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Age-dependent variations in the venom proteins of *Vipera kaznakovi* Nikolsky, 1909 and *Vipera ammodytes* (Linnaeus, 1758) (Ophidia: Viperidae)

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Abstract: Polyacrylamide disc gel electrophoresis and densitometry analysis were used to analyze venom extracts of *Vipera kaznakovi* (16.5, 30, and 55 cm) and *Vipera ammodytes* (28.5, 36.7, and 51.5 cm) specimens of 3 different lengths. *V. kaznakovi* specimens were collected from Hopa (Artvin Province); 2 *V. ammodytes* specimens were from Ağva (İstanbul Province) and the longest one was from Yuvacık (Kocaeli Province). The electropherograms of the venom protein samples showed age-dependent qualitative and quantitative variations.

Key words: *Vipera kaznakovi*, *Vipera ammodytes*, polyacrylamide disc gel electrophoresis, densitometry

1. Introduction

The Caucasus viper, *Vipera kaznakovi* Nikolsky, 1909, is known from the vicinity of Hopa and some other localities in the province of Artvin, Turkey. The main area is the Caucasus, including northeastern Turkey, Georgia, and Russia along the Black Sea coast (Mallow et al., 2003; Budak and Göçmen, 2008). The nose-horned viper, *Vipera ammodytes* (Linnaeus, 1758), is found in southern Europe through to the Balkans and Turkey. In Turkey, it is known to be distributed in the northwestern part, including Thrace and the Marmara Region (Tomovic, 2006).

Snakes include the largest of the venomous vertebrates, and they are the main source of natural toxins. Many snake species produce very large quantities of potent venoms (Mackessy, 2010). Snake species living in Turkey that produce venom are included in the families Viperidae, Elapidae, and the polyphyletic Colubridae (Budak and Göçmen, 2008).

Snake venoms are typically one of the most complex mixtures of primarily peptides and proteins among all natural venoms and very rich in enzymes (Tu, 1996; Chippaux, 2006; Mackessy, 2010). The venom of some species might contain more than 100 different toxic and nontoxic proteins and peptides. Snake venom also contains small organic molecules (e.g., carbohydrates, lipid groups, amines) and inorganic elements.

The sources of variation in venom composition include: (i) taxonomic differences, (ii) age, (iii) geography, (iv) diet, (v) seasonal variation, and (vi) sex-based variation (Chippaux et al., 1991; Chippaux, 2006; Mackessy, 2010). Age-dependent variations in biological and biochemical features of venom extracts have been frequently reported by various researchers and significant differences in toxicity, antigenic make-up, and biochemical composition, including enzyme activities, have been reported (Minton, 1967, 1975; Fiero et al., 1972; Theakston and Reid, 1978; Meier and Freyvogel, 1980; Meier, 1986; Mackessy, 1988; Furtado et al., 1991; Tun-Pe et al., 1995; Mackessy et al., 2003; Mackessy et al., 2006; Arıkan et al., 2006; Guércio et al., 2006).

Past studies related to snake venoms in Turkey mainly tended to deal with taxonomical comparisons (Arıkan et al., 2003, 2005, 2008; Göçmen et al., 2006). Recently, studies on biological and proteomic characterization of various viper venoms have been done in Turkey (Igci and Demiralp, 2012; Nalbantsoy et al., 2013; Yalcin et al., 2013).

The present study examined venom proteins of *Vipera kaznakovi* and *Vipera ammodytes* specimens collected from Hopa (Artvin Province), Ağva (İstanbul Province), and Yuvacık (Kocaeli Province). All venom extracts, obtained from 3 different specimens having different lengths for each species, were analyzed with polyacrylamide disc gel electrophoresis, and their venom electrophoretic patterns were compared.

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2. Materials and methods

Vipera kaznakovi specimens used in this study were collected from Hopa. Two specimens of *Vipera ammodytes* (28.5 and 36.7 cm) and the longest one (51.5 cm) were collected from Ağva and Yuvacık, respectively. The specimens were transported to the laboratory alive and kept in a terrarium. Their lengths were measured using a ruler. Venom samples were extracted from individuals as described by Tare et al. (1986), using a laboratory beaker and without applying pressure to the venom glands. Venom samples were centrifuged at 600 × *g* for 5 min, and 4 µL of a venom sample was used for each separation. The venom proteins were separated by polyacrylamide disc gel electrophoresis according to Davis (1964), slightly modified by Özeti and Atatür (1979) as previously described in detail (Arıkan et al., 2006). Briefly, venom samples were loaded onto the polyacrylamide gels and separated using a Canalco model 1200 electrophoresis apparatus. Gels were stained with 0.5% Amido black to visualize the protein bands and densitometric curves were obtained using a Gelman ACD-15 model 39430 densitometer at 500 nm.

The authors received ethical permission for venom sampling (Ege University Animal Experiments Ethics Committee, 2010-43) and special permission for field studies (from Republic of Turkey Ministry of Forestry and Water Affairs).

3. Results

The venom extracts of *Vipera kaznakovi* and *Vipera ammodytes* specimens had a viscosity higher than that of water. Although venom secretions of 2 specimens of *V. kaznakovi* (16.5 and 30 cm total lengths) were colorless, they were light yellow in the 55-cm-long specimen. In contrast, the venom extracts of 3 specimens of *V. ammodytes* were light yellow.

The gel photographs of the venom protein samples of *V. kaznakovi* specimens are presented in Figure 1. According to our results, the venom proteins of *V. kaznakovi* were separated into 12 fractions or fraction groups in the 2 shorter individuals (16.5 and 30 cm), while there were 13 fractions or fraction groups in the gel of the 55-cm-long individual (the longest one). Our comparative results of electrophoretic density curves (protein fractions) revealed significant quantitative differences especially between the 2 shorter individuals, while similar electrophoretic mobility patterns and fraction numbers were observed (Figures 1–4). Additionally, the densities of the venom proteins of the shortest individual differed strongly from the others (Figure 1).

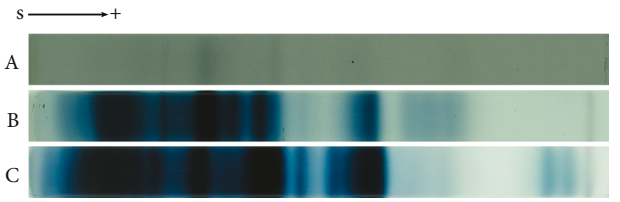

Figure 1. Polyacrylamide gel electrophoresis of venoms of *V. kaznakovi* of different lengths. **A.** 16.5 cm, **B.** 30 cm, **C.** 55 cm (**S:** Start, junction between the stacking and separation gels).

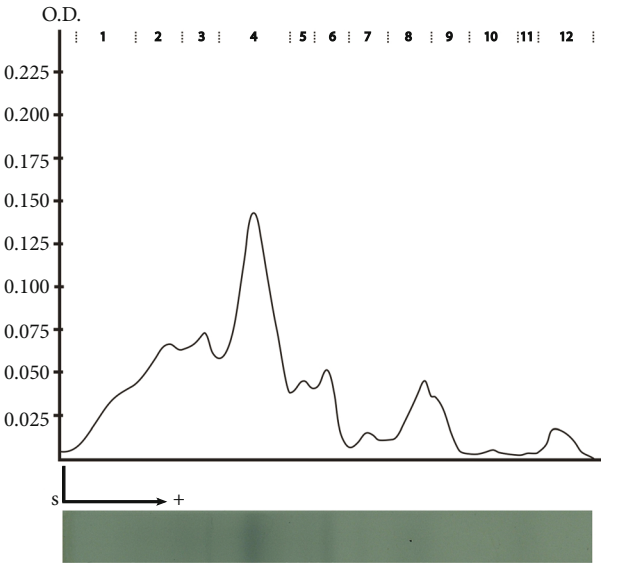

Figure 2. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from the 16.5-cm-long *Vipera kaznakovi* specimen, together with its densitometric tracing curve (**O.D.:** Optical density, **S:** Start, junction between the stacking and separation gels).

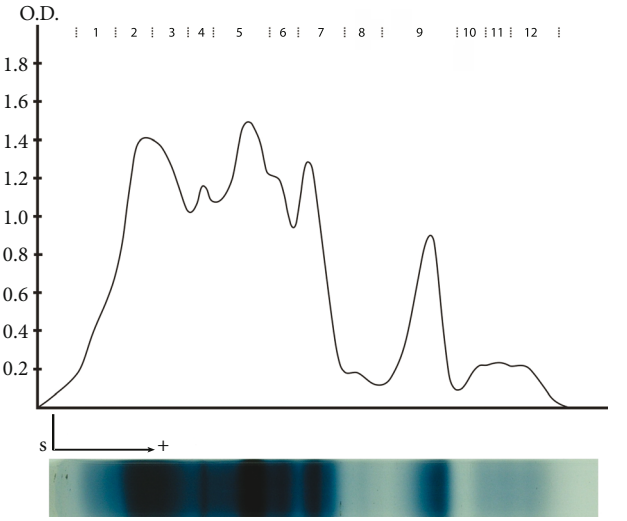

Figure 3. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from the 30-cm-long *Vipera kaznakovi* specimen, together with its densitometric tracing curve. For further explanation, see caption of Figure 2.

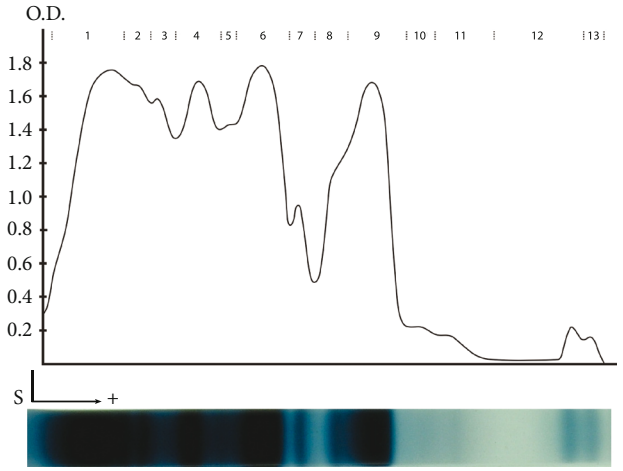

Figure 4. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from the 55-cm-long *Vipera kaznakovi* specimen, together with its densitometric tracing curve. For further explanation, see caption of Figure 2.

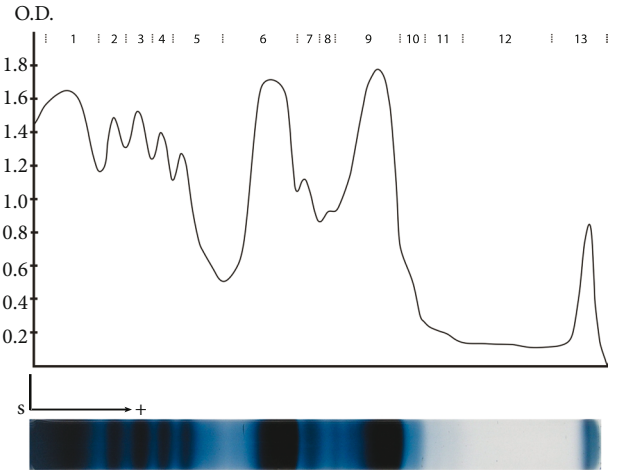

Figure 6. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from the 28.5-cm-long *Vipera ammodytes* specimen, together with its densitometric tracing curve. For further explanation, see caption to Figure 2.

The gel photographs of the venom protein samples of *V. ammodytes* are presented in Figure 5. Similar results were also found in *V. ammodytes*. According to our results of the electrophoretic and densitometric analyses, the venom proteins of the 28.5-, 36.7-, and 51.5-cm-long *V. ammodytes* individuals were separated into 13, 14, and 13 fractions or fraction groups, respectively. Our comparative results of electrophoretic protein fractions and densities revealed significant quantitative differences especially between the shortest (28.5-cm-long) and the longest (51.5-cm-long) individuals, while similar electrophoretic mobility patterns and fraction numbers were observed (Figures 5–8).

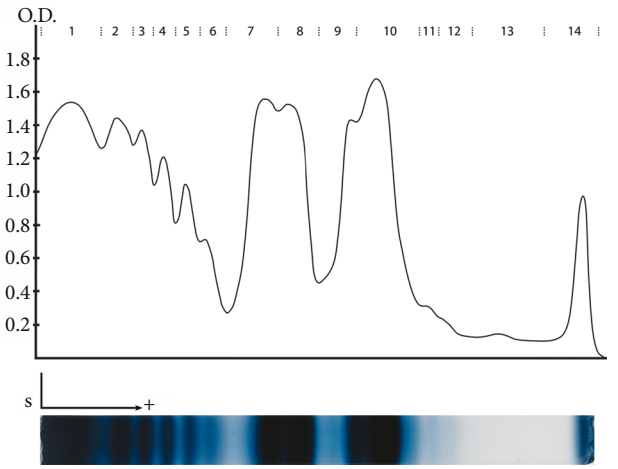

Figure 7. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from the 36.7-cm-long *Vipera ammodytes* specimen, together with its densitometric tracing curve. For further explanation, see caption to Figure 2.

4. Discussion

Many reports in the literature have documented variation in venom between different individuals of the same population of one species, during different ages of the animal (Chippaux, 2006; Mackessy, 2010). Various studies on age-dependent variations in snake venom proteins showed that younger individuals had more potent venom in terms of toxicity, and procoagulant and defibrinating activity (Bonilla and Horner, 1969; Fiero et al., 1972; Minton, 1975; Theakston and Reid, 1978; Meier and Freyvogel, 1980; Glenn and Straight, 1985; Furtado et al., 1991; Tun-Pe et al., 1995; Guércio et al., 2006; Mackessy et al., 2006).

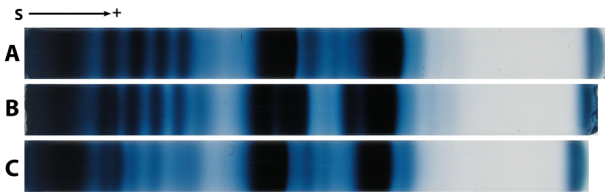

Figure 5. Polyacrylamide gel electrophoresis of venoms of *V. ammodytes* of different lengths. **A.** 28.5 cm, **B.** 36.7 cm, **C.** 51.5 cm (**S:** Start, junction between the stacking and separation gels).

Several parameters of venom are affected during ontogenetic development. As a result of the increased head size and so venom gland size, venom yields and total dry weight of adult snakes are generally greater than those of neonate or juvenile individuals and this can be regarded as a general trend for all venomous reptiles (Klauber, 1956; Meier and Freyvogel, 1980; Mackessy, 1985, 1988; Furtado et al., 1991; Mackessy et al., 2003, 2006, 2010). The total

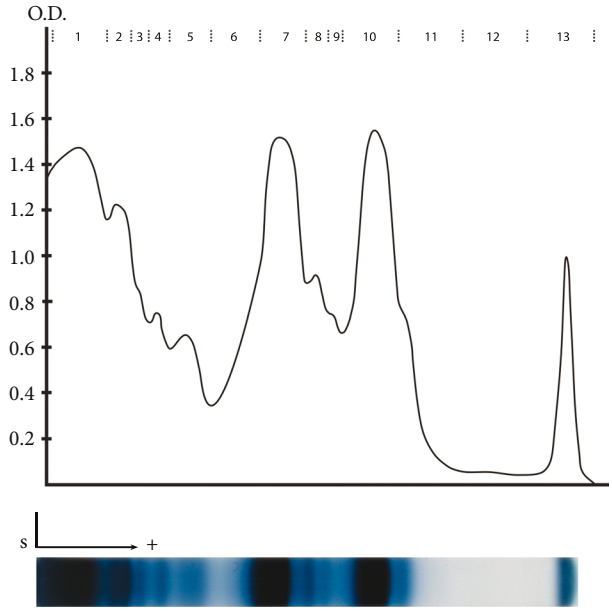

Figure 8. Gel photograph showing the electrophoretic separation of the venom protein sample obtained from the 51.5-cm-long *Vipera ammodytes* specimen, together with its densitometric tracing curve. For further explanation, see caption of Figure 2.

protein content of venom may also vary (generally increase) with age (Bonilla et al., 1973; Mackessy et al., 2006). Mackessy et al. (2006) reported that the lyophilized *Boiga irregularis* venom from neonate snakes had approximately one-half the total protein amount of venoms from adult snakes. Moreover, the biochemical composition of venom may vary with age (Meier, 1986; Guércio et al., 2006; Mackessy et al., 2006).

Mackessy (2010) stated that ontogenetic variations in venom had been noted for several snake species, including age-related differences in biochemical composition and enzyme activities (e.g., lower protease activity in the venoms of neonate or juvenile individuals). Tun-Pe et al. (1995) studied age-dependent variations in the venom of Russell's viper, *Daboia russelli siamensis*, by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They found that the venom of juveniles was colorless and had little L-amino acid oxidase activity. Similar results were reported in earlier studies on the venom of various viperid snakes (Bonilla and Horner, 1969; Theakston and Reid, 1978; Glenn and Straight, 1985; Furtado et al., 1991; Arıkan et al., 2006).

A study on age-related differences in the venom proteins of the common lancehead (fer-de-lance), *Bothrops atrox*, showed that the venom of the youngest individuals had fewer protein fractions and the number of the fractions increased with age (Meier, 1986). Similar results were reported also by Tun-Pe et al. (1995) and Arıkan et al. (2006).

Our observations on the color of the venom of *V. kaznakovi* and *V. ammodytes* are in accordance with the literature (Jiménez-Porras, 1964a; Tun-Pe et al., 1995; Arıkan et al., 2003, 2005, 2006). The yellowish color, which is a general characteristic of viper venoms, mainly originates from the flavin groups of L-amino acid oxidases (Chippaux, 2006; Tan and Fung, 2010). Although the brightness or intensity of the yellow may give an idea about the amount of enzyme (Jiménez-Porras, 1964b; Dimitrov and Kankonkar, 1968; Fiero et al., 1972; Tun-Pe et al., 1995), additional experiments must be carried out, such as western blotting and enzyme activity, in order to confirm the amount of the enzyme directly.

Among the *V. kaznakovi* and *V. ammodytes* specimens, during venom extraction, the most aggressive behavior was observed in the younger individuals, corroborating previous reports (Arıkan et al., 2006). The present study indicated that the number of fractions, which indicates the protein composition and concentration of the venom proteins, are higher in older than in the youngest specimens. Although the longest specimen of *V. ammodytes* is from a different locality and may reflect geographical variation, differences are observed well between the 2 shorter specimens and the longest specimen can also be included in our age-dependent comparison. Our results support those of Bonilla et al. (1973), Meier and Freyvogel (1980), Meier (1986), Tun-Pe et al. (1995), Arıkan et al. (2006), and Mackessy et al. (2006) on venom proteins of different viperid and crotalid snake species.

Documenting ontogenetic variations in snake venoms is important for antivenom production and understanding different pathologies. Age-dependent variation in the venom of *Montivipera xanthina* has been shown in Turkey previously (Arıkan et al., 2006). Our results demonstrate age-related variation in different age groups including a single individual per group, by using polyacrylamide disc gel electrophoresis. However, further investigation with more individuals to eliminate individual variations, including proteomic and toxicological studies, will reveal the level of variation and identify the proteins with altered expression levels. Our findings suggest that using venoms from individuals of *V. kaznakovi* and *V. ammodytes* at different ages for antivenom production is needed and will result in more potent antivenoms. Proteomic and biological characterization of venoms of snakes at different ages can give insight into the different pharmacological activities of their venoms and can lead to novel pathways of action.

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