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Toxicon 49 (2007) 995–1001

TOXICON

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Toxicity of venoms from vipers of *Pelias* group to crickets *Gryllus assimilis* and its relation to snake entomophagy

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Received 9 June 2006; received in revised form 23 January 2007; accepted 24 January 2007
Available online 6 February 2007

Abstract

The existing data indicate that snake venom is most toxic towards the natural vertebrate preys. Several species of snake include arthropods in their food. However, there is no available data on the toxicity of venom from entomophagous snakes towards their prey. We have studied the toxicity of venom from vipers of *Pelias* group towards crickets *Gryllus assimilis*. The *Pelias* group includes several closely related viper species inhabiting mainly the South European part of Russia, and they differ in their feeding preferences. Snakes from the *Vipera renardi*, *Vipera lotievi*, *Vipera kaznakovi*, and *Vipera orlovi* species feed on wide range of animals including insects, whereas snakes from *Vipera berus* and *Vipera nikolskii* species do not include insects in their diet. We have found that the venom from vipers that include insects in their diet possesses greater toxicity towards crickets. The greatest toxicity was observed for the venom from *V. lotievi*, which displays a preference for insects in its diet. Therefore, based on our data, we suggest that the viper entomophagy is not a result of behavior plasticity, but is probably determined at a genetic level.

Keywords: Viper; Food preferences; Venom; Toxicity; Entomophagy

1. Introduction

An overwhelming majority of snakes are predators, which feed on different taxa of animals, ranging from arthropods to mammals. It is well known that the poisonous snakes use their venom to restrain (or immobilize) the prey before ingestion. Recent data showed that the toxicity of venom could be prey specific. The venom from Brown Treesnake *Boiga irregularis* was more toxic to non-mammalian than mammalian preys (LD50 to chickens, geckos, skinks and mice was 1.75, 2.5, 4.5 and 31 μg/g body weight, respectively) (Mackessy et al., 2006). The greater toxicity of this venom towards birds and lizards may reflect the food preference of *B. irregularis*, which, being arboreal, feed mostly on these animals. The toxicity of venom from snakes belonging to 15 nominal taxa from *Micrurus* genus has been tested in native prey animals (Jorge da Silva et al., 2001). It was found that venom from nearly all *Micrurus,* for which prey preferences are known, are more toxic to natural prey than to non-prey species. Prey preference was the most important determinant of venom composition in *Micrurus*. It was also shown that the venom of coral snake *Micrurus nigrocinctus* was more toxic

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0041-0101/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2007.01.010

to its natural prey colubrid snake *Geophis godmani* than to any other colubrid species (Urdaneta et al., 2004).

Some snake species include arthropods in their diet (entomophagy); however, the number is small and the vast majority of species do not eat insects at all. It is not clear whether insect prey is killed by snake venom before ingestion. In addition, data on comparative toxicity of venom from entomophagous and non-entomophagous snakes towards insects are absent.

A group of shield-headed vipers (*Pelias* Merrem, 1820; Serpentes: Viperidae) (Garrigues et al., 2005) includes several closely related species that inhabit Central Europe and the South European part of Russia. Snakes from this group have a relatively broad range of nutrition, their usual diet consisting of small rodents, nestlings, lizards, and insects of Orthoptera order. Ophiophagy is also sometimes observed. Since this group of vipers includes species which feed on insects (e.g., East Meadow viper *Vipera renardi*) and those that do not (e.g., the common viper *Vipera berus*), it is a suitable model to study the relationship between entomophagy and venom toxicity towards insects. It should be noted that some of the species from this snake group have been described as individual taxa only very recently. Therefore, features of their behavior, including food preferences and venom properties, have not been studied in detail.

In the present work, we have studied the toxicity of the venom from seven shield-headed viper species towards crickets *Gryllus assimilis* (which mimic the natural insect preys), as well as the relationship between venom toxicity to insects and the ability of particular viper species for entomophagy.

2. Materials and methods

2.1. Snake and venom

Snakes from species of *Vipera nikolskii*, *V. berus*, *Vipera kaznakovi*, *Vipera orlovi*, *Vipera lotievi*, and *V. renardi* were captured in their natural habitat (Table 1). Snakes from each species were housed separately under uniform conditions in cages of $975 \times 480 \times 400$ mm allowing them to move freely. The temperature was maintained between 16 and 35 °C. Water was given ad libitum. Each species was bred for at least 3 years. *Viper* sp. is a newly identified species and is closely related to *V. renardi*. It also belongs to the *Pelias* group of vipers. Two pregnant females of *Viper* sp., captured in Baksan canyon (one in 2004 and other in 2005), gave birth to five siblings. Their behavior and feeding in captivity were studied for the first time. To determine the food preferences, the snakes were offered animals from different taxa that included mice (*Apodemus* and *Mus* sp.), lizards (*Lacerta* and *Eremias* sp.), European frogs (*Rana* sp.), locusts (*Locusta* sp.), and crickets (*G. assimilis*), the last two of Orthoptera order. Each prey animal was placed in the cage and snakes were observed until the prey was ingested. If the prey was not caught within several hours it was removed from the cage and new prey animal was placed.

For venom collection snakes were milked by manual gland massage, and the venom obtained was dried over anhydrous $CaCl_2$ and stored at −20 °C. The venom was obtained from several specimens of each species ranging from three snakes for *Viper* sp. to about 40 snakes for *V. berus* (Table 1).

Comparison of the venom derived from each viper species was performed by polyacrylamide gel

Table 1
Shield-headed viper species (*Pelias* Merrem, 1820) studied

Species	Capture area	Number of snakes used for pooling of venom
V. berus Linnaeus (1758)	Tver region, near Zubtsov	40
V. kaznakovi Nikolsky (1909)	Krasnodar Territory, near Adler	15
V. lotievi Nilson et al. (1995)	Karachaevo-Cherkes republic, near Khasaut village	17
V. nikolskii Vedmederya et al. (1986)	Penza region, near Zubrilovo village	25
V. orlovi Tuniyev and Ostrovskikh (2001)	Krasnodar Territory, Mikhaylovskiy mountain pass	15
V. renardi Christopher (1861)	Krasnodar Territory, near Beysugskiy firth	20
Viper. sp.	Kabardino-Balkar republic, Baksan canyon	3

electrophoresis (12% gel, thickness 1.5 mm) in the presence of sodium dodecyl sulfate under reducing conditions according to those of Smith (1994).

2.2. Toxicity determination

Toxicity determination was performed using crickets *G. assimilis* with body weights ranging from 0.3 to 0.9 g. Aqueous solutions of venom ranging from 1 to 6 μl were injected into the lateral region of the abdomen of each cricket. Concentrations of each venom used were 1, 5, or 10 μg/μl. Doses of 2.5, 5, 10, 15, 20, 25, 30, and 50 μg/g body weight were used for each venom. For *V. kaznakovi* and *V. lotievi* venoms a dose of 1.25 μg/g was also used. An equal volume of pure water was injected to control insects. Five insects were used for each dose and for a control. Live crickets were counted at 24, 48, and 72 h. LD_{50} was calculated by non-linear curve fit to the Levenberg–Marquardt equation using Origin 7.5 program (OriginLab Corporation, MA, USA). To find out the statistically significant differences, the data obtained was treated with the fit comparison tool of Origin 7.5 program. At the 0.05 significance level two datasets were considered statistically different.

3. Results

In breeding the shield-headed vipers (*Pelias* group) for many years (i.e. *V. berus*—15 years, *V. renardi*—5 years), we have observed that in captivity food preferences of vipers differ significantly. In general, vipers in captivity ate mice, lizards, frogs, and insects of Orthoptera order. Our long-term observations indicate that the main difference in feeding habits between shield-headed vipers is their ability to feed on insects. Only two (*V. nikolskii* and *V. berus*) of the seven species observed did not eat insects. The species included insects (locusts *Locusta migratoria* and crickets *G. assimilis*, Orthoptera) in their diet while snakes of *V. lotievi, V. renardi* sometimes preferred insects to other prey. The differences were more evident in neonate than adult vipers. This is illustrated in Table 2 for the two species with most difference in their diet. The neonate *V. renardi* vipers can be bred entirely on insects, whereas *V. berus* neonates cannot. The ability to feed on insects is preserved in adult *V. renardi* snakes; however, they can feed on other prey. According to our observations, the least selective vipers are from *V. orlovi* and *V. kaznakovi* species, which can eat mice, lizards, frogs, and insects without preference.

Table 2
Dietary differences in young *V. renari* and *V. berus* vipers

Species[a]	Quantity (%) of specimens eaten[b]		
	Insects	Neonate mice	Small lizards
V. renardi	98±2	3±1	97±2
V. berus	0	4±2	98±2

[a]Each group of species included 50 snakes. The experiment was started when the snakes were 1 month old.

[b]For mice and lizards the following feeding schedule was used: 25 prey animals were given on the first day; if all the animals were consumed, 15 animals were given on the second day and then 10—on the third. On the fourth day the number of the non-consumed animals was counted. Feeding was performed every 10 days. For insects: 25 insects were supplied each day. Before each feed, the number of remaining insects was counted.

Our observations confirmed that, for viper neonates, small lizards are the most common prey when first hunting initiates their predatory instinct. For *V. berus* and *V. nikolskii* neonates small young frogs (*Rana* sp.) can also be included; however, for these species insects do not appear to be included (according to our observation). On the contrary, we often observed that insects served as initial hunting prey for *V. renardi*, *V. lotievi*, *V. kaznakovi*, and *V. orlovi* neonates.

Taking into account the above observations as well as current data on the food composition of the shield-headed vipers (Drobenkov, 2005; Garanin et al., 2004; Ananjeva et al., 1998) we consider *V. renardi* and *V. lotievi* vipers to display pronounced entomphagy; *V. kaznakovi*, *V. orlovi* and *Viper* sp. with moderate entomophagy; and *V. berus* and *V. nikolskii* as non-entomophagous species.

We have observed that when hunting locusts or crickets, the viper bites the insect at the dorsal region, thus avoiding injury by the prey extremities. The insect is then usually consumed without being released. Such a process can create the impression that venom is not used for hunting. However, in some cases it was observed that the insect was released and escaped from the viper jaws but death occurred within a few seconds. Thus for example, a locust of about 0.5 g body weight, was bitten at its abdomen by a young *V. lotievi* snake, then released, and was completely immobilized in 6 s. This example clearly shows that entomophagous viper

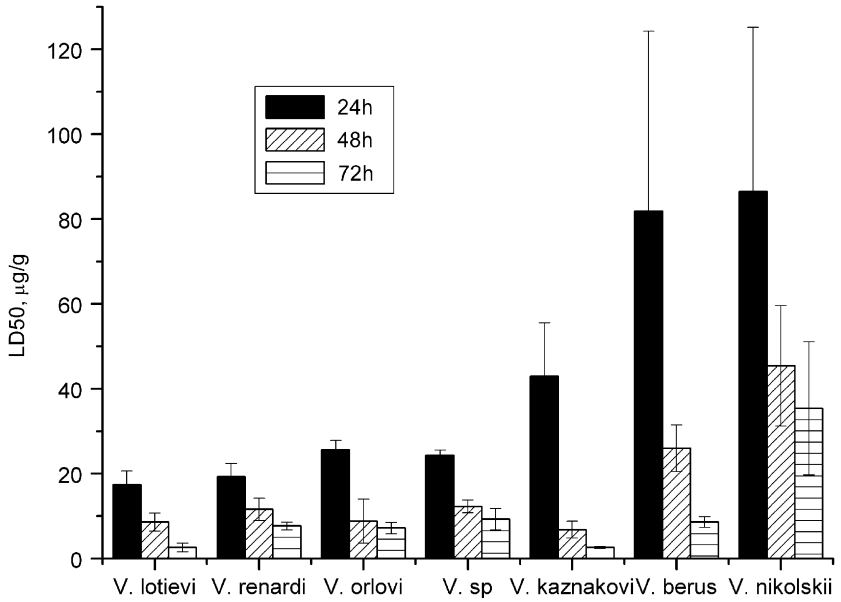

Fig. 1. Toxicity of venom from different viper species towards cricket *G. assimilis*. Aqueous solutions of venom ranging from 1 to 6 μl were injected into the lateral abdominal region of crickets. Five insects were used for each dose and for a control. The number of live crickets was counted at 24, 48, and 72 h. LD_{50} was calculated by non-linear curve fit of the experimental data to Levenberg–Marquardt equation using Origin 7.5 program (OriginLab Corporation, MA, USA).

venom is used to kill or at least immobilize the insect.

Based upon these observations we studied the toxicity of venom from different viper species towards cricket *G. assimilis*. The cricket species was chosen because it belonged to the same order of orthoptera as common insect prey for entomophagous vipers. A large ontogenetically homogenous population of *G. assimilis* necessary for toxicity studies can be easily obtained in laboratory. For the purpose of this study viper specimens were collected in areas where the possibility for cross-contamination was minimal (Table 1). Pooled samples of venom from each species were analyzed by polyacrylamide gel electrophoresis to confirm that no cross-contamination of species occurred. Venoms from several populations of *V. renardi* were analyzed and were identical in composition. Similar results were obtained for venom from two phenotypical morphs within a single population of *V. lotievi*. It should be noted that the pooling of the venoms was very important for the goals of the study. This minimized subtle intraspecific variation in venom composition which may be influenced by ontogenic, seasonal, sexual, and geographic factors (Creer et al., 2002 and references therein).

We determined the toxicity (expressed as LD_{50}) of venom from seven species of vipers which differ in their ability to feed on insects. The data given in Fig. 1 and Table 3 clearly show that venom from entomophagous snakes (*V. renardi* and *V. lotievi*) possesses considerably (more than four times) greater toxicity to crickets than venom from non-enterophagous species (*V. nikolskii* and *V. berus*). This difference was statistically significant ($p = 0.05$) at almost all observation times, but was most evident at 24 h after venom injection. *V. lotievi* venom was the most toxic to crickets (Fig. 1, Table 3). Insects were observed for 3 days post-injection and toxicity for all venoms increased with time. However, the observed increase was not equal

Table 3
Toxicity of the viper venom towards *Gryllus assimilis* cricket

Species	LD_{50} (μg/g)			Display of entomophagy
	24 h	48 h	72 h	
V. lotievi	17.4	8.6	2.6	Pronounced
V. renardi	19.3	11.6	7.7	Pronounced
V. orlovi	25.6	8.8	7.2	Moderate
V. sp.	24.3	12.3	9.3	Moderate
V. kaznakovi	42.9	6.8	2.6	Moderate
V. berus	81.9	26.0	8.6	Absent
V. nikolskii	84.8	45.4	35.4	Absent

$n = 5$, two independent experiments.

for all venoms tested. The greatest increase in toxicity was observed for *V. kaznakovi* venom. It was the least toxic when compared to the other enthomophagous viper venoms at 24 h but the most toxic after 72 h. The lowest increase in toxicity was observed for *V. nikolskii* and *V. renardi* venom. Venom from the newly identified *V.* sp. had toxicity characteristics similar to other vipers manifesting entomophagy.

4. Discussion

The data obtained clearly show that the venom from seven viper species differ significantly in their toxicity towards crickets (Fig. 1). In this study, we chose seven species belonging to the group of shield-headed vipers (*Pelias* group) (Garrigues et al., 2005). Some authors regard this group as a subgenus of the *Vipera* genus (Kalyabina-Hauf et al., 2004). The taxonomy within the *Pelias* group has not yet been completely established. However, most authors agree that at least three separate taxa can be identified in this subgenus: *V. berus*, *V. kaznakovi* and *V. ursinii* complexes (Nilson et al., 1994, 1995; Nilson and Andren, 2001; Kalyabina-Hauf et al., 2004; Garrigues et al., 2005). Along with well-known species, the new ones have been recently identified within all three complexes. *V. nikolskii* was isolated from the *V. berus* complex (Vedmederya et al., 1986; Bakiev et al., 2005), *V. orlovi* from the *V. kaznakovi* complex (Tuniev and Ostrovskikh, 2001) and *V. renardi* (Nilson and Andren, 2001) and *V. lotievi* (Nilson et al., 1995) from the *V. ursinii* complex. Phylogenetic studies (Nilson et al., 1994; Kalyabina-Hauf et al., 2004; Garrigues et al., 2005) showed that *kaznakovi–orlovi* and *ursinii—renardi–lotievi* are more closely related to each other than to *berus–nikolskii*. There are several sets of data confirming such classification with presence of different species within complexes. Thus, maximum parsimony analysis of the *Cyt b* gene revealed that *V. nikolskii* and *V. berus* are different taxa (Garrigues et al., 2005). Recently, we have shown (Gao et al., 2005) that heterodimeric phospholipases A_2 are the main constituents of *V. nikolskii* venom. Similar proteins were not found in the well characterized *V. berus* venom. Such differences in the venom composition support the classification of these vipers into two species. Phylogenetic analysis has clearly shown that *V. kaznakovi* and *V. orlovi* are distinct species (Kalyabina-Hauf et al., 2004). Similar analysis also demonstrated the difference between *V. ursinii, V. renardi* and *V. lotievi* species (Nilson et al., 1994; Nilson and Andren, 2001). All these data indicate that taxonomical studies of the *Pelias* group are continued; nevertheless, one can consider at least three taxa within this group as well established (*V. berus*, *V. kaznakovi* and *V. ursinii* complexes). These taxa inhabit different geographical zones (Ananjeva et al., 1998). *V. berus* inhabits a wide geographical region including Europe and northern part of Asia (predominantly Siberia). These snakes prefer forests and steppe forests characterized by a wet and moderately cold climate. The preferred habitat for all meadow vipers (*V. ursinii* complex) is dry grassland which varies from alpine meadows to low land grass steppe or dry puszta (Nilson and Andren, 2001). *V. kaznakovi* is distributed in the moist and warm low lands of western Caucasus as well as the mountain valleys to the east (Nilson et al., 1994, 1995). In different habitat zones the composition of food available for snakes differ significantly which is probably reflected in food preferences of these species. From the group of shield-headed vipers inhabiting the South European part of Russia, only *V. berus* has been studied in detail with respect to its food preferences. Its diet consists of mice, field-voles, frogs, chicks of small passerines, and sometimes lizards, lizards forming the basis for feeding of young *V. berus* (Drobenkov, 2005). There have been reports of unexpected objects in viper stomachs including cockchafers, mollusks, earthworms, sheep excrements and grass sprouts. However, under the experimental conditions used it was not possible to include these objects as dietary matter for vipers. It is most likely that they were contained in ingested preys. This "secondary ingestion" was well demonstrated (Creer et al., 2002) for arthropod remnants found in stomachs of some pit vipers *Trimeresurus stejnegeri*. For *V. kaznakovi* the predominant prey is small vertebrates and sometimes insects, whilst for *V. ursinii* insects form a large part of their diet (Ananjeva et al., 1998). For *V. renardi*, orthopteran insects may constitute more than 40% of their diet (Garanin et al., 2004). Our observations on young snakes from *V. berus* and *V. renardi* species (Table 2) confirm this data.

It was suggested that the type of animal preys and (as a result) the feeding habits of the snakes are directly reflected in venom composition (Daltry et al., 1996) and vice versa (Li et al., 2005). Our study on the toxicity of venoms from different *Pelias* vipers including entomophagous and

non-entomophagous snakes confirms this suggestion. This is the first time that toxicity of entomophagous and non-entomophagous viper venom towards different preys were compared. *V. berus* venom toxicity towards mice has been shown to vary. Following intravenous injection the toxicity measured as LD_{50} was reported to be 0.55 μg/g (Tu, 1977), 0.86 μg/g (Calderon et al., 1993) or 1.33 μg/g (Orlov et al., 1990). For *V. renardi* LD_{50} is equal to 0.77 μg/g (Orlov et al., 1990; herein this viper is regarded as *V. ursinii* in accordance with a classification adopted at that time). Therefore, for mice the toxicity for both of these venoms is almost equal. For insects venom toxicity of entomophagous *V. renardi* exceeded that of *V. berus* more than four times at 24 h after injection. However, 48 h after injection this ratio was approximately three times and at 72 h only 1.1 times. All these differences were statistically significant at $p = 0.05$. It could be suggested that *V. renardi* venom contains a component which acts fast on insects since its greater toxicity is more pronounced at 24 h after injection; but at 72 h this difference is negligible probably due to other toxins common to both viper venoms.

Based upon the differences in food preferences and choice of prey initiating the predatory instinct of neonate vipers, we suggest that the viper species belonging to *Pelias* group which inhabit the Southern European part of Russian Federation are indeed heterogeneous by feeding habits, and differences in venom toxicity emphasize a genetic determination of this phenomenon. The current literature on snake phylogeny supports this suggestion. As discussed earlier, at least three separate taxa can be found within the *Pelias* group: *V. berus*, *V. kaznakovi* and *V. ursinii* complexes. Snakes from the *V. berus* complex (*V. berus* s.s. and *V. nikolskii*) are non-entomophagous, and their venoms possess the lowest toxicity towards insects (Fig. 1). Snakes from the *V. ursinii* complex (*V. renardi* and *V. lotievi*) demonstrate pronounced entomophagy and their venom is the most toxic to crickets. *V. kaznakovi* s.s. and *V. orlovi* demonstrate moderate entomophagy and their venom is less toxic than that of snakes from *V. ursinii* complex.

Our results clearly show that the venom toxicity towards insects correlates with viper enthomophagy (but not with available data about venom toxicity to mice). The reason why some head-shielded vipers acquired the capacity to feed on insects is not clear. An interesting example of evolutionary link between venom composition and dietary adaptation is given by Li et al. (2005). In *Aipysurus eydouxii* (Marbled Sea Snake) the loss of venom toxicity towards its "conventional" prey is a result of gene mutation that brought about an essential shift from its usual prey to fish eggs exclusively. However, all the *Pelias* vipers studied have preserved the ability to feed on vertebrates (conventional viper prey). Most probably in the areas inhabited by entomophagous vipers insects are more abundant and/or available than vertebrate prey. These factors may induce a shift in food preferences of entomopagous vipers that could in turn be reflected in venom composition. Therefore, one can suggest that the snake entomophagy is not a result of behavioral plasticity but a phenomenon probably determined at a genetic level and reflected in interspecies differences in venom composition.

For the first time, we have shown that venom of entomophagous vipers is more toxic to insects than venom of vipers which do not feed on insects. Vipers of *Pelias* group, which manifest enthomophagy, display a pronounced enhancement in venom toxicity to insects. It is quite plausible to suggest that the venom of entomophaguos vipers contain toxins specific to insects. The identification of these toxins is currently being carried out by our group.

Acknowlegments

The authors are grateful to Dr. N.L. Orlov (Institute of Zoology RAS, Sankt-Petersburg) for help in species identification, Prof. V.I. Tsetlin for fruitful discussion, Dr. V. Wong (McGill University, Montreal, Canada) and D.I. Rodinov for the help with manuscript preparation. This work was supported in part by Russian Foundation for Basic Research (Grant # 06-04-48982).

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