to nerve stimulation, there were little alterations in the responses to ACh, carbachol and KCl (**Fig. 14**). The effects of venom from *V. nikolskii* were similar (**Fig. 14**).

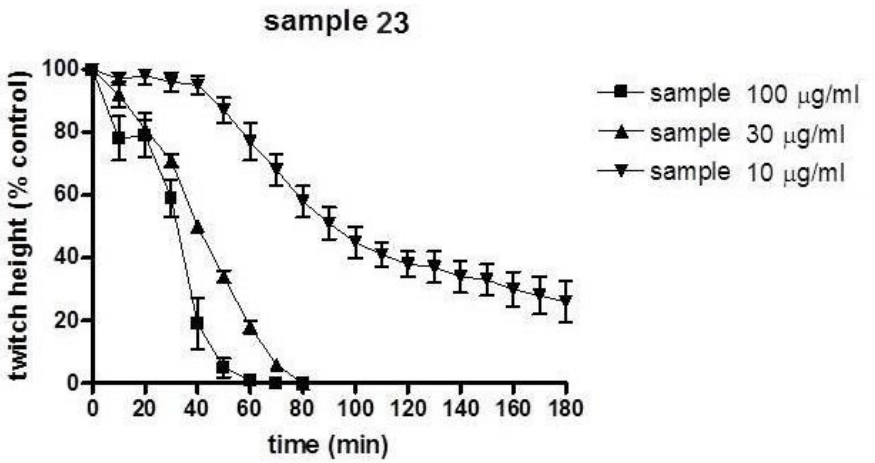



**Figure 13.** The effects of venom of an adult male Eastern Hungarian *V. b. berus* on chick *biventer cervicis* nerve-muscle preparation. The same venom sample was used that numbered as "23" in table 1.

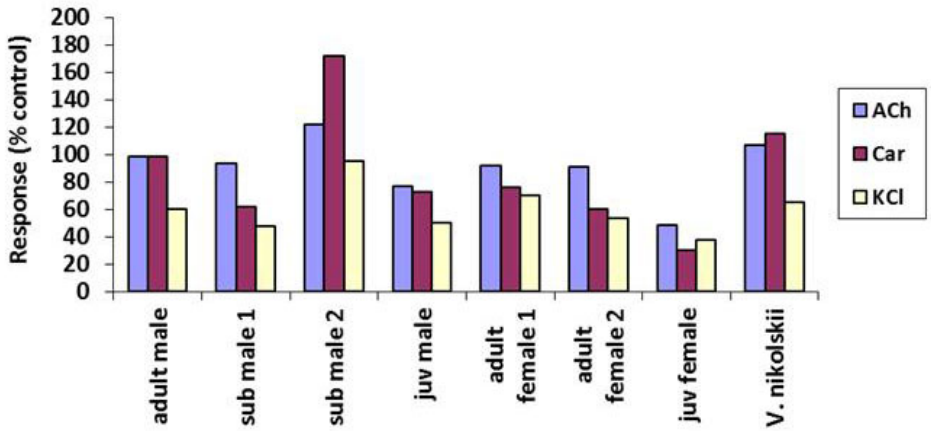



**Figure 14.** Responses of chick *biventer cervicis* preparations to acetylcholine (ACh; 1 mM), carbachol (Car; 20 µM) and potassium chloride (KCl; 40 mM) after exposure to 100 µg of venom per ml. The samples are the same as shown in figure 9.

In order to test the possibility that the effects of the venoms on neuromuscular function might be associated with phospholipase $A_2$ activity of the venoms, some experiments were conducted in Krebs-Henseleit solution in which the $CaCl_2$ had been replaced by $SrCl_2$ since $Sr^{2+}$ can replace $Ca^{2+}$ in the release of ACh but it reduces the enzyme activity of phospholipase $A_2$. The effects of the venom (20 µg/ml) from one of the Hungarian snakes (specimen number is "23" in table 1) were greatly reduced in Krebs-Henseleit solution in which the $CaCl_2$ had been replaced by $SrCl_2$: there was little reduction in the responses to nerve stimulation compared to that in time-matched control preparations in $Ca^{2+}$-containing physiological salt solution (**Fig. 15**).

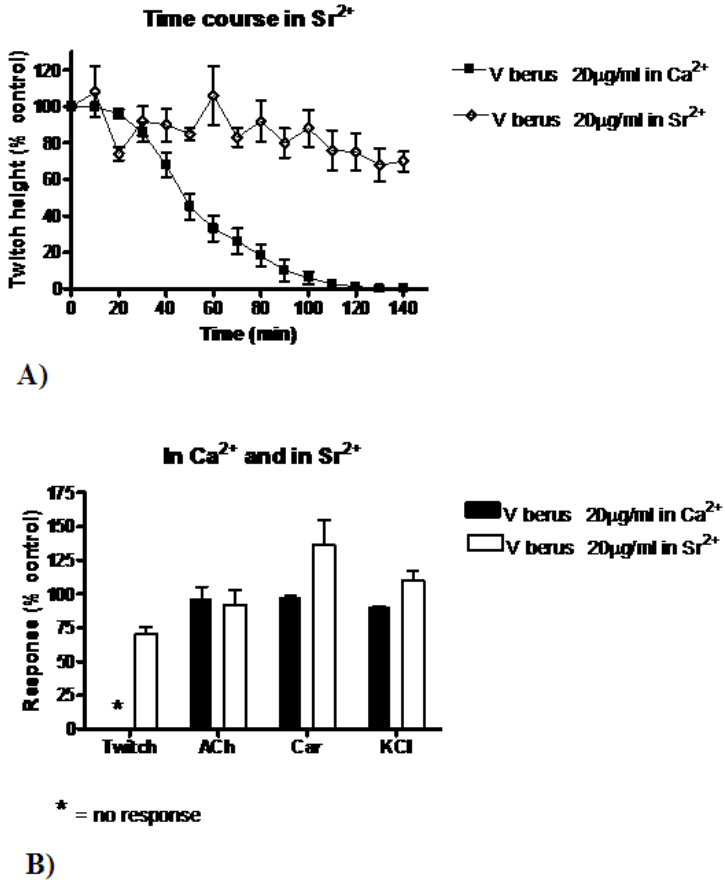



**Figure 15.** Comparison of the effects of *V. b. berus* venom on chick *biventer cervicis* preparations in $Ca^{2+}$- and $Sr^{2+}$-containing physiological salt solution. The same venom sample was used that numbered as "23" in table 1. **Legend: A)** Twitch responses to nerve stimulation decreased following addition of venom to the preparations in the normal (calcium-containing) solution. **B)** Only very little twitch block could be observe on the preparations in the strontium-containing solution. Closed squares: in $Ca^{2+}$; open diamonds: in $Sr^{2+}$.

### *5.3.9. Inhibitory venom effect on a glutamatergic synapse of the rat brainstem slice preparation*

Further experiments were performed with whole-cell patch-clamp to investigate the effects of venom on glutamatergic synaptic neurotransmission in the central auditory pathway of the rat. When the auditory nerve fibres

were stimulated, a prominent excitatory postsynaptic current was recorded (in these experiments the inhibitory neurotransmission was inhibited by the application of 1 µM strychnine and 10 µM bicuculline, **Fig. 16A**). When the brain slices were exposed to the venom (9.74 µg/ml) for 5 min, the amplitude of the first postsynaptic current decreased from -1069 ± 20.8 pA to -302 ± 83.8 pA (**Fig. 16B**). The ratio of the second and first PSC amplitude (PPR) was changed from 0.95 ± 0.03 to 0.78 ± 0.06 (p= 0.00045 for the amplitude and 0.008 for the paired pulse ratio, n= 3). The effect was irreversible.

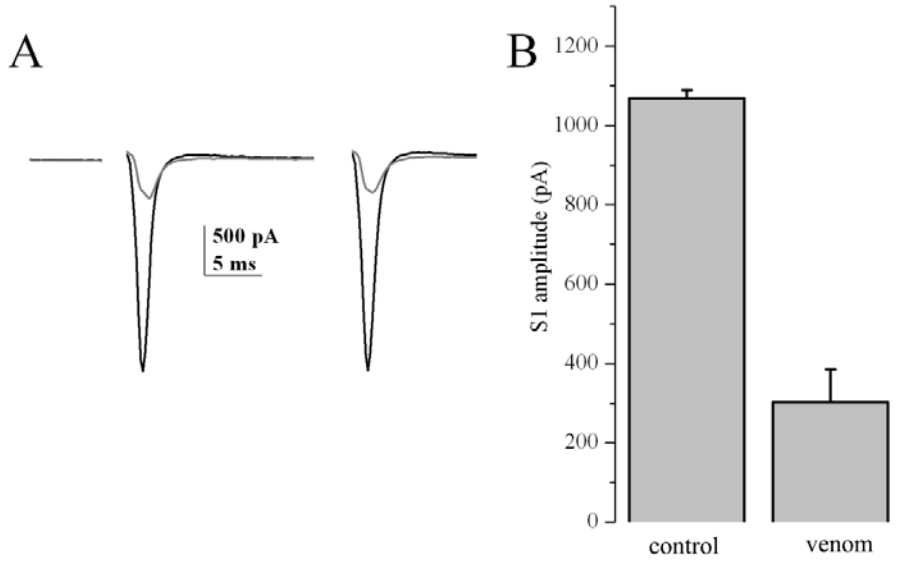



**Figure 16.** Inhibitory effect of *V. b. berus* venom on the glutamatergic synapse of the end-bulb of Held (A) and amplitudes of excitatory postsynaptic currents during venom administration after repeated excitation (B). The same venom sample was used that numbered as "23" in table 1.

**Legend: A:** The black current trace is the control recording; the grey curve is the postsynaptic current during venom exposure at 9.74 µg/ml venom concentration in the presence of two blockers (1 µM strychnine and 10 µM bicuculline). The inhibition revealed by the difference between the two curves is the consequence of the venom application. **B:** Summary of the data recorded under control conditions and in the presence of the venom.

# 6. General Conclusions and Implications

Snake venoms are highly complex biologically active mixtures and quite plastic: their composition can be influenced by several extrinsic and intrinsic factors. These can affect their biochemical and pharmacological properties, resulting in variations in venom composition within the same species. This is well-documented phenomenon for several species, particularly for medically important tropical and subtropical taxa (i.e. *Echis*, *Bitis*, *Bothrops* genera), while few studies have dealt with these venom aspects of the European *Vipera*.

In particular, there have been only two venom-related studies, which addressed the intraspecific variations of *V. berus* venom (i.e. NEDOSPASOV & RODINA 1992; MALENEV et al. 2007), the most clinically important member of the genus of *Vipera* in Central and Northern Europe. Venom individualities potentially affect the venom protein-profiles and activities and can have consequent clinical implications (WARRELL 1997). Therefore, the primary focus of the dissertation was in determining the individual variation in venom composition and certain venom features of a defined population of *V. b. berus* in eastern Hungary, where neurotoxic envenomings had been already reported previously (i.e. MALINA et al. 2008a, 2013).

### The eastern Hungarian *V. b. berus* and their venom yield

AGÓCSY (1958) was the first who recorded the taxon from Upper Tisza River valley, around Vámosatya and Nagyar, eastern Hungary. In 1960, MARIÁN collected 9 specimens (8 males and 1 female; 1 juvenile, 2 subadults and 6 adults) from this territory (Lónya, Vámosatya, Csaroda). According to MARIÁN's (1960) records, the average total length of the collected specimens was 57.6 cm (27.6-71.0 cm). The average length of the milked specimens in this research lags behind a bit compared to MARIÁN's (1960) data.

It is not easy to determine the venom yield of a given species because it is influenced by several intrinsic but also extrinsic factors (WILLEMSE et al. 1979; NAULLEAU 1984; KOCHVA 1987; CHIPPAUX et al. 1991; CHIPPAUX 2006; MIRTSCHIN et al. 2006). The published data about the secreted and

milked venom amount of this species vary in the available literature. According to BUBALO et al. (2004), it is 14 mg in dry weight. THEAKSTON & REID (1976) give this venom amount between 10 and 18 mg (dry weight), but BROWN (1973) determined it only in 6 mg (dry weight). Average amount of the collected 35 individual venoms in this research, is very closed to BROWN's (1973) data. Interestingly, venom yield averages is also similarly low – 5 mg with a maximum of 10 mg, dry weight – in case of the specimens derived from the south-western Hungarian lowland *V. b. bosniensis* populations (MALINA et al. 2011b), while SCHÖTTLER (1938) determined the venom yield of *V. b. bosniensis* (derived from higher elevations) as 15 mg.

Although, we can only hypothesise that elevation can be a determining factor of venom yield in *V. berus*, consequently, specimens from mountain regions may have higher venom yield. On support of this, NAULLEAU (1976) has already showed that lowland populations of the Asp viper (*V. aspis*) have lower venom yield than that of the mountainous *V. aspis* populations. However, the injected venom amount univocally influences the clinical picture of envenomed patients and also the severity of envenomings, it is not the primary determining factor in the severity of envenomings as this latter is influenced by other extrinsic factors, as well (CHIPPAUX et al. 1996; CHIPPAUX 2006; WARRELL 1997). Consequently, the specimens from the Hungarian lowland *V. b. berus* and *V. b. bosniensis* populations have low venom yield that based on our results, and these vipers are able to inject small venom dose in a single bite but capable to cause quite severe envenomings (MALINA et al. 2008b, 2011b, 2013; MALINA 2011) contrary to the bite of the mountainous *V. b. berus*, living in the Zemplén Hills (MALINA et al 2011a; MALINA 2011). If we take into account only their venom composition with disregarding to the extrinsic factors (e.g. weight and age of the bitten patient, actual health state, anatomical location of bite, etc. (PERSSON 1995; CHIPPAUX 2006)), it is obvious that there can be marked quantitative differences in their venom composition and thus, venom toxicity. It has now been proven in certain *V. aspis* population that their more complex venom composition is responsible for the more variable clinical manifestations and the severe course of envenomings (FERQUEL et al. 2007).

**Neurotoxic envenomings and the discussion of the two clinical case reports**

A large number of reports have been issued about the clinical aspects of *V. berus* bites since Reid's pioneering work and most of them are about the envenomings by the nominate subspecies. While the information about envenomings inflicted by the Bosnian subspecies (*V. b. bosniensis*), is limited.

Reuss was the first, who reported that the envenomings by the Serbian *V. b. bosniensis* - populations in the Valley of the Sava River - can result ptosis and external ophthalmoplegia with complete ocular immobility and reduced visual acuity, dysphagia as a result of palatal weakness, speaking difficulties, the loss of balance with uncoordinated movements, and dyspnoea (MALINA et al. 2011b). The victims' systemic condition resembles to drunken sensation or alcoholic intoxication due to the evolved diplopia, which usually develops within 7 h (MALINA et al. 2011b). Following Reuss, other authors i.e. SCHÖTTLER (1938), CYRÉN (1941), and SCHIEMENZ (1987) mentioned that the venom of *V. b. bosniensis* is possibly mainly neurotoxic. Although, the first authenticated case reports and clinically oriented papers about unambiguous neurotoxic envenomings by *V. b. bosniensis* were published only in the last few years (i.e. WESTERSTRÖM et al. 2010; MALINA et al. 2011b). From Hungarian perspective, the earliest indication for the neurotoxic envenomings inflicted by adders of Somogy County, documented by Fejérváry in 1934, who mentioned that the eyes of victim lost their function shortly post-bite (FEJÉRVÁRY 1934), which is very likely the drop of the eyelids, namely ptosis. A more recent study confirmed (MALINA et al. 2011b) that the initial phase of neurotoxic manifestations following the bite of lowland popualtions of *V. b. bosniensis* in south-western Hungary and Croatia, was always expressed in cranial nerve disturbances, i.e. ptosis, external ophthalmoplegia, diplopia, and sometimes reduced focusing capability and/or blurred vision.

On the other hand, the neurotoxic effects of *V. b. berus* envenoming are poorly documented in the literature and so far, such cases were recorded only from north-western, northern, and central Europe (OTTO 1929; REUSS 1930; FRANCKE 1937; MALINA et al. 2008a). Individual case reports on

neurotoxic signs and symptoms indicate that isolated neurotoxic *V. b. berus* populations might exist in Europe, mostly within the Carpathian basin (MALINA et al. 2008a, 2013; GAFENCU et al. 2012) and in certain parts of Germany (OTTO 1929; REUSS 1933; FRANCKE 1937). Subjective symptoms such as the respiratory distress and paraesthesiae cited by certain authors, are far too non-specific to be acceptable as evidence of neurotoxic envenoming (KARLSON-STIBER et al. 2006). Patients severely envenomed by *V. berus* may become unconscious (WARRELL 2003; MEIER et al. 2003), but this is attributable to the early, profound, persistent or recurrent hypotension that is such a marked clinical feature of these envenomings. Coma certainly does not imply a central neurotoxic effect of the venom that would be inherently implausible since the molecular size of snake venom neurotoxins allows minimal penetration of the blood–brain barrier (GUBENŠEK et al. 1982). 'Lip paralysis' is readily confused with angioedema, a characteristic feature of *V. berus* bite (KARLSON-STIBER et al. 2006), and should not be considered a neurological sign (MEIER et al. 2003).

***Case report 1.*** This case was the firstly documented neurotoxic envenoming inflicted by *V. b. berus* in eastern Hungary. The patient's diplopia resolved rapidly, 11 h after the bite (MALINA et al. 2008a). After ptosis, diplopia resulting from paralysis of extra-ocular muscles is usually the next effect of the descending paralysis typical of snakebite neurotoxicity (MALINA et al. 2008a). It was the first clinical report of this feature after *V. b. berus* envenoming but it has been observed, together with other neurological signs such as ptosis, ophthalmoplegia and dysphagia, following bites by some populations of *V. aspis* and *V. ammodytes*, whose venoms are known to contain neurotoxins (MEIER et al. 2003; JAN et al. 2007). The relatively rapid resolution of diplopia suggests that it was caused by a postsynaptic neurotoxin, i.e. Vaspin in the venom of this *V. b. berus* population. Clinical effects of postsynaptic neurotoxins tend to resolve more rapidly than those caused by presynaptic neurotoxins which damage the nerve terminals. It is presumed that the neuromuscular junctions innervating the extraocular muscles (*m. rectus*, *m. obliquus*) were reversibly blocked by venom neurotoxin causing external ophthalmoplegia and hence double vision, which resolved when the neurotoxin dissociated from its receptor. Drowsiness is a puzzling and unexplained feature of neurotoxic and other snakebites (MEIER et al. 2003), but in our patient this may have been attributable to the analgesic

he had been given. Neurotoxicity and the resulting diplopia, dizziness or high blood pressure may have contributed to our patient's dizziness, positional vertigo and unsteady gait.

This patient showed another extraordinary sign, pronounced and protracted high blood pressure as opposed to hypotension, which is the most dangerous effect of *V. berus* envenoming (WARRELL 2003, 2005; KARLSON-STIBER et al. 2006). In a case of *V. b. bosniensis* bite in south-western Hungary in 1979, an abnormally high blood pressure was recorded (200/120 mmHg), which peaked 32 h after the bite and the patient developed bilateral ptosis, as well (MALINA et al. 2008a). KRUE & HANSEN (1999) reported a case from Denmark, when a 6-year-old boy developed high blood pressure secondary to renal damage following *V. b. berus* bite, while GARKOWSKI et al. (2012) also recorded transient hypertension in three patients bitten by *V. b. berus* in eastern Poland. Anxiety can raise the blood pressure, but this patient denied that he was anxious on admission. His high blood pressure fluctuated, responded promptly to the angiotensin-converting enzyme vasodilator captopril, but later recurred. It has been suggested that the venom of certain *V. berus* population might contain cardiotoxins (KARLSON-STIBER et al. 2006), capable of acting at autonomic synapses to affect blood pressure. Hypertension without neurotoxic symptoms was observed in patients envenomed by Western Russell's vipers (*D. siamensis*) in Taiwan (HUNG et al. 2002). Neuromyotoxic signs caused by bites of Eastern Russell's vipers (*D. russelii*) and attributable to $PLA_2$ neurotoxins in Sri Lanka and India are not associated with hypertension (WARRELL 2003). It seems likely, therefore, that distinct toxins are responsible for these neurological and cardiovascular symptoms.

Scorpion ion channel toxins release noradrenaline, dopamine and other catecholamines causing hypertension with tachycardia as seen in human victims of envenoming by *Androdoctonus spp.*, *Leiurus quinquestriatus* and other taxa (ISMAIL 1995). Perhaps a venom constituent of this *V. b. berus* population releases catecholamines from adrenergic nerve endings to cause similar effects on blood pressure.

Some authors suppose that Vaspin genes may occur in all the species of Palaearctic vipers (JAN et al. 2007). Ammodytoxin genes have been demonstrated in different viperid species from south-eastern France to Slovenia (JAN et al. 2007). It seems likely that Vaspin genes are expressed in

the populations of *V. b. berus* in eastern Hungary, while the venom of *V. b. bosniensis* in south-western Hungary may contain Ammodytoxins, as well. The presence of Ammodytoxins would support the hypothesis of some Hungarian authors' that Slovenia is a possible origin of this population (KORSÓS & KRECSÁK 2005). Alternatively, the neurotoxic activity of the venom of eastern Hungarian *V. b. berus* populations may have evolved in parallel with *V. b. bosniensis* populations in the Balkans, or have been retained after the recolonisation from the Balkans, since the Balkan Peninsula was a possible refugia (JAN et al. 2007). This could explain the occurrence of neurotoxicity after bites by *V. b. berus* in Bihar County – summarized by MALINA et al. (2008a) –, which is close to the area where this patient was bitten.

***Case report 2.*** This second case presents further unequivocal evidence for the existence of a neurotoxic population of *V. b. berus* in eastern Hungary. In this patient, oculomotor palsy manifested as partial bilateral ptosis, gaze palsy, and consequent diplopia. The patient tried to compensate her gaze palsy by turning her head instead of moving her eyes. These neurological signs and symptoms suggest that cranial nerves III, IV, and VI were affected by the paresis. The patient's ptosis persisted through the second day. Ptosis is one of the early signs of neuromuscular paralysis in snakebites, although it is easily missed in neurotoxic *Vipera* spp. envenomings. Patients often develop ptosis lasting less than a day as was reported in *V. b. bosniensis* (WESTERSTRÖM et al. 2010; MALINA et al. 2011b) or *V. a. francisciredi* bites (BEER & PUTORTI 1998). Gaze paresis, dysphagia, and dysarthria are the most documented and frequent neurological signs of neurotoxic viper envenomings in Europe (GONZÁLEZ 1982; BEER & PUTORTI 1998; FERQUEL et al. 2007; LUKŠIĆ et al. 2006; LONATI et al. 2009; DE HARO et al. 2002, 2009; MALINA et al. 2008a, 2011b; GAFENCU et al. 2012).

Photophobia was documented only in *V. aspis* bites from a limited area in south-eastern France, where neurotoxic envenomings are frequent (DE HARO et al. 2002). In our case, this particular clinical feature was assessed subjectively while relying on the description of the patient. According to her, she experienced it only on the first day after the incident. We deem it was related to neurotoxicity, although, the origin of drowsiness in the envenomings by European viperids is debated. It is considered to be either caused by venom-induced endorphin release (WARRELL 1986), vasovagal

responses, and central nervous system depression (PERSSON 1995) or regarded as a neurotoxic symptom, occasionally reported in *V. a. aspis*, *V. a. zinnikeri* (DE HARO et al. 2002, 2009), and *V. a. francisciredi* envenomings (BEER & PUTORTI 1998). Drowsiness also developed in a neurotoxic envenoming by *V. berus* in south-eastern Romania in 2012 (GAFENCU et al. 2012) where the taxon was erroneously recorded as *V. b. bosniensis* by the authors, but there is no doubt that only *V. b. berus* occurs in Romania (URSENBACHER et al. 2006; ZINENKO et al. 2010). While the weakness and prostration of the girl was most noticeable during the first day of hospitalization. Likely it was a consequence of dehydration owing to repetitive vomiting. Her inability to stand was most probably caused by her weakness, intensive dizziness, and the slight disturbances of fluid retention.

The culprit adder of this incident belongs to an eastern Hungarian *V. b. berus* population different from the one in which the first neurotoxic envenoming was reported in 2008 (i.e. MALINA et al. 2008a). This recent envenoming occurred a mere 22 km from the previous location, mentioned in case report I. The two adder populations are isolated from each other by the River Tisza. Such geographical isolation may lead to venom variability (CHIPPAUX et al. 1991; CHIPPAUX 2006; BELT et al. 1997; MUKHEREJEE et al. 2003; ALAPE-GIRÓN et al. 2008) and therefore, physicians need to pay more attention to uncommon features of envenoming that may be attributed to geographic variability. The case report of two patients described here presented with moderate local symptoms followed by neurological disturbances primarily manifested as dysfunction of certain cranial nerves and provides unambiguous clinical evidence for the existence of neurotoxic *V. b. berus* populations in a restricted geographical area in eastern Hungary.

**Studies on neurotoxic effects of venom**

A number of observations reveal that the effects of the bites inflicted by snakes of the same population can vary because of the variability of venom composition (BELT et al. 1997; FERQUEL et al. 2007; ALAPE-GIRÓN et al. 2008; ÖHLER et al. 2010). This intrapopulation venom variability may derive from several different causes such as prey specificity, ontogenetic variation, and other ecological pressures (WILLIAMS et al. 1988; SASA et al. 1999; WÜSTER et al. 1999). In south-eastern France, some specimens of *V.*

*aspis* may produce more varied clinical manifestations – mainly in neurological disturbances – on envenomed humans than others as a result of their more complex venom composition, especially because of their higher $PLA_2$ arsenal variability (FERQUEL et al. 2007). Interestingly, regional venom variations of *V. berus* have already been predicted earlier in the literature (READING 1996) but no robust research has been performed on its venom composition variability till now; except for the study of NEDOSPASOV & RODINA (1992) and MALENEV et al. (2007). NEDOSPASOV & RODINA (1992) showed age-related variations associated with the amidolytic activity of venom, while the more recent study of MALENEV et al. (2007) confirmed that venom toxicity ($LD_{50}$) varies between populations of the Russian *V. berus*.

Venom composition is highly influenced by the available native prey species of snakes and the physiological responses of prey animals for the venom, correlating with the geographical distribution of snakes (CHIPPAUX et al. 1991; DA SILVA & AIRD 2001; AGUILAR et al. 2007; BARLOW et al. 2009). ANDRE & ABE (1999) have showed that the venom of adult individuals of the Jararaca (*Bothrops jararca*), is roughly fifteen times more toxic for rodents (mouse) than for frogs, as frogs do not compose the nutrition regimen of adult *B. jararaca*. Take into account that *Pelophylax* kl. *esculentus*, which was used in our study, does not belong to natural prey species of *V. berus* (NILSON et al. 2005), we also applied high venom doses (50 times of murine $LD_{50}$ i.v. with *in vivo* injection) in our frog experiments.

Isolated frog nerve-muscle preparations prepared from different frog species were (SCHÖTTLER 1938; MOHAMED & ZAKI 1958; LOOTS et al. 1973; RODRIGOUES-SIMONI et al. 1983), but also still are in use today (SHELKE et al. 2000; RAMANAZOVA et al. 2008) to study the neurotoxic activity of venoms of different venomous snake taxa. Nowadays, frog nerve-muscle preparations used in snake venom related studies, typically requires collection of wild-caught frogs (e.g. RAMANAZOVA et al. 2008), although captive-bred specimens of a given species would have to be used as a standard, of which would be enormous benefit in snake venom research.

After direct local venom administration to the frog neuromuscular junction on *ex vivo* preparations (prepared from intact specimens), the preparation developed unusual phasic and augmented tetanic muscle contractures in response to nerve stimulation. The underlying cause of these tetanic contractures will require further investigation, although

prejunctionally acting snake venom neurotoxins are known to increase transmitter release before complete blockage of neurotransmission and venom $PLA_2$ exerts enzymatic activity in frog nerve-muscle preparations (ROWAN & HARVEY 1998). On the other hand, the unresponsiveness of preparations to the exogenously applied ACh is evidence for the prejunctional venom action (HARVEY et al. 1994) as well as the ineffective washing with venom-free physiological solution which did not led to recovery. The *in vivo* nerve-muscle preparations isolated from envenomed frogs were refractory to stimulation through the nerve, although the muscle was able to contract in response to direct electrical excitation. The envenomed live frogs developed muscle fasciculations before the onset of flaccid paralysis. Paralytic phenomenon could be additionally observed on the venom injected mice and also progressed in flaccidity before death. Flaccid paralysis is an unambiguous indication for the neurotoxic activity of snake venoms (KARALLIEDDE 1995) and was described on mice in other viperid taxa, e.g. in the venom of *Azemipos feae* (VEST 1985), or in case of *Bothrops neuwiedi* venom, as well (BORJA-OLIVEIRA et al. 2007). Flaccid paralysis is the most dreadful clinical sign of neurotoxic snakebites (WARRELL 2010) and has been reported on humans bitten by certain big-bodied viper species, i.e. by *Daboia russelli pulchella* (formerly *Vipera russelli pulchella*) (PHILLIPS et al. 1988; WARRELL 2010). It has been recently shown that neurotoxic $PLA_2$s are responsible for the botulinum-like flaccid paralysis in viper envenomations (RIGONI et al. 2008). Some European taxa from the genus *Vipera*, where the venom possesses neurotoxic activity, are able to cause progressive descending paralysis in patients where ptosis is often the first sign, then facial and bulbar involvement characterize these neurotoxic viper envenomings (PERSSON 1995; FERQUEL et al. 2007; DE HARO et al. 2009; MALINA et al. 2008a, 2011a, 2011b). The significantly lower venom yield of the European viperid species can be one of the most likely explanations for the absence of generalised flaccid paralysis on human victims, in contrast patients bitten by large vipers with neurotoxic venom components, e.g. in case of *D. r. pulchella* (PHILLIPS et al. 1988). Snake venoms with myotoxic activity can cause an initial increase in twitch tension but (HARVEY et al. 1994), in the concentration used in the present study the venom has no myotoxic activity as shown by the responses of preparations to exogenous KCl application. However, the recorded mild fall in the muscle

tension in the preparations from envenomed frogs, suggests myotoxicity albeit only at very high doses. Since the $PLA_2$-dependent skeletal neuromuscular junction specific neurotoxins are the most toxic venom components (DOLEY et al. 2009), the lower murine $LD_{50}$ value of our examined adder venom can be in connection with its neurotoxic activity, whereas neurotoxins promote rapid prey death.

*Vipera b. berus* venoms from the same area of eastern Hungary where the bite by this subspecies caused neurotoxicity in human victims (MALINA et al. 2008a, 2013) rapidly produced cranial nerve involvement and limb paralysis progressing to complete flaccidity in envenomed mice. These symptoms were also observed in mice injected with the control venom of *V. nikolskii*, but not in the case of the Austrian *V. b. berus* venom. All adder venoms from eastern Hungary had neuromuscular effects - except one sample - although each sample was different in the intensity of neuromuscular effects, mirrored in response of CBC preparations to the exogenous chemical and electrical stimuli. Some venom showed negligible myotoxic activity, based on the reduced twitches to direct electrical stimulation and/or the reducing response to KCl (HARVEY et al. 1994), while all the Hungarian venoms (except one sample and the Austrian *V. b. berus*) caused rapid and irreversible prejunctional block of neuromuscular transmission on CBC preparations; receptor sensitivity was unchanged when responses to nerve stimulation were abolished, and the muscles responded to direct stimulation, implying a selective block of acetylcholine release from motor nerve endings. However, in case of certain venoms, the rate of onset of twitch block was unusually fast for prejunctionally acting snake venom. Taken together, these data clearly demonstrated that the venoms of the eastern Hungarian *V. b. berus* have unambiguous neurotoxic activity, in which individualities also mirror. The markedly reduced responses to all three stimuli (ACh, carbachol and KCl) and the initial augmentations that were also observed on the preparations exposed to the control Austrian *V. b. berus* venom, indicating myotoxic effect. On the bases of the preparation-responses, the venom of *V. nikolskii* also had neurotoxic activity that was prejuntionally active in nature corresponding to the report of RAMAZANOVA et al. (2008), and also had some myotoxic activity, as well.

The strong, irreversible inhibitory venom effect on excitatory postsynaptic currents in the glutamatergic synapse of brainstem is a further

evidence of the effect on synaptic neurotransmission. The ratio of the second to the first PSC amplitude (PPR) was reduced, indicating presynaptic inhibition (ZUCKER & REGEHR 2002). Snake venom neurotoxins predominantly affect the peripheral nervous system but our results show that certain viper neurotoxins can act on non-cholinergic synaptic transmission in the CNS *in vitro*; e.g. venom of the Russell's viper (*D. russelii*) is able to block non-cholinergic (dopamine, serotonin, norepinephrine) synaptic transmissions (HARVEY 1984). While we have no direct evidence that venom components cross the blood-brain-barrier *in vivo*, inhibition of non-cholinergic synaptic transmission in the rat aVCN may explain the symptoms of vertigo, occasionally reported in certain neurotoxic envenomings by vipers (FRANCKE 1937; MALINA et al. 2008a; DE HARO et al. 2009; GAFENCU et al. 2012) in Europe. However, the fall in blood pressure, may contribute to the development of this symptom.

**Electrophoretic pattern of venoms**

Electrophoretic investigations of snake venom proteins are quite effective and broadly applied methods for comparative studies at each taxonomic level (SAINT GIRONS &DETRAIT 1992; BARLOW et al. 2009). The profile of protein bands of our *V. b. berus* venoms showed deviations from those that were previously demonstrated by SAINT GIRONS & DETRAIT (1992) with snakes from Brittany (France) and by RAMAZANOVA et al. (2008) from Russia. Venom pattern of lowland adders from Hungary also differ from the venom of the Austrian mountainous *V. berus* used as a control in our study, a strong evidence for the existence of geographical venom variations of this species. Obvious diversity reflects in the venom pattern of the Hungarian adders, involving mostly the higher molecular mass components (probably because of glycosylation) that corresponds to the molecular mass range of P-I metalloproteinases - 20-24 kDa - and P-III metalloproteinases - 45-70 kDa - (MACKESSEY 2009). The relative amounts of some secreted peptides - based on their intensity - in the venoms appear to be also highly variable among the individuals. Gender-specific variability could be detected in these venoms, which has been proved in other species of genus *Vipera*, e.g. in *V. latastei* (AREZ et al. 1994). Venom pattern of the juvenile adders is clearly separated from the adults and subadults. Ontogenetic shifts in their diet can be a

plausible explanation for this phenomenon, since the feeding behaviour of juveniles in many habitats (mainly consists of lizards; genus *Lacerta* and *Zootoca*) differs from the adults (especially small rodents) (NILSON et al. 2005). However, the cause of development of these ontogenetic variations is not completely clear in several taxa and resulted distinct conceptions last decades (BARLOW et al. 2009). According to recent knowledge, venom composition can be influenced by environmental but also by genetic factors at individual levels (DALTRY et al. 1997). We suggest that dynamics in protein production and peptide maturation in juveniles probably differ from the adults, can also result such variations in their venom protein profiles.

**Protease activity**

Basically the studied *V. berus* venoms showed a relatively low protease activity on gelatin substrate and more than half of the samples were devoid of gelatinolytic activity. Protease activity was also absent in case of the Austrian *V. b. berus* and *V. nikolskii* venom. The degradation profile of venoms correlates with detected variability in the venoms' protein expression profile on SDS-PAGE, associating with the metalloproteinases, mainly in the range of 45-70 kDa (MACKESSEY 2009). Individual variations in the gelatinolytic activity of venoms can be also observed; the venom of juvenile and adult males and certain subadult female had higher enzymatic activity than that of the venom of the remainder samples. Interestingly, all the tested adult female venoms showed low protease activity. Our results, namely that the majority of the tested venoms possessed relatively poor protease activity or lacked this venom activity, can be associated with certain local signs and symptoms such as tissue necrosis and blood-blisters, which are absolutely an unexpected and very rare clinical features of *V. berus* envenomings (REID 1976; WARRELL 2005). Certain viper venom proteases play a toxic role associated with the above-mentioned local pathophysiological effects, while other proteases are responsible for the local and/or systemic haemorrhages and coagulopathy (GUTIÉRREZ et al. 2009). Here, we would like to emphasize that haemorrhages and blood coagulation disturbances are not characteristic feature and rare events of *V. berus* envenomings within its whole Hungarian territory (MALINA 2011, MALINA et al. 2012). We could not conclude that juvenile adders have lower degradative activity on this substrate

corresponding to the approximate range of 45 kDa proteins than that of the adult specimens, contrary to NEDOSPASOV & RODINA's (1992) work where unambiguous age-dependent venom variations were demonstrated on kallikrein substrate regarding the proteolytic activity of the Russian *V. berus* venoms.

## Venom lethality, $PLA_2$ activity and $PLA_2$ spectra of venoms detected by MALDI-TOF MS

Venom toxicity is often used in venom variation related studies (CHIPPAUX et al. 1991). In our study, there were no considerable individual differences in lethality of the Hungarian adder venoms. This can be associated with the detected non-significant differences in the $PLA_2$ activity of their venoms. MALENEV et al. (2007) have also showed some $LD_{50}$ differences concerning to the venoms of *V. b. berus* collected in different Russian localities. The eastern Hungarian adder venoms proved to be more toxic than that of the myotoxic Austrian *V. b. berus* ($LD_{50}$: 0.72 μg/g i.v.), and the venom of *V. nikolskii* ($LD_{50}$: 0.83 μg/g i.v.). The difference was significant between certain Hungarian samples and the two controls, which can explain with the neurotoxic activity and/or the distinct level of venom neurotoxins (this latter especially in case of *V. nikolksii* and the Hungarian adder venoms) as the $PLA_2$-dependent skeletal neuromuscular junction-specific neurotoxins are the most toxic snake venom components (DOLEY et al. 2009).

The investigation of BALIJA et al. (2005) lead to similar results regarding the $PLA_2$ activity of the Croatian *V. ammodytes* venoms but inter-populations and not among the specimens. However, one sample was significantly different from the other two in its toxicity. Therefore, we conclude that the toxicity of *V. berus* venom is only partly dependent on the activity of $PLA_2$s and other factors, e.g. level and/or type of various $PLA_2$s may account for the lower lethal toxicity of these individual venoms. This seems to be confirmed by the spectra of $PLA_2$ arsenal of their venoms, whereas several and various types of $PLA_2$s have been found, indicating their different molecular weights, detected by MALDI-TOF MS in our study. In addition there was little change in the responses to agonists in the CBC preparations in the strontium-containing solution, therefore our conclusion is

that the prejunctional block of twitch responses to nerve stimulation is associated with the activity of a $PLA_2$-dependent neurotoxin.

Intra-population variability in the expression level of venom of neurotoxins was already observed in other taxa, i.e. genus *Naja* (CHIPPAUX et al. 1991). Individual quantitative and qualitative heterogeneity of the $PLA_2$ content is clearly manifested in the venoms of the Hungarian adders as showed their spectrograms that has already been a well-known phenomenon in other species of *Vipera* (e.g. *V. aspis*; FERQUEL et al. 2007), where the intraspecific venom variation is reflected mainly in their $PLA_2$ composition of specimens derived from different localities but also intra-populations. In other taxa, the variation of this toxin group (group II $PLA_2$s) is thought to be responsible for the different symptoms in patients envenomed by snakes from different regions and has great relevance from clinical and toxicological point of view (WARRELL 1986, 1997; MUKHERJEE et al. 2000; FERQUEL et al. 2007).

**Taxonomic and phylogenetic implication of their venom neurotoxins**

The taxonomy of certain members within the *V. berus*-complex is not yet completely established and there are still some open questions (JOGER et al. 1997). REUSS (1937) emphasized that most of the adder populations in the Balkan Peninsula – included by him in a new genus, *Mesocoronis* Reuss 1927; today is recognised as *V. b. bosniensis* – have neurotoxic venom and considered this characteristic as a distinguishing and ancestral venom feature of these adder populations that can be used as a basis for adder taxonomy. Later, the neurotoxic venom of *V. b. bosniensis* was also mentioned by several authors (e.g. SCHÖTTLER 1938; SCHIEMENZ 1987; DETRAIT & SAINT GIRONS 1978) and was used as a special venom character that is not proper to the venom of *V. b. berus*, while newer papers suggested that certain *V. b. berus* populations might possess venom with neurotoxic activity (MALINA et al. 2008a). On the basis of our results, the neurotoxin-content of venom of the members from *V. berus*-species group is not a consistent indicator of taxonomic sub-divisions as others, e.g. RAMAZANOVA et al. (2008) mentioned it, e.g. in case of *V. nikolskii* and *V. berus* and, in addition, the protein pattern of *V. nikolskii* is very similar to that of *V. b. berus.* Although, we could use only one *V. nikolskii* sample as a control – and also one

Austrian *V. b. berus* sample –, we do believe that the taxonomic revision of the sister taxon, *V. nikolskii* (formerly *V. b. nikolskii*) might be indicated in the near future despite the potential existence of considerable venom variability at intraspecific levels.

Neurotoxic populations of other taxa from the monophyletic *Vipera* in Europe (i.e. *V. aspis* and *V. ammodytes*) also derive from an ancestral lineage and compose the basal sub-clade of the given species (WESTERSTRÖM et al. 2010). In addition, the $PLA_2$-dependent skeletal neuromuscular junction specific toxins, i.e. ammodytoxins, also form a basal linage and evolved earlier during the venom evolution of European viperids (JAN et al. 2007). In case of *V. berus*, both the Balkan clade (includes the subspecies of *V. b. bosniensis*) and the Carpathian sub-clade from the Northern clade, represent a basal and ancestral phylogenetic lineage of the species (URSENBACHER et al. 2006). The lowland adder populations in eastern Hungary are recognized as subspecies of *V. b. berus* (MARIÁN 1960; DELY & MARIÁN 1960; KORSÓS & KRECSÁK 2005) and belong to the Carpathian lineage that forms a cluster with those adders that are native to the Transylvanian lowlands (KORSÓS 2007). It might be possible that the neurotoxic content of their venom can be in connection with their early venom evolution, similarly as it was suggested in *V. b. bosniensis*, previously (SAINT GIRONS & DETRAIT 1978; MALINA et al. 2008; WESTERSTRÖM et al. 2010). It is still an open question whether which ammodytoxin isoform(s), or a totally different and still unknown but also an ancient and less complex molecule structured neurotoxic venom component from the group of $PLA_2$s have been retained in the early evolutionary stages of *V. berus*. The above hypothesis may explain the sporadic reports of the insular-like distribution of neurotoxic envenomings inflicted by certain population of *V. b. berus* as well as *V. b. bosniensis*. However, our data emphasize the need for further work in order to confirm this hypothesis about the evolutionary and phylogenetic aspects of *V. berus* venom.

# 7. Summary – In English and Hungarian

## In English

The results of the present Ph.D. dissertation demonstrate that the specimens of *V. b. berus* from a lowland population in a limited area in eastern Hungary have developed marked variable individual venom phenotypes, which are reflected not only in their electrophoretic venom pattern but also in protease activity, and certain pharmacological and biological activities that may influence the symptoms manifested on envenomed patients. This latter was demonstrated by clinical case reports

The electrophoretic profile of our *V. b. berus* venom samples showed deviations from those that were previously demonstrated by other authors with French and Russian *V. berus* venoms. Venom pattern of lowland adders from Hungary also differ from the venom of the Austrian mountainous *V. berus* used as a control in our study, can be evidence for the existence of potential geographical venom variations of this species. The relative amounts of some secreted peptides - based on their intensity - in the venoms appear to be also highly variable among the individuals. Gender-specific variability could be detected in these venoms, which has been proved in other species of genus *Vipera*. The protein pattern of *V. nikolskii* is very similar to that of *V. b. berus.*

Our experimental studies confirmed that the venom of the adders derive from this population, shows different individual neuromuscular effects on nerve-muscle preparations. The neuromuscular paralysing effects consistent with facilitate of the release of acetylcholine (ACh), while they had little effect on ACh receptors or directly on skeletal muscle function, unlike the venom from the Austrian *V. b. berus*. The venom of *V. nikolskii* was also neurotoxic similarly as the Hungarian *V. b. berus* venoms. Typically, prejunctional block is associated with phospholipase $A_2$ activity in the venoms and this was consistent with the loss of paralysing activity in experiments in which $Ca^{2+}$ was replaced with $Sr^{2+}$. Envenomed frogs, chicks, and mice generally displayed limb paralysis that progressed to complete flaccidity, and these paralytic signs are unambiguously associated with

neurotoxicity. Neurological disturbances dominated by the cranial nerve dysfunctions on envenomed patients by viperid species whose venom possesses neurotoxic activity. We successfully showed evidence for the neurotoxic activity of these venoms that is unambiguously associated with the neurological deficits developed by patients who suffered the adder-bite within this region. The first patient in case report 1, had diplopia resulting from paralysis of extra-ocular muscles, is usually the next effect of the descending paralysis (ptosis is often the first sign) typical of snakebite neurotoxicity. Diplopia is a known neurotoxic symptom, together with other neurological signs and symptoms such as ptosis, ophthalmoplegia and dysphagia, following bites by some populations of *V. aspis* and *V. ammodytes*, whose venoms are known to contain neurotoxins, as well. The other patient in case report 2, also developed unambiguous cranial nerve disturbances; manifested in bilateral impairment characterized by oculomotor paralysis with partial ptosis, gaze paresis, and diplopia. There is no doubt that the venom of further specimens belong to other eastern Hungarian populations within this area, can be also neurotoxic and associated with this type of activity, as the case reports are demonstrated here about patients who were bitten by specimens derived from two different locality within this region.

We could demonstrate a strong, irreversible presynaptic inhibitory venom effect on excitatory postsynaptic currents in the glutamatergic synapse of rat brainstem in case of the Hungarian adder venom, which is a further evidence of the effect on presynaptic neurotransmission. Although, snake venom neurotoxins predominantly affect the peripheral nervous system, but our results show that the neurotoxic activity of the tested Hungarian *V. berus* venom can act on the non-cholinergic synaptic transmission in the CNS *in vitro*, similarly as the venom of the Russell's viper (*D. russelii*), which is proved to be an inhibitor of non-cholinergic synaptic transmissions, as well. While we have no direct evidence that venom components cross the blood-brain-barrier *in vivo*, but the inhibition of non-cholinergic synaptic transmission in the rat brainstem neurons, may explain the symptoms of vertigo (it is not the same as the "simple" dizziness) occasionally reported in certain neurotoxic envenomings by European vipers.

We used MALDI-TOF MS to study and represent the heterogeneity of the $PLA_2$ content of venoms. Analyses of the whole venoms revealed

considerable variations in electrophoretic profiles but diversity was also reflected in the $PLA_2$-spectrogram of venoms. All of the venoms had many molecular species in the mass ranges of typical phospholipases detected by MALDI-TOF MS. On the bases of our results and the recent data available in the literature, we conclude that some toxic and non-toxic phospholipases must be present in the studied venoms. On the other hand, we showed non-significant individual differences in the $PLA_2$ activity of the Hungarian samples and between the Hungarian and the two control samples. Although, there were no considerable individual differences in the *in vivo* toxicity of the Hungarian adder venoms but the difference was significant between certain Hungarian samples and the two controls, which can explain with the neurotoxic activity and/or the distinct level of venom neurotoxins of the Hungarian venom samples. The high toxicity of these Hungarian adder venoms most probably is in connection with their prejunctionally acting $PLA_2$ content since the $PLA_2$-dependent skeletal neuromuscular junction specific neurotoxins are the most toxic venom components, of which main function is the promoting of rapid prey death through predominant prey-immobilizing mechanism. Thus, the lower murine $LD_{50}$ value of our examined adder venoms can be in connection with its neurotoxic activity, as well.

The studied *V. berus* venoms had a relatively low protease activity and more than half of the samples were devoid of this type of activity. Our observation regarding the low and/or lacking protease activity also corresponds to certain and very rare clinical manifestations (i.e. local blisters containing blood, necrosis) of *V. berus* envenoming. The degradation profile of venoms correlates with detected variability in the venoms' protein expression profile on SDS-PAGE, associating with the metalloproteinases. We could also show individual variations in case of protease activity of certain samples; the venom of certain males had higher enzymatic activity than that of the venom of certain adult females. While the Austrian *V. b. berus* and *V. nikolskii* venoms were completely devoid of protease activity. We could not unambiguously prove the age-related variability in the protease activity for the venom of juveniles, contrary to the work of other authors who showed this type of variability in case of *V. berus* with Russian origin.

Take into consideration the evolution of $PLA_2$s of the European *Vipera* and the phylogeography of *V. berus* – as the Carpathian Basin was

one of refugia of *V. berus* and the eastern Hungarian adders belong to an early evolutionary subclade – it can be hypothesized that the neurotoxic activity of their $PLA_2$(s) can be an ancient venom character, which maybe "lost" at most of the centre and the northern geographic distribution of the species during venom evolution. While some of these genes (e.g. neurotoxin encoding genes) recruited or retained in those *V. berus* populations, i.e. Carpathian subclade and the Balkan sublcade, which compose the basal linage of the phylogenetic hierarchy. This hypothesis could be an explanation for those convincing evidence as neurotoxic envenomings inflicted by *V. b. berus* have been mainly reported from the Carpathian basin and the territory of *V. b. bosniensis*. On the basis of our results, we strongly presume that the neurotoxin-content of venom or the venom neurotoxic activity in the *V. berus*-species group, is not a consistent indicator of the taxonomic sub-divisions as other authors mentioned it, for example in case of *V. nikolskii* and *V. berus*. On account of the above, the taxonomic revision of the sister taxon, *V. nikolskii* (formerly *V. b. nikolskii*) might be considered in the near future.

**In Hungarian**

A jelen Ph.D. disszertáció eredményei egy adott kelet-magyarországi területen élő, síkvidéki *V. b. berus* populációhoz tartozó viperapéldányok méregösszetételben rejlő jelentős egyedi változékonyságot mutatják be, amely azonban nem csak mérgük elektroforetikus futtatása során kapott mintázatban tükröződik, hanem jelen van a méreg proteáz aktivitásban, továbbá megnyilvánul mérgük bizonyos farmakológiai és biológiai aktivitásban is. Ez a méreg-összetételbeli változékonyság egyértelműen befolyásolhatja a megmart személyeken a mérgezés során kibontakozó klinikai képet. A disszertációban ez utóbbiak, klinikai esetismertetések és klinikai tanulmányokon keresztül kerültek bemutatásra.

Az általunk vizsgált *V. b. berus* méregminták elektroforetikus profilja eltérést mutat a már korábban, más szerzők által bemutatott francia és orosz eredetű *V. berus* mérgek méregmintázatától. A kelet-magyarországi síkvidéki *V. b. berus*ok méreglenyomata szintén különbözik az általunk kontrolként felhasznált hegyvidéki, osztrák *V. b. berus* példány mérgétől, amely bizonyítékul szolgálhat a fajnál potenciálisan előforduló földrajzi méreg-

összetételbeli változékonyság jelenlétére. A szekretált fehérjék mennyiségének tekintetében – intenzitásuk alapján – a minták ugyancsak meglehetősen változatos egyedi különbségeket mutattak. Ivar-függő variabilitást is sikerült kimutatnunk, amely azonban már bizonyított egyéb, szintén a *Vipera* genusba tartozó fajoknál.

Kísérleteink megerősítették, hogy e populációból származó viperák mérge az ideg-izom preparátumokra egyedileg jellemző mértékben, de aktivitásukban többnyire megegyező neuromuszkuláris hatást gyakorolnak. Ezek a paralitikus neuromuszkuláris hatások összeegyeztethetők az acetilkolin (ACh) felszabadulás méreg általi elősegítésével, míg a minták csak csekély mértékben befolyásolták az ACh receptorok, vagy közvetlenül a vázizomzat funkcionalitását, ellentétben az osztrák *V. b. berus* méreggel. A *V. nikolskii* mérge a hazai *V. b. berus* mintákhoz hasonlóan neurotoxikusnak bizonyult. A kimutatott prejunkcionális gátlás jellegzetesen e mérgek foszfolipáz $A_2$ aktivitással függ össze. Ez utóbbi ugyanis összeegyeztethető paralitikus hatásuk elvesztésével; amikor a kísérletet $Ca^{2+}$ ionokat tartalmazó oldat helyett $Sr^{2+}$-ot tartalmazó oldatban végeztük. A méregmintákkal leinjekciózott békák, csirkék és egerek általánosan mutatták a végtagbénulás jeleit, mely végül a teljes petyhüdt bénulás beálltáig súlyosbodott. Ezek a bénulásos jelek egyértelműen összefüggnek a neurotoxicitással. A mérgükbe neurotoxinokat is szekretáló viperafajok által megmart személyeken a neurológia zavarok közül az agyidegek diszfunkciója dominál. Kísérletekkel sikerült alátámasztanunk, hogy ezek a méregminták neurotoxikus aktivitással is rendelkeznek, és amely egyértelműen kapcsolatban áll azon megmart személyek neurológiai tünetegyüttesével, akik a viperamarást az adott régióban szenvedték el. Az elsőként bemutatott, 1-es esetismertetésben szereplő megmart személynek diplopiája alakult ki a külső szemmozgató izmok bénulása következtében. Ez általában a következő jele a descendáló paralízisnek (az első sokszor a ptosis), amely tipikusan a neurotoxikus kígyómarások ismérve. A diplopia egy már ismert neurotoxikus tünete némely *V. aspis* és *V. ammodytes* állományhoz tartozó viperák által okozott marásoknak, ideértve az egyéb neurológiai jeleket és tüneteket is, mint például a ptosis, az ophthalmoplegia és a dysphagia. E viperák mérgéről már ismert, hogy neurotoxinokat is tartalmaz. A 2. esetismertetésben szereplő megmartnál, szintén az agyidegek félreérthetetlen diszfunkciója jelentkezett; oculomotorius bénulással, kétoldali de részleges ptosissal, következményes

tekintetbénulással és diplopiával. Kétségtelen, hogy a régióban élő, de egyéb kelet-magyarországi populációhoz tartozó példányok mérge szintén neurotoxikus aktivitással rendelkezhet, tekintettel az értekezésben bemutatott esetismertetésekben szereplő megmart személyek tüneteire, akik két különböző és nem azonos populációba tartozó példánytól szenvedték el a marást az adott régión belül.

A magyarországi viperamérgeknél sikerült kimutatnunk egy erős és irreverzibilis gátlást az excitatorikus posztszinaptikus kurrensen, patkány agytörzsi glutamáterg szinapszisban. Ez szintén alátámasztja a méreg szinaptikus transzmisszióra gyakorolt preszinaptikus hatását. Ugyan a kígyómérgek neurotoxinjai elsősorban a perifériás idegrendszerre hatnak, eredményeink azt mutatják, hogy egyes viperamérgek neurotoxinjai képesek hatni a központi idegrendszer (CNS) nem-kolinerg idegátvitelére *in vitro*; hasonlóan, mint a Russell vipera (*D. russelii*) mérge, amelynek a nem-kolinerg szinaptikus transzmisszió gátló hatása már igazolt. Noha nincs közvetlen bizonyítékunk a méregkomponensek vér-agy gáton való *in vivo* penetrációjára, de a patkány agytörzsi neuronokban található nem-kolinerg szinaptikus transzmisszió méreg általi gátlása, esetleg magyarázatul szolgálhat a vertigo (nem keverendő össze az „egyszerű" szédüléssel) megjelenésére, amelyet bizonyos neurotoxikus európai viperamarások alkalmával olykor jelentenek.

Vizsgálataink során MALDI-TOF tömegspektrometriás mérést alkalmaztunk, amellyel a mérgek $PLA_2$ tartalmának heterogenitását kívántuk demonstrálni. A mérgek elemzése során számottevő variabilitást sikerült kimutatnunk, amely mind az elektroforetikus méreg-lenyomatokban, mind a mérgek $PLA_2$-spektrogramjában is tükröződik. Az összes méregmintának számos molekulatípusa megtalálható volt a $PLA_2$-kre olyannyira jellemző molekulatömeg tartományban, melyeket MALDI-TOF MS módszerrel detektáltunk. Eredményeinkből és a jelenleg rendelkezésre álló irodalmi adatok alapján arra következtettünk, hogy néhány toxikus és nem-toxikus foszfolipáznak is jelen kell lennie a bevizsgált méregmintákban. Másrészről jelentéktelen egyedi különbségeket találtunk a mérgek $PLA_2$ aktivitásnak tekintetében, mind a magyar méregmintákat egymással összevetve, mind a magyarokat a két kontrollal összehasonlítva. Bár nem volt számottevő individuális különbség a hazai viperamérgek között toxikusságukat tekintve *in vivo*, de néhány magyar minta szignifikánsan toxikusabb volt, mint a két

kontrol méregminta, amely magyarázható a hazai méregminták neurotoxikus aktivitásával és/vagy eltérő neurotoxin tartalmukkal. A hazai viperamérgek magas toxicitása minden valószínűség szerint a prejunkcionálisan ható $PLA_2$ tartalmukkal áll kapcsolatban, mivel a $PLA_2$-függő és a neuromuszkuláris kapcsolatra specifikus neurotoxinok a legtoxikusabb méregösszetevők. Fő feladatuk a zsákmányállatok gyors elpusztításának elősegítése, túlnyomóan bénító mechanizmuson keresztül. Ekképpen a bevizsgált viperamérgeink egereken detektált alacsony $LD_{50}$ értéke szintén kapcsolatban állhat neurotoxikus aktivitásukkal.

A tanulmányozott *V. berus* mérgeknek aránylag alacsony volt a porteáz aktivitása és több mint a minták fele mentesnek bizonyult a proteáz aktivitástól. Az általunk megfigyelt alacsony proteáz aktivitás és/vagy annak hiánya, szintén összhangban áll a *V. berus* marásoknál igen ritkán megjelenő, bizonyos klinikai tünetekkel (i.e. helyileg kialakult vérrel telt hólyagok, nekrózis). A mérgek degradációs lenyomata korrelál az SDS-PAGE során detektált variabilitással, és amely a metalloproteinázok jelenlétével lehet kapcsolatos. Egyedi variabilitást sikerült azonosítanunk egyes minták proteáz aktivitásában is; némelyik hím példány mérge magasabb proteáz aktivitással rendelkezett szemben a kifejlett nőstények mérgével. Ugyanakkor mind az osztrák *V. b. berus*, mind a *V. nikolskii* mérge teljesen mentes volt a proteáz aktivitástól. A juvenilis egyedeknél azonban nem tudtuk egyértelműen bizonyítani a kor-függő proteáz aktivitásban megmutatkozó méreg-összetételbeli változékonyságot, ellentétben más szerzők munkájával, akik orosz eredetű *V. berus* mérgek esetén ezt a típusú variabilitást kimutatták.

Tekintettel az európai *Vipera genus*ba tartozó fajok $PLA_2$-inek evolúciójára és a *V. berus* filogeográfiájára – minthogy a Kárpát-medence volt a faj egyik refúgiuma, továbbá a kelet-magyarországi viperák egy ősi evolúciós alcsoporthoz tartoznak –, ezért feltételezzük, hogy mérgük neurotoxikus $PLA_2$ tartalma egy ősi méregkarakter, amely a faj közép és északi elterjedési területein élő populációinak mérgéből az evolúció során "elveszett". Míg azok a *V. berus* populációk, amelyek pl. a kárpáti és a balkáni alcsoporthoz tartoznak és a faj két fő filogenetikai vonalát képezik, az egyéb méregkomponensek expressziójáért felelős géneket (pl. a neurotoxinokat kodoló gének) mérgük evolúciója során vagy megtartották, illetve visszanyerték. Ez a hipotézis meggyőző magyarázattal szolgálhat, hogy eddig a *V. b. berus* által okozott neurotoxikus marásokat leginkább csak

a Kárpát-medencéből és a *V. b. bosniensis* elterjedési területéről jelentettek. Eredményeink alapján erősen feltételezzük, hogy a méreg neurotoxin-tartalma illetve neurotoxikus aktivitása a *V. berus*-fajcsoportnál nem kizárólagos meghatározója az egyes rendszertani alcsoportoknak és besorolásoknak, ahogyan azt más szerzők említik, például a *V. nikolskii* és a *V. berus* kapcsán. Az iménti tényeket szem előtt tartva, a testvér-taxon, azaz a *V. nikolskii* (korábban *V. berus nikolskii*) rendszertani revíziója a közel jövőre nézve megfontolandó.

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# Present Ph.D. dissertation is based on the author's following publications


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European adder (*Vipera berus berus*) in Eastern Hungary: second authenticated case. *Wilderness & Environmental Medicine*, 24: 378–383.

# List of figures

**Figure 8.** Mechanogram of the frog nerve-muscle preparation (*in vivo*) from envenomed specimens, after the development of neuromuscular block.
**Legend: A)** control twitch (made from non-envenomed specimens); **B)** Response of the muscle during direct muscle stimulation; **C)** Responses of the muscle during direct neural excitation. Black arrow shows the washing with physiological solution (venom-free).

**Figure 9.** Isolated frog neuromuscular preparation (*ex vivo*) prepared from intact animals, exposed to *V. b. berus* venom.
**Legend: A)** administration of venom (120 μg/100 μl). Scale is 1 min. **B)** Rectangle: Expanded view of the repetitive phasic tetanic muscle contractures developed in every 1-2 sec. interval.

**Figure 10.** Responses of frog nerve-muscle preparation (*ex vivo*) to direct muscle stimulation (A), exogenous ACh (0.01 μM) (B) and to KCl (67 mM) (C) after the development of complete flaccidity. Scale is 10 sec.

**Figure 11.** Venom effects of eastern Hungarian and the Austrian *V. b. berus* on chick *biventer cervicis* nerve-muscle preparation.
**Legend: A)** Reduced twitch responses to nerve stimulation. Venom concentrations: 10 μg/ml. After 180 min in venom at 10 μg/ml, twitch height was about 20% of control (pre-venom) height. **B)** Reduced twitch responses to nerve stimulation with venom concentration 100 μg/ml for sample 23 and 37, while 30 μg/ml for sample 25. **C)** Responses of chick *biventer cervicis* preparations to acetylcholine (ACh; 1 mM), carbachol (Car; 20 μM) and potassium chloride (KCl; 40 mM) after exposure to venoms. Sample 23 and 37=eastern Hungarian adder venoms, 25=Austrian *V. b. berus*. The same samples were used that presented in table 1.

**Figure 12.** Effects of *Vipera* venoms on twitch responses of chick *biventer cervicis* preparations. Columns show the time (min) for the responses to nerve stimulation to be reduced to 50% of control, pre-venom heights (n= 2-4).
**Legend:** Seven samples from the east Hungarian *V. b. berus* specimens are shown: adult male and female, sub-adult males, juvenile male and females, and *V. nikolskii*. * = this sample of venom from an adult female adder did not cause twitch blockade to 50% in <120 min.

**Figure 13.** The effects of venom of an adult male Eastern Hungarian *V. b. berus* on chick *biventer cervicis* nerve-muscle preparation. The same venom sample was used that numbered as “23” in table 1.

**Figure 14.** Responses of chick *biventer cervicis* preparations to acetylcholine (ACh; 1 mM), carbachol (Car; 20 μM) and potassium chloride (KCl; 40 mM) after exposure to 100 μg of venom per ml. The samples are the same as shown in figure 9.

**Figure 15.** Comparison of the effects of *V. b. berus* venom on chick *biventer cervicis* preparations in $Ca^{2+}$- and $Sr^{2+}$-containing physiological salt solution. The same venom sample was used that numbered as "23" in table 1.
**Legend: A)** Twitch responses to nerve stimulation decreased following addition of venom to the preparations in the normal (calcium-containing) solution. **B)** Only very little twitch block could be observe on the preparations in the strontium-containing solution. Closed squares: in $Ca^{2+}$; open diamonds: in $Sr^{2+}$.

**Figure 16.** Inhibitory effect of *V. b. berus* venom on the glutamatergic synapse of the end-bulb of Held (A) and amplitudes of excitatory postsynaptic currents during venom administration after repeated excitation (B). The same venom sample was used that numbered as "23" in table 1.
**Legend: A:** The black current trace is the control recording; the grey curve is the postsynaptic current during venom exposure at 9.74 μg/ml venom concentration in the presence of two blockers (1 μM strychnine and 10 μM bicuculline). The inhibition revealed by the difference between the two curves is the consequence of the venom application. **B:** Summary of the data recorded under control conditions and in the presence of the venom.

# List of tables



**Legend:** From 1 to 27: venom patterns of the same specimens are presented in figure 2. Number 25 and 26 are the control venoms; 25=Austrian *V. b. berus* and 26=*V. nikolskii*; *=length and sex were not recorded; ‡=unknown dry weight, 20 µl fresh-milked undiluted venom. ‡‡= unknown dry weight, 23 µl fresh-milked undiluted venom.

# List of appendices

**Appendix 1.** Molecular weights of the different proteins in the molecular mass range of $PLA_2$s, detected in the individual adder venoms (n=25) and the control samples (n=2) by MALDI-TOF MS. The same samples are presented here, which also listed in table 1 (from 1-27) and showed by figure 2.
**Legend:** *=control sample I. (Austrian *V. b. berus*); ‡= control sample II. (*V. nikolskii*)

# Appendix

**Appendix 1.** Molecular weights of the different proteins in the molecular mass range of $PLA_2$s, detected in the individual adder venoms (n=25) and the control samples (n=2) by MALDI-TOF MS. The same samples are presented here, which also listed in table 1 (from 1-27) and showed by figure 2.

**Legend:** *=control sample I. (Austrian *V. b. berus*); ‡= control sample II. (*V. nikolskii*)

| Molecular mass ranges of the main protein-groups | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Group I** | **Group II** | **Group III** | **Group IV** | **Group V** | **Group VI** | **Group VII** | **Group VIII** | **Group IX** |
| **13500-13595 Da** | **13600-13695 Da** | **13700-13795 Da** | **13800-13895 Da** | **13900-13995 Da** | **14000-14095 Da** | **14100-14195 Da** | **14200-14295 Da** | **14300-14395 Da** |
| 13548.35 | 13654.60‡ | 13700.65 | 13842.53‡ | 13909.08 | 14004.33 | 14100 | 14200.50 | 14300 |
| 13549.18 | 13665.44 | 13705.36* | 13874.31 | 13914.09* | 14004.91 | 14101.97 | 14282.36 | 14302.66 |
| 13554.91 | 13665.77 | 13724.53 | 13875.59 | 13916.70 | 14018.60 | 14102.12 | 14291.53 | 14304.03 |
| 13555.48 | 13665.87 | 13770.59 | 13875.66 | 13941.70 | 14022.63 | 14102.59 | | 14305.20 |
| 13555.49 | 13671.17 | | 13877.13 | 13951.32 | 14043.29‡ | 14104.44 | | 14305.42 |
| 13555.52 | 13672.19 | | 13878.40 | 13955.67 | 14084.54 | 14105.52 | | 14305.74 |
| 13555.61 | 13672.42 | | 13880.36 | 13971.57 | 14085.41 | 14106.38 | | 14307.13 |
| 13555.81 | 13672.64 | | 13880.38 | 13984.55 | 14091.58 | 14107.32 | | 14310.11 |
| 13556.07 | 13673.50 | | 13880.43 | 13984.77 | 14093.40 | 14109.43 | | 14311.49 |
| 13556.07 | 13674.76 | | 13885.18 | 13986.22 | 14093.70 | 14112.52 | | 14315.69* |
| 13557.77 | 13676.09 | | 13886.53 | 13987.37 | 4094.54 | 14117.50* | | 14320.71 |
| 13558.54 | 13677.21 | | 13886.73 | 13988.96 | 14096.58 | 14121.61 | | 14322.08 |
| 13558.84 | 13677.41 | | 13886.89 | 13993.27 | 14096.62 | 14121.96 | | 14323.60 |
| 13559.24 | 13677.66 | | 13887.55 | 13996.17 | 14097.37 | | | 14340.17 |
| 13561.72 | 13679.11 | | 13889.11 | 13998.02 | 14098.00 | | | |
| 13562.91 | 13679.42 | | 13889.30 | | 14098.17 | | | |
| 13563.24 | 13680.64 | | 13889.81 | | 14098.99 | | | |
| 13565.25 | 13680.77 | | 13891.32 | | | | | |
| 13567.79 | 13682.02 | | 13892.11 | | | | | |
| 13571.10 | 13682.39 | | 13892.89 | | | | | |
| 13571.77 | 13684.87 | | 13893.93 | | | | | |
| 13571.78 | 13687.30 | | 13897.15 | | | | | |
| 13578.27* | 13687.35 | | 13897.19 | | | | | |
| 13587.67 | | | | | | | | |