Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution

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The processes that drive the evolution of snake venom variability, particularly the role of diet, have been a topic of intense recent research interest. Here, we test whether extensive variation in venom composition in the medically important viper genus \textit{Echis} is associated with shifts in diet. Examination of stomach and hindgut contents revealed extreme variation between the major clades of \textit{Echis} in the proportion of arthropod prey consumed. The toxicity (median lethal dose, LD\textsubscript{50}) of representative \textit{Echis} venoms to a natural scorpion prey species was found to be strongly associated with the degree of arthropod feeding. Mapping the results onto a novel \textit{Echis} phylogeny generated from nuclear and mitochondrial sequence data revealed two independent instances of coevolution of venom toxicity and diet. Unlike venom LD\textsubscript{50}, the speed with which venoms incapacitated and killed scorpions was not associated with the degree of arthropod feeding. The prey-specific venom toxicity of arthropod-feeding \textit{Echis} may thus be adaptive primarily by reducing venom expenditure. Overall, our results provide strong evidence that variation in snake venom composition results from adaptive evolution driven by natural selection for different diets, and underscores the need for a multi-faceted, integrative approach to the study of the causes of venom evolution.

**Keywords:** snake venom; adaptation; diet; coevolution; \textit{Echis}

1. INTRODUCTION

Variation in venom composition is ubiquitous among venomous snakes, occurring at all taxonomic and biological levels (Chippaux \textit{et al}. 1991). Although it is generally accepted that the primary function of snake venom is to facilitate immobilization and/or digestion of prey, the extent to which adaptive processes drive the evolution of snake venom diversity has been widely debated. Several authors have supported an ‘overkill’ hypothesis of venom evolution, which postulates that, due to the apparent high toxicity of many snake venoms and the large doses injected, variation in venom composition is unlikely to be subject to natural selection for lethality to prey, and that venom diversity largely results from neutral evolutionary processes (\textit{Sasa} 1999\textit{a},\textit{b}; \textit{Mebs} 2001). By contrast, other authors argue that snake venom composition is subject to strong natural selection, and that venom diversity results from adaptation to specific diets (\textit{e.g.} Daltry \textit{et al}. 1996; \textit{Wüster et al}. 1999; Kordiš \\& Gubenišek 2000).

Well-documented instances of resistance to envenoming in prey species (Poran \textit{et al}. 1987; Heatwole \\& Poran 1995; Biardi \textit{et al}. 2006) demonstrate the potential of selection for increased venom toxicity in snakes, and the possibility that reciprocal coevolutionary ‘arm’s races’ may occur between snakes and their prey. Further indication that venom composition is subject to natural selection comes from evidence that venom production is metabolically costly, representing a trade-off between the metabolic costs of venom synthesis and increasing foraging efficiency (McCue 2006). This contradicts the assumption that snakes inject many orders of magnitude more venom into prey than the lethal dose required, which is further challenged by evidence that at least some snakes ‘meter’ the amount of venom injected depending upon prey size (Hayes \textit{et al}. 1995). The selective consequences of the metabolic cost of venom production are also demonstrated by an example of adaptive venom loss following an evolutionary shift to a diet of fish eggs in the sea snake \textit{Aipysurus eydouxii} (Li \textit{et al}. 2005).

Other studies have highlighted apparent coincident variation in venom composition and diet as evidence of adaptive venom evolution. Geographical variation in venom composition, as revealed by electrophoretic profiles or mass spectrometry, has been shown to be correlated with geographical variation in diet in some pitvipers (Daltry \textit{et al}. 1996; Creer \textit{et al}. 2003). At the molecular level, venom-coding genes show extremely high rates of sequence evolution and an excess of non-synonymous over synonymous substitutions, suggesting that selection drives rapid toxin diversification by favouring mutations that alter protein structure (Kordiš \\& Gubenišek 2000; Lynch 2007).

While compositional and molecular analyses of snake venoms provide evidence of adaptation, the functional
significance of these adaptations remains unknown. Ultimately, this can only be tested by measuring the effects of venom on natural prey (Sasa 1999b; Wüster et al. 1999). Naturalistic prey models have been used to investigate the adaptive significance of ontogenetic venom variation, revealing instances of ontogenetic shifts in prey preference accompanied by corresponding shifts in prey-specific venom toxicity (Mackessy 1988; Andrade & Abe 1999; Mackessy et al. 2006). Previous studies have also compared venom toxicities to natural prey between related snake species with variable diets, and have typically found the venom of a given species to be more toxic to its preferred prey than that of congeneres with different diets (Jorge da Silva & Aird 2001; Starkov et al. 2007). These results are certainly consistent with the hypothesis that natural selection for different diets has driven the evolution of interspecific venom variation. However, without knowledge of where evolutionary shifts in diet and venom composition have occurred during the evolutionary history of these species, the alternative hypothesis of phylogenetic constraint cannot be rejected: similarity in venom characteristics and diet may simply be the result of common ancestry rather than selection.

To provide a more robust test of hypotheses on the causes of variation in venom composition, approaches are required which examine venom characteristics and diet within a phylogenetic framework. This requires an analysis of closely related venemous snake species, some of which feed on radically different prey types with different physiology. The saw-scaled vipers (Viperidae: *Echis*) fulfil these criteria. This genus of small vipers is distributed throughout much of Africa north of the Equator, the Arabian Peninsula, India and Sri Lanka (Wüster et al. 1997), and represents the most medically significant group of snakes in several parts of this distribution (Warrell et al. 1977). Previous studies document considerable interspecific variation in venom composition (Schaefler 1987), which is further supported by a lack of antivenom cross-reactivity against envenoming by different *Echis* species (Gillissen et al. 1994). Great variation in diet is also reported within the genus. Some species consume predominantly vertebrate prey (Tsairi & Bouksila 2004), while others prefer arthropods such as scorpions and centipedes (Gasperetti 1988; Revault 1996). Arthropods can be considered atypical viper prey (Shine et al. 1998), thus arthropod feeding most probably represents a derived condition arising at some point during the diversification of *Echis*. We hypothesized that this shift to physiologically distinct, well-defended and potentially dangerous arthropod prey would impose considerable selective pressure, resulting in adaptive evolution of venom composition for increased toxicity to arthropods.

We tested our hypothesis of adaptive venom evolution by integrating evidence of phylogeny, venom composition, diet and venom activity. This involved reconstructing phylogenetic relationships between the major clades of *Echis* and verifying that variation in venom composition occurs among them. Determining the extent of arthropod versus vertebrate feeding for each clade then allowed the inference of evolutionary shifts in diet occurring during the diversification of *Echis*. Finally, to investigate the functional significance of variation in *Echis* venom composition, the toxicity of representative venoms from each *Echis* clade was measured against a naturalistic arthropod prey model, the scorpion *Scorpio maurus*. Use of a phylogenetic framework allows an assessment of the extent to which dietary shifts are associated with adaptive venom evolution for increased toxicity to novel prey types, and a more rigorous test of the factors driving snake venom evolution than previous studies.

2. MATERIAL AND METHODS

(a) Phylogenetic analysis

Although the intrageneric taxonomy of *Echis* is currently in a state of flux (Wüster et al. 1997), ongoing molecular phylogenetic work (W. Wüster & C. E. Pook 2008, unpublished data) has shown that previously described species (Cherlin 1990) form four monophyletic species complexes: (i) *Echis carinatus* group, including *E. carinatus* and *Echis multisquamatus*; (ii) *Echis pyramidum* group, including *E. pyramidum*, *Echis leucogaster* and *Echis khasazkhi*; (iii) *Echis ocellatus* group, including *E. ocellatus* and *Echis jogerii*; and (iv) *Echis coloratus* group, including *E. coloratus* and *Echis omanensis*. The phylogenetic relationships among the four major species groups were reconstructed using a single representative of each: *E. carinatus* socureki (Sharjah, United Arab Emirates); *E. pyramidum* leakeyi (Baringo, Kenya); *E. ocellatus* (northern Cameroon); and *E. coloratus* (Israel). Corresponding sequences for the vipers *Cerastes cerastes* (Egypt) and *Bitis arietans* (Agadir, Morocco) were included as outgroup taxa (Wüster et al. 2008).

Total DNA was extracted from tissue (ventral scale clipping) or blood samples using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich). Parts of four mitochondrial genes and one nuclear gene were amplified using the polymerase chain reaction (PCR) and sequenced. These were cytochrome b (cytb), NADH dehydrogenase subunit 4 (ND4), 12s rRNA, 16s rRNA and the nuclear recombination activating gene 1 (RAG1). Details of PCR protocols, primers and reaction conditions are provided in the electronic supplementary material.

All protein-coding gene sequences were aligned manually. 12s and 16s rRNA sequences were aligned using CLUSTALW (Thompson et al. 1994), with later corrections by eye. Protein-coding genes were translated into amino acid sequences and checked for unexpected indels or stop codons to detect the possible presence of non-coding copies or pseudogenes (Zhang & Hewitt 1996).

Phylogenetic analysis was carried out using Bayesian inference (BI) methods. We first partitioned the data into eleven biologically relevant partitions: all protein-coding genes were partitioned separately into first, second and third codon positions, and 12s and 16s were also treated as separate partitions. The most appropriate model of sequence evolution for each partition under the Akaike information criterion was estimated using MrModelTest v. 2.2 (Nylander 2004). The final BI analysis, using MrBayes v. 3.1 (Ronquist & Huelsenbeck 2003), involved four parallel runs of five million generations, each with one cold and three heated chains. The number of generations prior to burn-in was determined through visual inspection of a plot of likelihood scores against generation number.

Previous analyses have suggested that *E. coloratus* may form the sister taxon to the remaining *Echis* (Lenk et al. 2001). To determine whether our data significantly rejected this phylogenetic hypothesis, we calculated the proportion of
post-burn-in Bayesian trees consistent with it by filtering the trees in PAUP^* v. 4b10 (Swofford 2002). The hypothesis was rejected if fewer than 5 per cent of post-burn-in trees were consistent with it.

(b) Electrophoresis of venom
To examine whether variation in venom composition exists among the major *Echis* species groups, we produced electrophoretic venom profiles for the same representative *Echis* taxa used for phylogenetic analysis, in addition to the outgroup taxa *B. arietans* and *C. cerastes*. The snakes (*E. c. sochureki*, United Arab Emirates; *E. p. leakeyi*, Kenya; *E. ocellatus*, Nigeria; *E. coloratus*, Egypt; *B. arietans*, Ghana; and *C. cerastes*, Egypt) were maintained in the herpetarium at the Liverpool School of Tropical Medicine. Venoms were extracted from at least 10 specimens of each species, pooled, lyophilized and stored at 4°C. The phosphate buffered saline-reconstituted venom samples were mixed with an equal volume of reduced protein-loading buffer (1 mg ml\(^{-1}\)), boiled for 10 min and loaded onto a 15 per cent reduced SDS–PAGE gel and electrophoresis performed at 200 constant voltage for 57 min. The fractionated venom proteins were visualized by staining with Coomassie blue.

(c) Dietary analysis
To determine the extent and variation of arthropod feeding among the major *Echis* species groups, preserved museum specimens (*E. carinatus* group \(n=22\); *E. pyramidum* group \(n=65\); *E. ocellatus* group \(n=46\); and *E. coloratus* group \(n=99\)) were dissected and the stomach and hindgut contents examined. Intact prey items were identified visually. Partially digested prey remnants were examined using a dissecting microscope (\(\times 10–40\) magnification), and identified by the criteria shown in the electronic supplementary material. Very small arthropods or exoskeleton fragments were not recorded owing to the possibility of secondary ingestion via the gut contents of the actual prey (Creer et al. 2002). The snout-vent length (SVL) of each specimen was also measured.

Chi-squared tests were used to test for significant differences in the proportion of arthropods and vertebrates consumed between the four species groups. ANOVA was used to test for significant differences in SVL between snakes that had consumed arthropods and those that had consumed vertebrates in each species group. Analyses were performed using SPSS v. 12.

(d) Venom toxicity to scorpions
The scorpion *S. maurus* (Egypt) was selected as a suitable naturalistic arthropod prey species with which to compare *Echis* venom toxicities, as this species inhabits semi-arid and arid environments (Prendini et al. 2003) that are also typical for *Echis*, and has a very large distribution sympatric with several *Echis* species. The body mass of scorpions ranged from 0.6 to 2.7 g.

Venom toxicities were measured for each of the representative *Echis* taxa used in phylogenetic analysis and venom electrophoresis: *E. p. leakeyi* (Kenya); *E. c. sochureki* (Pakistan); *E. ocellatus* (Nigeria); and *E. coloratus* (Saudi Arabia). In order to infer the ancestral level of arthropod-specific venom toxicity that existed before the evolutionary shift to arthropod feeding, the venom toxicity of the vertebrate-feeding outgroup taxon *B. arietans* (Ghana) was also measured. Lyophilized venoms were obtained as described for venom electrophoresis, and dissolved in insect Ringer's solution to achieve specific concentrations used in toxicity tests. Venoms were administered to scorpions by injection into the lateral posterior pre-abdomen, through the pleural membrane.

Initially, median lethal doses (LD\(_{50}\)) were determined by injecting groups of scorpions with varying venom doses and recording mortality after 24 hours. The range of doses tested and the number of scorpions in each group \((n)\) are shown in table 1. All doses were mass \((\mu g \cdot g^{-1} \cdot body \ weight)\) and volume \((0.05 \ ml \ g^{-1} \ body \ weight)\ adjusted. Additionally, to test the possibility of mortality due to the injection procedure, control groups were injected with 0.05 ml g\(^{-1}\) \((n=3)\) and 0.1 ml g\(^{-1}\) \((n=3)\) of insect Ringer's solution only, representing, respectively, the same volume and double the volume used in venom injections. LD\(_{50}\) was calculated by probit analysis using Minitab v. 15. Dose was the stimulus and venom the factor. Response was in event (mortality)/trial \((n)\) format. Weibull distribution was assumed, as this provided the best fit to the data (Pearson \(\chi^2_{18}=19.15, p=0.692\); deviance \(\chi^2_{18}=9.41, p=0.994\)). The test for equal slopes indicated that the assumption of equal regression slopes among venoms was not violated \((\chi^2=8.09, p=0.088)\).

As the standard LD\(_{50}\) is of questionable biological relevance (a snake requires a much greater probability of prey death in a much shorter duration), we also investigated the time taken for scorpions to become incapacitated (defined as inability to self-right when turned on to its back) and die (defined as complete lack of movement when touched) following a biologically realistic venom dose. We selected 3 mg g\(^{-1}\) as an appropriate dose, as this represents two-thirds of the average total venom yield (9 mg dry weight, R. A. Harrison 2007, personal observation) from a medium-sized *E. ocellatus* for the envenomation of a scorpion with a mass of 2 g. Times to incapacitation and death were recorded to the nearest minute. Five scorpions received each *Echis* venom \((n=5\) per venom\) and four received *B. arietans* venom \((n=4)\). ANOVA with post hoc tests (Tukey) was used to test for significant differences between venoms in the time to scorpion incapacitation and death. Analyses were performed using SPSS v. 12.

### Table 1. Scorpion mortality 24 h after venom injection. (Total group size \((n)\) is shown in parentheses.)

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<tr>
<th>venom</th>
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<td><em>E. p. leakeyi</em></td>
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<td><em>E. c. sochureki</em></td>
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<tr>
<td><em>E. ocellatus</em></td>
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<tr>
<td><em>E. coloratus</em></td>
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<td><em>B. arietans</em></td>
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To test whether these doses of venom constitute a biologically realistic representation of natural predator–prey interactions between Echis and scorpions, we conducted feeding trials, in which captive E. c. sochureki (Sharjah, United Arab Emirates; n = 2) were offered live S. maurus. Snakes were placed in a transparent enclosure and left undisturbed for a minimum of 24 hours before a scorpion was introduced. Behaviour was recorded using a video camera located outside the enclosure, and the time between scorpion envenomation and onset of feeding measured using the video camera time counter.

3. RESULTS

(a) Phylogenetic analysis

A total of 3145 bp of sequence (cyt b: 789 bp; ND4: 644 bp; 12s rRNA: 404 bp; 16s rRNA: 472 bp; and RAG1: 836 bp) was aligned. A 24 bp section of 16s and a 48 bp section of 12s were excluded due to alignment difficulties. No unexpected indels, frameshifts or non-sense codons were detected in protein-coding gene sequences. Nucleotide sequences were deposited in GenBank (accession numbers: EU852294–EU852305 and EU852312–EU852329). The final dataset contained 738 variable sites, of which 339 were parsimony informative.

Models of sequence evolution for data partitions used in the BI analysis are shown in the electronic supplementary material. Examination of likelihood plots revealed that stationarity was reached after approximately 50 000 generations, but as a ‘safety margin’ we discarded the first million generations. The BI tree with Bayesian posterior probabilities is shown in figure 1, and yielded the topology (carinatus (ocellatus (coloratus, pyramidum))). The monophyly of the clade consisting of E. coloratus, E. pyramidum and E. ocellatus was supported by high Bayesian posterior probability values, but support for the monophyly of E. pyramidum and E. coloratus fell just short of statistical significance. Out of 32 004 post-burn-in BI trees, only 4 (0.012%) supported the position of E. coloratus as the sister taxon of the remaining Echis. We therefore consider this hypothesis rejected.

(b) Electrophoresis of venom

Subjecting venom samples to reduced SDS–PAGE (figure 2) revealed considerable variation in venom protein composition between representatives of each major Echis species group, which was comparable with that found between Echis and the more distantly related vipers B. arietans and C. cerastes. From this preliminary analysis, there was no obvious association between venom protein profile and diet.

(c) Dietary analysis

In total, 152 identifiable prey items were recovered. Chi-squared tests showed significant differences between Echis species groups in the proportion of arthropods consumed ($\chi^2_{51}=28.54, p<0.001$). Arthropods constitute more than half the diets of the E. carinatus and E. pyramidum groups. The E. ocellatus group is less reliant on arthropod prey, and the E. coloratus group feeds almost exclusively on vertebrates (figure 3; more detailed dietary data are provided in the electronic supplementary material). In all species groups, the mean SVL of snakes that consumed arthropods was less than those that had consumed vertebrates, the difference being significant in the E. coloratus group ($F=8.25, d.f.=1, p=0.007$), the E. ocellatus group ($F=7.60, d.f.=1, p=0.009$) and the E. pyramidum group ($F=9.93, d.f.=1, p=0.003$), but not in the E. carinatus group ($F=0.49, d.f.=1, p=0.49$). However, there was extensive overlap in SVL between snakes that had consumed arthropods and those that had consumed vertebrates in all Echis species groups, indicating that no clear ontogenetic shift in diet occurs in Echis, and both vertebrates and arthropods are consumed at all life stages (see the electronic supplementary material).

(d) Venom toxicity to scorpions

Scorpion mortality for the doses tested is shown in table 1. Control experiments with insect Ringer solution only resulted in zero mortality. Probit analysis revealed that

![Figure 1. Bayesian phylogeny of the major Echis species groups, inferred using a single representative taxon from each. Bayesian posterior probabilities are shown by relevant nodes. Columns to the right indicate the proportion of arthropod prey consumed (diet) and venom toxicity to scorpions (venom): +, +++; , moderate; –, low; nt, not tested. Instances of dietary shifts in prey type accompanied by coevolution of venom activity are indicated by vertical bars along branches.](Image 304x642 to 485x765)

![Figure 2. Venom protein profiles; venom from B. arietans (Ba), C. cerastes (Cc), E. c. sochureki (Ecs), E. ocellatus (Eo), E. p. leakeyi (Epl) and E. coloratus (Ec) were fractionated using 15% reduced SDS–PAGE.](Image 324x318 to 528x520)
E. p. leakeyi and E. c. sochureki had the most scorpion-lethal venoms (lowest LD₅₀), followed by, in order of diminishing toxicity; E. ocellatus, B. arietans and E. coloratus. Significant differences in LD₅₀ were evident in all pairwise venom comparisons, except between E. p. leakeyi and E. c. sochureki (figure 3).

Following administration with a biologically realistic venom dose, mean times to scorpion incapacitation (27–43 min) and death (43–64 min) were longer than expected (figure 4). ANOVA revealed significant differences in both the time to incapacitation (F = 5.49, d.f. = 4, p = 0.004) and death (F = 5.80, d.f. = 4, p = 0.003) between the five venoms. Post hoc tests revealed significant differences between B. arietans venom and that of E. c. sochureki (incapacitation p = 0.028 and death p = 0.022), E. coloratus (incapacitation p = 0.007 and death p = 0.024) and E. p. leakeyi (incapacitation p = 0.035 and death p = 0.002). There were, however, no significant differences in either the mean time to scorpion incapacitation or death between any of the four Echis venoms, or between E. ocellatus venom and B. arietans venom.

During feeding trials, successful feeding was observed on three occasions. Snakes delivered a rapid strike to the scorpion and then quickly released it. One or two subsequent bites were delivered to the scorpion in all trials. These were spaced at variable time intervals (3–40 min between bites), but never delivered in rapid succession. Feeding did not commence until shortly after the scorpion stopped moving completely. The mean time from the initial bite to commencement of feeding was 43.1 min (s.e. = 2.4 min; 3 trials; n = 2 snakes), which is comparable with the time to scorpion death following a biologically realistic E. c. sochureki venom dose (figure 4), supporting our assumption that the dose selected was representative of natural predatory behaviour.

4. DISCUSSION

The results provide strong evidence for the role of natural selection in shaping venom composition in the genus Echis. Electrophoresis of representative venoms verified the existence of considerable variation in venom protein composition between the four major Echis species groups (figure 2). Examination of stomach and hindgut contents also confirmed that significant variation occurs between species groups in the proportion of arthropod prey consumed. Measuring the effects of representative venoms to a naturalistic arthropod prey model reveals a strong association between venom toxicity (in terms of LD₅₀) and diet: venoms from the frequent arthropod-feeding E. carinatus group and E. pyramidium group are significantly more toxic to scorpions than venoms from the occasional arthropod-feeding E. ocellatus group, which in turn is significantly more toxic than the venoms from the vertebrate-feeding E. coloratus group and the more distantly related B. arietans (figure 3). Comparing scorpion LD₅₀ values with those reported for mice (E. p. leakeyi 0.7 μg g⁻¹, E. c. sochureki 1.9 μg g⁻¹, E. ocellatus 0.41 μg g⁻¹ and E. coloratus 1.25 μg g⁻¹—Theakston & Reid 1983) indicates that the relative lethality ranking of the Echis venoms is different for scorpions than for mice. This demonstrates that the increased scorpion toxicity associated with the venoms of arthropod-feeding Echis is prey specific, rather than simply the result of a venom that is generally more lethal. A similar association between diet and arthropod-specific venom toxicity is reported among the Eurasian vipers, Vipera (Pelias group), which also show marked interspecific variation in the amount of arthropod prey consumed (Starkov et al. 2007). Increased venom toxicity to natural prey species is also well known in vertebrate-feeding snakes (Jorge da Silva & Aird 2001; Pawlak et al. 2006; Mackessy et al. 2006).

Mapping the degree of arthropod feeding and venom lethality to scorpions onto the phylogeny of the major Echis species groups further reinforces the hypothesis of a coevolutionary relationship between venom and diet (figure 1). Both outgroup taxa are, like the majority of vipers (Mallow et al. 2003), vertebrate predators, suggesting that vertebrate feeding is the ancestral viper condition. The low scorpion toxicity of B. arietans venom

Figure 3. Scorpion LD₅₀ for Echis and B. arietans venoms. Species abbreviations are consistent with figure 2. Error bars show 95% confidence intervals. Pairwise statistical comparisons are shown by asterisks (* = p < 0.05; *** = p < 0.001; n.s., not significant) and superscripts (a: Z = 0.0, p = 1.0; b: Z = 3.48, p < 0.001; c: Z = 2.47, p = 0.014; d: Z = 2.02, p = 0.043). The pie charts above show the proportion of arthropods (black portions) and vertebrates (grey portions) consumed by each Echis species group.

Figure 4. Mean time to scorpion incapacitation (white bars) and death (grey bars) following a biologically realistic venom dose. Species abbreviations are consistent with figure 2. Error bars show standard error.
that the principal driver for increased arthropod-specific lethality may not be the need for rapid killing times, but selection for venom economy. These conclusions underscore the need for a broad, integrative approach to the study of the causes and correlates of the evolution of venom composition. In particular, the use of natural prey items is essential to illuminate the interaction between venom and diet, and this interaction may be considerably more complex than suggested by the reliance on standard LD50 tests in much of the literature. The genus Echis, with its extreme variation in diet composition, is shown to be a promising model for further studies on the basis and mechanisms for adaptive evolution of venom composition in snakes.

Procedures involving animals were approved by the Bangor University Animal Ethics Committee.

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