**Venom evolution in snakes is driven by trophic and macroecological factors.**

**Kevin Healy a, b, Chris Carbone c and Andrew L. Jackson a, b.**

a School of Natural Sciences, Trinity College Dublin, Dublin 2, Ireland. b Trinity Centre for Biodiversity Research, Trinity College Dublin, Dublin 2, Ireland. c Institute of Zoology, Zoological Society of London, London, UK

**Significance**

Snake venom is best known for its ability to kill prey, a property that both makes it of biomedical interest and a public health concern. However, snake venom also offers a novel opportunity to understanding the evolution of predator traits. As a predator trait venom can be quantified by measuring its potency and quantity allowing the importance of trophic drivers, such as predators-prey arms race dynamics, and macroecologcal drivers, such metabolic scaling and habitat dimensionality, on its evolution can be tested. Here, using comparative analysis we show that trophic drivers result in venom evolving higher potency towards species closely resembling their natural diet and macroecological drivers shape the quantity of venom available due to habitat dimensionality and metabolic constraints.

**Abstract**

Snake venom is well known for its ability to incapacitate prey. This property of snake venoms is the source of not only interest in its biomedical uses but also

results in the major it a source of both novel biomedical compounds and as a health concern. However, despite this snake venom the evolutionary drivers behind its ability to incapacitate prey is still poorly understood. Even the role of predation as a driver of venom evolution is under question due to the range of venom potency across species, ranging from the almost nonvenomous xxx to the inland taipan to incapacitate 100,000’s mice, suggesting venom is effectually under neutral selection. Whatever this drivers, venom, like all tissues, is subject to the forces of evolution, whether this is as selection to incapacitate prey, reduce the energetic costs associated with its production or through neutral drift over evolutionary time. Using a dataset of over 200 measures of Ld50 values for over 100 snake species we support the importance of predation in driving venom evolution by showing that venom is more affective in incapacitating prey species phylogenetically closer to those found in a species diet. Further to this we show that venom

We also show that venom evolution is driven by prey size and type an. Finally, we find that the scaling relationship between

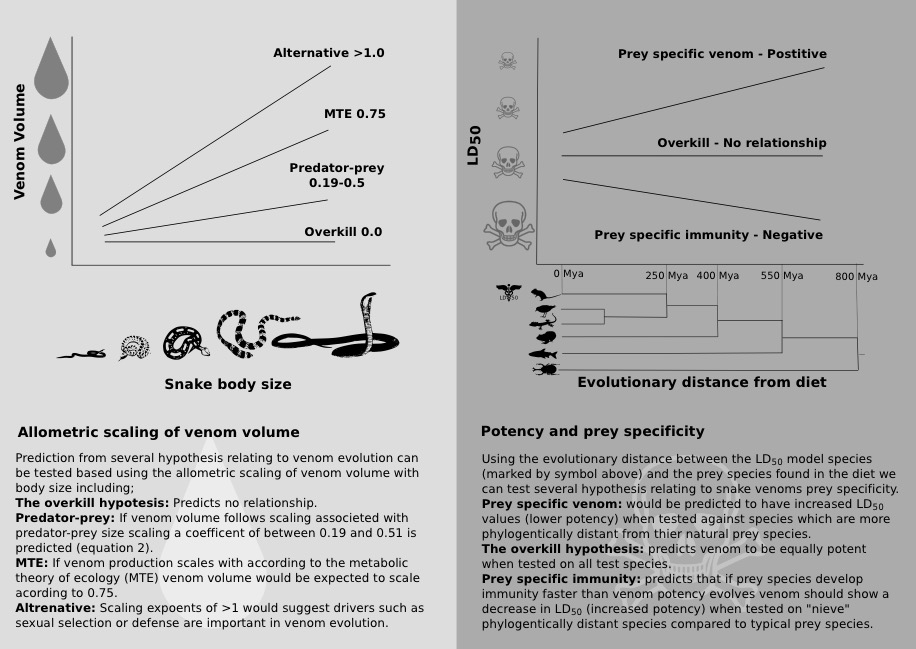
Keywords: Venom Body size, Comparative analysis, Scaling, trophic ecology, Macroecology, LD50, phylogenetic analysis

**Introduction**

Snake venom is perhaps best known for its ability to incapacitate biological systems, a property which has made it both a source of novel biomedical compounds ([1](#_ENREF_1)) and also a major health concern due to the estimated millions of human envenomation cases each year ([2](#_ENREF_2)). However, snake venom is also an ideal system of predatory trait evolution ([1](#_ENREF_1)), particularly as its predatory functionality, the ability to subjugate prey, can be quantified by measured venom potency and the quantity a species possess. Despite significant developments in our understanding of the evolutionary origin ([3-5](#_ENREF_3)) and the subsequent evolution of its complex compounds ([1](#_ENREF_1), [6](#_ENREF_6)), surprisingly little is known about the evolutionary pressures that drive this ability to incapacitate prey. For example, many species, such as the Gold-ringed cat snake (*Boiga dendrophila*), are incapable of subduing laboratory test models (ref) while other species, such as Russel’s viper (*Daboia russelii*), possess enough potent venom to incapacitate hundreds of thousands of potential prey items (ref). This lack of a clear pattern relating to the ability to subdue prey using venom suggests drivers assumed to be important in driving predator trait evolution, such as trophic factors, are less important than expected (ref). One potential explanation for this lack of a pattern is that weak selective pressures are caused by an “overkill” effect, were due the capacity of many species venoms to kill prey numbers far in excess of any biological requirement, such as in the case of Russel’s viper, neutral evolutionary process becoming the main driver of trait variation ([7](#_ENREF_7), [8](#_ENREF_8)). Such a scenario predicts that traits relating to venom, such as potency and quantity, would be idiosyncratic in nature and also raises the question of the general importance of ecological factors on the evolution of predatory traits. Here, we use a comparative approach that accounts for both snake phylogenetic history and the evolutionary naivety of species which venom potency is measured on to test whether snake venom traits follow idiosyncratic patterns expected from neutral selection or patterns predicted from macroevolutionary and trophic theory.

As predation plays a key role in both ecosystems dynamics ([9](#_ENREF_9)) and the evolution of the species within these ecosystems ([10](#_ENREF_10)) much attention has been given to understanding the general patterns related to these trophic interactions and the drivers behind them. These include macroecological patterns derived from body mass and habitat complexity which predict allometric scaling relationships for traits underlining trophic interactions such as search and encounter rates ([11-13](#_ENREF_11)); the ability to spot and track prey ([14](#_ENREF_14), [15](#_ENREF_15)); and ingestion rates ([13](#_ENREF_13), [16](#_ENREF_16)). Other general predictions for predatory traits stem from the co-evolution between both predators and their prey, were arms race dynamics may drive the evolution of predatory traits that increase the ability to capture prey and conversely the evolution of traits in prey species that increases their ability to escape ([17](#_ENREF_17), [18](#_ENREF_18)). While these two general drivers are likely to have important influences on trophic trait evolution they can be difficult to decouple as predatory traits are often multifaceted, for example claws can be used for predation, climbing, digging, defence etc., and difficult to compare across the large number of species required for macroecological studies (ref). Snake venom however, offers a primarily predatory system which can be quantitatively compared across a large group of diverse species allowing the importance of both trophic and macroecological factors on its evolution to be tested.

Predatory foraging traits are expected to be adapted towards their main target prey species. For example, jaw morphology in cichlid fish is strongly selected by factors such as prey type ([19](#_ENREF_19)). Similar selection pressures from prey type is also seen in snake venom. For example, a switch in diet from fish to eggs has resulted in the almost complete atrophy of the venom apparatus in the egg eating marble sea snake *Aipysurus eydouxii* (refs), demonstrating the importance of predation in venom evolution (ref). Further such trophic selection is also seen in cases of prey-specific venoms were potency is higher when tested on species commonly found within the snakes diet, such as observed in Malayan pitvipers ([20](#_ENREF_20)), coral snakes ([21](#_ENREF_21)), the viper genus *Echis* ([22](#_ENREF_22)), saw-scaled vipers ([23](#_ENREF_23)) and insect eating *Pelias* vipers ([24](#_ENREF_24)). However, such co-evolution is not apparent in all snake species, such as in tiger snakes where variation in venom across populations is independent of dietary differences ([25](#_ENREF_25)). Furthermore, prey species can also respond in such co-evolutionary arms races through the development of immunity. Evidence for this is seen in venoms that show weaker potencies in potential prey items such as in the cases of Opossums and Neotropical pitvipers ([26](#_ENREF_26)); eels and *Laticauda colubrine* ([27](#_ENREF_27)); and between ground squirrels and rattlesnakes ([28](#_ENREF_28), [29](#_ENREF_29)) . This idiosyncratic pattern of some species displaying prey-specific venom while others do not may mirror a pattern predicted from the Overkill hypotheses were neutral processes are the main driver of venom potency ([8](#_ENREF_8)). However, many measures of venom potency are not typically tested on natural prey species leading to the question of whether these examples represent an idiosyncratic pattern expected under weak selection or whether a general pattern will emerge under appropriate analysis (ref). While measures of potency using natural prey species are becoming more common, these are taxonomically restricted making large comparative analysis difficult ([21](#_ENREF_21), [22](#_ENREF_22)). Here we account for the species a venom is tested on and measure the phylogenetic distance of this species to the snake’s species natural diet which, in turn, allows us to test whether venom is generally adapted towards common target prey species. However, while venom potency is an important aspect of these species ability to incapacitate prey, the volume of venom and the role of other macro-ecological drivers are also likely to be important when considering venom evolution.



The ability to subdue prey is not only dependent on the potency of a species venom but also the amount of venom available. As such, venom volume is also expected to be under positive selection from potential trophic and macroecological factors. As venom production incurs a metabolic cost ([30](#_ENREF_30)) (although the level of this cost is debated ([31](#_ENREF_31))) and requires storage, the volume of venom a snake species can produce is likely to be heavily linked to one of the strongest determinants of trophic ecology; body size. In general, larger predators eat larger prey ([16](#_ENREF_16)). If venom is under selection for its ability to kill prey it would be expected that larger snake species would need to produce larger quantities of venom to keep pace with the subsequent increased in prey size. Such an increase of venom volume to compensate for larger prey is supported by the metering of venom in response to prey size seen in several species ([32](#_ENREF_32), [33](#_ENREF_33)). However, while bigger snakes are known to have larger amounts of venom in general ([34](#_ENREF_34)) little is known about the interspecific scaling of this trait. One prediction is that venom volume increases with snake body size with an exponent relating to their predator-prey body size scaling as described in equation 1;

Were the scaling exponent *a* is approximately 0.88 ([16](#_ENREF_16)). However, venom volume would not be expected to scale according to this exponent as the effects of toxicological agents also follows an allometric relationship ([35](#_ENREF_35)) were the amount of venom required (*V*) to induce the same incapacitating effect on a prey of mass (*Mprey*) would follow (equation 2);

were *b* is the scaling coefficient of venoms toxicological effects ([35](#_ENREF_35)). Hence to calculate the expected allometry of venom volume with snake body mass in a case were venom volume increases at a rate to match predator-prey size scaling after accounting for scaling of toxicological effects we substitute from equation 1 into equation 2 to get (equation 3);

If we take the commonly used value of 0.75 for the interspecific scaling of drug dosages ([35](#_ENREF_35)) for *b* and the value of 0.68 for *a* from the scaling predator-prey mass relationship for snakes ([16](#_ENREF_16)) we would expect a scaling exponent of approximately 0.51 between snake venom volume and snake mass. Other predictions relating to venom volume scaling include the overkill hypothesis which predicts no relationship between venom volume and prey size, or alternatively venom volume may scale according to constraints such as metabolic costs, were a scaling of 0.75 would be expected ([36](#_ENREF_36)). At the other extreme superlinear allometeries would suggests patterns associated with drivers such as sexual selection, such as proposed by the weapons hypothesis ([37](#_ENREF_37)), or defenses requiring increased effectiveness with size, such as seen in the allometry of horn growth in horned lizards ([38](#_ENREF_38)) (Figure 1).

Finally, an overlooked feature that may also drive both venom volume and toxicity evolution is habitat structure ([39](#_ENREF_39)). The structural complexity of a habitat, such as whether it's a 2-dimensional terrestrial surface or a complex 3-dimensional forest canopy, can influence both encounter rates ([12](#_ENREF_12), [16](#_ENREF_16)) and the escape rates of prey, with higher dimensional spaces increasing both ([40](#_ENREF_40), [41](#_ENREF_41)). Hence predators in high dimensional habitats with associated increased escape rates may compensate through larger volumes of more potent venom in order to increase capture rates. For example, strike and release behaviors may be less successful in either 3-dimensional arboreal or aquatic environments requiring higher toxicities to incapacitate prey quickly. Conversely there may be less of a requirement for high potencies and venom volumes due to increases in encounter rates, and hence feeding opportunities, in high dimensional habitats which may compensate for possible increases in escape rates.

Here we test the importance of these multiple potential drivers of both venom quantity and potency in a phylogenetically corrected comparative analysis of ninety-nine species of venomous snakes. We test the importance of snake body mass; habitat dimensionality; prey type; and prey size on the variation of both venom toxicity, as measured using the median lethal dose (LD50), and venom volume. We achieve this by using a novel metric of the evolutionary distance between the model animal on LD50 is measured and the typical species found in each snakes’ diet in order to test the general pattern of snake venom prey-specificity which predicts higher potencies when tested on species phylogenetically close to natural target prey. This approach also allows us to control for the variance associated with the LD50 model used and hence test the general influence of macroecological factors on factors such as venom volume.

Using these corrections and a series of models accounting for phylogenetic similarity between snake species we test; (1) the overkill hypothesis: that there is no relationship between venom volume or toxicity with prey size; (2) the importance of trophic drivers on venom evolution including; that venom potency is higher (lower LD50) when tested on model species phylogenetically closer to species found in the diet; and that snake species which include eggs in their diets have lower venom potencies (higher LD50) or volumes; (3) the importance of macorecological drivers on venom evolution including; that venom volume is higher in species with larger prey, with a predicted scaling of approximately 0.51 or that (4) venom volume scales according to 0.75 as expected if metabolic cost is the main driver; (5) the importance of habitat dimensionality on venom evolution, in particular that; species in high dimensional habitats show either higher or lower potencies or venom volumes depending on encounter and escape rates; and finally (6) the importance of other potential drivers such as superlinear scaling of venom volume or potency as expected with the weapons hypothesis or sexual selection drivers. We show that both trophic and macroecological factors are important in driving venom evolution with patterns supporting prey-specific venom in general and venom volume scaling as predicted by metabolic cost constraints. We also find that much of venom evolution remains to explained, suggesting other mechanisms such as neutral evolution still play a significant role in its evolution.

**Methods**

**Data**

We collected data on venom volume and toxicity from the literature, along with our predicted drivers. We used mean dry weight (mg) as a measure of venom volume as it represents the amount of active ingredients available and is the most available reported measure. As a measure of venom lethality we used median lethal dose (LD50). We only included intravenous (IV), subcutaneous (SC), Intraperitoneal(IP) or intramuscular routes (IM) of injecting the venom as other routes were too uncommon to include within the analysis. We include LD50 values measured on all animal models as we were interested in including variation relating to the potential prey specific nature of venom. While other measures of venom toxicities are available, only measures of LD50 were numerous enough to carry out a large scale comparative analysis.

To test whether venom is prey specific we calculated the phylogenetic distance between the model animal species used to measure LD50 for each snake species and the species naturally present in its diet. We calculated this as the sum of the phylogenetic distance, using mean estimates from TimeTree ([42](#_ENREF_42)), between each prey group and the LD50 model multiplied by the proportion of each prey group reported in each snake species diet. For example, a species with a diet comprising of 20% mammals, 50% fish and 30% reptiles with a LD50 measured using mice would have a diet with an average phylogenetic distance of 0.2(0) + 0.5(400.1) + 0.3(296) = 288.85 million years from the common ancestor of the LD50 model.

Diet data was collated from the literature using studies with quantitative estimates of prey proportions, mainly from studies of stomach contents. As prey items were rarely identified to lower taxonomic levels diet was categorized into six prey categories; invertebrates, fish, amphibians, lizards, birds and mammals.

Species habitat was categorized as either terrestrial, fossorial, aquatic or arboreal based on accounts in the literature. In order to directly test the expected effect of the dimensionality of habitat environment each environment was scored, as in Pawar et al (2012)need ref, with terrestrial and fossorial environments scored as two-dimensional and arboreal and aquatic scored as three-dimensional. As some venomous species also engage in constriction behavior we collected data on any observation of constriction behavior in capturing prey from the literature ([43](#_ENREF_43)).

For snake body size we used total length values from the literature ([44-50](#_ENREF_44)) and field guides ([46-48](#_ENREF_46), [51-56](#_ENREF_51)) as these were the most common measures available. All lengths were then converted to mass using family-level allometric scaling ([57](#_ENREF_57)). Prey size data was included from dietary studies when available. When prey size was not reported in the dietary studies and were prey species were identified to the species level, we used mean prey species body mass from available databases ([44](#_ENREF_44), [57](#_ENREF_57), [58](#_ENREF_58)). In cases were only body lengths were available for prey species allometric scaling were used to convert to mass ([44](#_ENREF_44), [59](#_ENREF_59)). For species that were only identified to the genus level the genus mean body mass was used if possible. The estimate mean prey size for each snake species was then calculated using a weighted mean based on the proportion each prey species/genus or group within the diet.

Snake mass, prey mass, LD50, venom volume and phylogenetic distance between diet and model were all log10 transformed, mean centered and expressed in units of standard deviation prior to analysis. Significance was determined for the fixed effects when 95% of the data is greater or less than 0. The phylogeny from Pyron RA & Burbrink ([60](#_ENREF_60)) was included in all analyses to account for similarities in traits due to common descent.

**Analysis**

To test our hypotheses we fit Bayesian multivariate phylogenetic mixed models using the MCMCglmm package ([61](#_ENREF_61)) in R v 3.2.4 ([62](#_ENREF_62)). As venom volume and LD50 are likely to have co-evolved, both were included as correlated (??) multivariate normal response variables in all models. Phylogeny was controlled by including it using the animal term in the MCMCglmm model while variation due to multiple measures on individual species was included using a separate random term. For the main model snake body mass; LD50 inoculation method (SC, IM, IV, IP); habitat dimensionality (2D, 3D); the presence of eggs in the diet (absent, present); and the phylogenetic distance of diet species to LD50 model were included as explanatory variables to give the analysis;

1. Volume + LD50 = *f*(Snake mass + LD50 method + Presence of eggs in diet + Phylogenetic distance between diet and model species + Habitat dimensionality) (275 observations over 99 species).

A similar model was also fit including prey size as an explanatory model which resulted in a smaller dataset of 177 observations across 68 species. To estimate the direct scaling exponents relating to prey mass, venom volume and predator mass as referred to in equations (1 and 3) we also ran the following model;

1. Volume = *f*(Prey mass)
2. Prey mass = *f*(Predator mass)

Finally, we also fitted a final set of sensitivity analysis including the main model with constriction behavior included as a categorical factor (absent, present) and a model with habitat type included instead of habitat dimension with the levels of terrestrial; aquatic and arboreal.

All models were fitted with parameter expanded priors (Hedfield 2010) with standard non-informative priors also tested separately to ensure that choice of prior had no affect on model results. A burn-in, thinning and number of iterations was determined for each model separately to ensure effective sample sizes exceeded 1000 for all parameter estimates. We tested for convergence using the Gelman-Rubin statistic over three separate chains ([63](#_ENREF_63)).

**Results**

**Predator-prey coevolution**

Only eight species in the dataset were found to include eggs within their diet with eggs consisting more than 10% in only four of these species. Despite these small sample sizes LD50 was found to be significantly higher in species with eggs in their diet in both the main and constriction models, while venom volume showed a non-significant negative association with the presence of eggs in a species diet in all models (Table 2; Tables A2-4).

Of the species included within the analysis only 14 species have a diet completely matching that of the LD50 model, i.e. the LD50 of a species with a diet including 100% mammals tested using a mouse model. Most species in the dataset have a diet with a least some component not matching the LD50 model as reflected by a median evolutionary distance of 211.3 million years between the diet and the LD50 species. In all models species LD50 increased with mean phylogenetic distance between the diet and the LD50 model indicating species with diets phylogentically close to the LD50 model have higher toxicities (table 2; tableA2-3; Figure 1B). From the main model, after back transforming the mean centred log10 value, LD50 was found to increase by 1.44 for every 100 million years between the species in the diet and that of the LD50 model. This was after correcting for LD50 injection route were intravenous and Intraperitoneal routes were found to have lower LD50 values in comparison to a subcutaneous route (table 2; tableA2-3).

Figure%202%2018%20may.pdf

Figure 1. (A) Relationship between log10 mass (g) against log10 venom volume (mg). Red points and fitted line (intercept = -0.58, slope = 0.0016) represent species in 2D habitats and the blue points and fitted line (intercept = -1.14, slope = 0.75) represent species in 3D habitats. Hollow points represent silhouette species which are from left to right *Atractaspis bibronii*; *Emydocephalus annulatus*; *Naja\_melanoleuca*; *Agkistrodon piscivorus*; *Ophiophagus hannah*. (B) Mean phylogenetic distance between diet species and LD50 model (Myr) and log10 LD50 against log10 venom volume (mg) (intercept = -0.58, slope = 0.75). Hollow points represent silhouette species which are from left to right; *Bungarus multicinctus*; *Oxyuranus microlepidotus*; *Echis carinatus*; *Causus rhombeatus*.

Table 2. Estimates and higher and lower 95% credibility intervals (CI) for LD50 and mean volume. Fixed factors include mass; LD50 method (subcutaneous (SC), intravenous (IV), intrapulmonary (IP) and intramuscular (IM)); habitat dimensionality (Dim- 2D and 3D); Presence of eggs in diet (Eggs in Diet) and the mean phylogenetic distance between diet species and the LD50 model (Diet-LD50 Dist). The random terms and the co-variance (CV) between LD50 and volume are also presented. The model was run with 12,000,000 iterations with a 2,000,000 burn-in and a thinning of 5000.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **LD50** | | | | | **Mean Volume** | | | |
|  | Estimate | | | Lower CI | Upper CI |  | Estimate | Lower CI | Upper CI |
| **Fixed Terms** | |  | |  |  |  |  |  |  |
| Intercept | | 0.200 | | -0.161 | 0.567 |  | 0.200 | -0.161 | 0.567 |
| Mass | | **0.134** | | **0.016** | **0.262** |  | **0.510** | **0.442** | **0.564** |
| LD50 methodSC | |  | |  |  |  |  |  |  |
| *IV* | | **-0.624** | | **-0.842** | **-0.435** |  | -0.011 | -0.052 | 0.030 |
| *IP* | | **-0.537** | | **-0.746** | **-0.309** |  | -0.010 | -0.049 | 0.033 |
| *IM* | | -0.228 | | -0.455 | 0.049 |  | -0.009 | -0.056 | 0.042 |
| Dim2D | |  | |  |  |  |  |  |  |
| *3D* | | -0.202 | | -0.670 | 0.243 |  | **-0.829** | **-1.286** | **-0.396** |
| Eggs in Diet | | 0.448 | | -0.162 | 0.021 |  | **-0.741** | **-1.325** | **-0.206** |
| Diet-LD50 Dist | | **0.360** | | **0.248** | **0.463** |  | -0.003 | -0.029 | 0.019 |
|  | |  | |  |  |  |  |  |  |
| **Random Terms** | | |  |  |  |  |  |  |  |
| Phylogeny | | 0.909 | | 0.479 | 1.452 |  | 0.456 | 0.145 | 0.847 |
| Phylogeny CV | | -0.003 | | -0.290 | 0.301 |  | -0.003 | -0.290 | 0.301 |
| Species | | 0.055 | | 0.001 | 0.170 |  | 0.308 | 0.156 | 0.462 |
| Species CV | | 0.030 | | -0.040 | 0.118 |  | 0.030 | -0.040 | 0.118 |
| Residuals | | 0.268 | | 0.215 | 0.328 |  | 0.009 | 0.007 | 0.011 |
| Residuals CV | | 0.003 | | -0.004 | 0.011 |  | 0.003 | -0.004 | 0.011 |

**Macroecological drivers; body size and habitat dimensionality**

Body size, prey size and habitat dimensionality were correlated with venom volume. The mean volume of venom, as measured using dried weight, ranged from 0.15 mg in the egg-eating sea snake (*Emydocephalus annulatus*) to 571 mg in the forest cobra (*Naja melanoleuca*). The main correlate with venom volume was body size with a log-log scaling of between 0.74 and 0.76 across all models after back transforming (Table 2, A2-4; Figure 1). Venom volume also showed a positive increase with prey body, with a log10-log10 increase of 0.139, however only 90% of the posterior samples are above the zero threshold (Table A2). In the model which only included venom volume and prey mass, we found a significant exponent of 0.37 (Table A5) and similarly in the model of predator prey body mass scaling we found an exponent of 0.52 (Table A6). Whether using the scaling exponent of 0.51 as calculated in equation 2 or using an exponent of 0.19 as calculated using a value of 0.37 found here between venom volume and prey size for *b* in equation 2 and 0.52 found here between prey and predator scaling for a, both values are far lower than the exponent of 0.75 found seen across each model (Table 2, A2-4; Figure 1).

The next most significant driver of venom volume was the dimensionality of the habitat with the 27 species in high dimensional environments (arboreal = 9, aquatic = 18) showing lower venom volumes in comparison to species in lower dimensional habitats (Table 2). A sensitivity analysis were habitat was include as terrestrial, arboreal and aquatic also showed similar significant reductions in both arboreal and aquatic habitats (Table A4).

In all models there is was a high phylogenetic signal for both LD50 and venom volume with a higher signal for LD50 in all models (Table 2, A2-4).

**Discussion**

Predator traits are likely to be heavily shaped by both predator-prey evolutionary dynamics and macroecological forces imposed by the limitations of body size and habitat structure. Here we show that, as predicted, venom potency is prey specific, with higher potencies towards species naturally found in a snakes diet, and venom volume is related to both snakes and prey body size and the dimensionality of the environment. These results show that, contrary to some claims ([8](#_ENREF_8)), snake venom evolution is influenced by not only predator-prey evolutionary dynamics but also macroecological factors. However, while our results heavily support the importance of both these drivers in the evolution of predator traits, the predator-prey body size scaling relationships found here suggests that venom evolution is under different constraints in comparison to more general predator-prey scaling patterns.

The generality of prey specific venom in snakes has attracted much commentary mainly in the form of the overkill hypothesis ([8](#_ENREF_8)). As supported by the reduced potency of venom in species with eggs in their diet as found here, the presence of venom in snakes is almost certainly linked with its predatory function. Whether selection relating to predation similarly extends to venom potency in general has been less clear. Our results show that predation is an important driver of venom potency, with venom typically more potent when tested against species closer to that typically found in a snakes diet. Much of the difficulty to date in identifying the general prey specific nature of snake venom has stemmed from the use of lab species, such as mice, to test potency which may not reflect the predators natural prey ([21](#_ENREF_21)). Here by flipping the usual inconvenience towards comparative analysis due to the use of none natural prey species we show that a snakes venom potency is lower for test species phylogenetically more distant from the snakes natural prey.

This effect of reduced potency with phylogenetic distance from natural diet is likely to underestimate the prey specific nature of venom in general due to our use of LD50. While LD50 measures the ability of a venom to kill prey, it is more likely that venom is under selection to simpley subdue prey in order to reduce the chances of prey escaping or retaliating. Even though killing prey does achieve this, other measures of venoms ability to subdue prey, such as the speed at which a venom affects prey, may be a clearer reflection of the aspects of venom which selection acts on ([22](#_ENREF_22)). In any case, despite the use of the courser but more available LD50 measure, we find a clear pattern of prey specificity. Hence, while a number of studies have suggested that prey specific venom is at least common among snake species ([21](#_ENREF_21), [22](#_ENREF_22), [24](#_ENREF_24), [64-66](#_ENREF_64)), these results confirm that prey specific venom is a general pattern in snakes with groups showing less specific venom ([25-29](#_ENREF_25)) more likely to be the exception.

While predator-prey dynamics has received the most attention with regards to predator trait evolution the importance of macroecological factors, namely body size and habitat complexity, are also likely to be important drivers of these traits. Our results support their importance with venom volume in particular showing relationships to both snake body size and habitat dimensionality. Unsurprisingly, we found that larger snakes have larger quantities of venom. However while snakes with larger prey items in their diets show trends of increased venom volumes as expected, the general increase in venom volume did not follow increases expected from a predator-prey scaling perspective ([16](#_ENREF_16)), with venom volume increasing faster with prey body size than expected. Even considering potential variation in the allometric scaling of venom dosage ([35](#_ENREF_35)), an exponent far in excess of 1 is required for the relationship between required dosage and prey size (equation 2) in order to match the scaling of 0.75 between venom volume and snake mass found here. Furthermore, the scaling exponent relating predator and prey size was found to be far lower in venomous snakes in comparison to snakes as a whole group ([16](#_ENREF_16)), suggesting that venomous species are more generally feed on smaller prey than expected. In the absence of the expected predator-prey scaling a more likely explanation for the scaling of 0.75 between venom volume and snake body size is that it follows limitations relating to the scaling of metabolic rate ([67](#_ENREF_67)), which increases with a scaling coefficient of 0.75 with respect to body mass ([36](#_ENREF_36), [67](#_ENREF_67)), it might be expected if the production of a potentially metabolically costly material such as venom ([30](#_ENREF_30)), were maintained at a constant proportion of overall energy budgets across snake species. The scaling coefficient found we find hence suggests that metabolic constraints may potentially be a more important driver of venom volume than predator-prey size relationships.

Another potential macroecological factor shaping the available volume of venom to a species is habitat dimensionality. While we expected that species in high dimensional habitats may have higher venom volumes to compensate for higher escape rates of prey ([39](#_ENREF_39)) we found that, counter to our expectation, these species had lower venom volumes in comparison species in low dimensional habitats (terrestrial and fossorial). This may be associated with differences in prey capturing behaviour as it might be expected that high dimensional environments require more holding behaviours during attacks in order to prevent the loss of prey, hence allowing a more accurate delivery of venom. However, the presence of constriction in venomous snakes ([43](#_ENREF_43)), the most extreme form of prey holding behaviours, is present in both arboreal and terrestrial species and was found to have no effect when included within the analysis. Furthermore bite and release behaviours are known in arboreal species such as the black mamba (*Dendroaspis polylepis*) suggesting this behaviour is not fully restricted to low dimensional environments ([51](#_ENREF_51)). Another potential explanation is that higher encounter rates in high dimensional environments ([12](#_ENREF_12)) may reduce the missed opportunity of feeding cost associated with replenishing venom. Rates of replenishing venom can be substantial with estimates of replenishment rate ranging from 3-7 days ([68](#_ENREF_68)) to 30-50 days ([33](#_ENREF_33), [69-71](#_ENREF_69)). These long periods of replenishment may hence select for larger reserves in species where encounter rates with prey are lower in order to minimise potential missed opportunity costs. While further research on the role of habitat dimensionality will allow more detailed understanding of the mechanisms driving this trend our results highlight that venom may not only be selected according to metabolic constraints but that factors relating to encounter rate may also have important influences.

Our analysis shows that both predator-prey dynamics and macroecological factors shape the evolution of venom in snakes. Through establishing these patterns we reject the strongest form of the overkill hypothesise which posits that positive selection plays a minor role in shaping venom evolution. However, while the above factors play an important role in the evolution of venom, much of the variation relating to the ability to incapacitate prey is still unexplained. Outside selection pressures related to ecology and physiology other evolutionary mechanisms, such as gene duplication events, play an important role in venom evolution ([66](#_ENREF_66)). In fact, such mechanisms are likely to be reflected in the high phylogenetic signal found for both venom volume and potency, were rare duplication and mutation events result in closely related species showing similar trait values ([72](#_ENREF_72)). While future research will continue to unravel the huge variation associated with snakes ability to kill prey, our analysis shows that both predatory-prey dynamics and macroecological factors are important drivers in the evolution of venom. These results show that by testing general drivers of predatory traits we can further understand the evolution of not only unusual physiologies, such as the venomous system, but also through understanding the evolution of predator traits in general, trophic ecology as a whole.

**References**

1. Casewell NR, Wüster W, Vonk FJ, Harrison RA, & Fry BG (2013) Complex cocktails: the evolutionary novelty of venoms. *Trends in ecology & evolution* 28(4):219-229.

2. Kasturiratne A*, et al.* (2008) The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. *PLoS Med* 5(11):e218.

3. Fry BG*, et al.* (2006) Early evolution of the venom system in lizards and snakes. *Nature* 439(7076):584-588.

4. Fry B*, et al.* (2015) The origin and evolution of the Toxicofera reptile venom system. *Venomous Reptiles and Their Toxins: Evolution, Pathophysiology and Biodiscovery*:1.

5. Reyes-Velasco J*, et al.* (2015) Expression of venom gene homologs in diverse python tissues suggests a new model for the evolution of snake venom. *Molecular biology and evolution* 32(1):173-183.

6. Dowell NL*, et al.* (2016) The deep origin and recent loss of venom toxin genes in rattlesnakes. *Current Biology* 26(18):2434-2445.

7. Mebs D (2001) Toxicity in animals. Trends in evolution? *Toxicon* 39(1):87-96.

8. Sasa M (1999) Diet and snake venom evolution: can local selection alone explain intraspecific venom variation? *TOXICON-OXFORD-* 37:249-252.

9. Pace ML, Cole JJ, Carpenter SR, & Kitchell JF (1999) Trophic cascades revealed in diverse ecosystems. *Trends in ecology & evolution* 14(12):483-488.

10. Palkovacs EP & Post DM (2009) Experimental evidence that phenotypic divergence in predators drives community divergence in prey. *Ecology* 90(2):300-305.

11. Domenici P (2001) The scaling of locomotor performance in predator–prey encounters: from fish to killer whales. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 131(1):169-182.

12. Pawar S, Dell AI, & Savage VM (2012) Dimensionality of consumer search space drives trophic interaction strengths. *Nature* 486(7404):485-489.

13. Kane A*, et al.* (2016) Body Size as a Driver of Scavenging in Theropod Dinosaurs. *The American Naturalist* 187(6):706-716.

14. Healy K, McNally L, Ruxton GD, Cooper N, & Jackson AL (2013) Metabolic rate and body size are linked with perception of temporal information. *Animal behaviour* 86(4):685-696.

15. Kiltie R (2000) Scaling of visual acuity with body size in mammals and birds. *Functional Ecology* 14(2):226-234.

16. Carbone C, Codron D, Scofield C, Clauss M, & Bielby J (2014) Geometric factors influencing the diet of vertebrate predators in marine and terrestrial environments. *Ecology letters* 17(12):1553-1559.

17. Dawkins R & Krebs JR (1979) Arms races between and within species. *Proceedings of the Royal Society of London B: Biological Sciences* 205(1161):489-511.

18. Van Valen L (1973) A new evolutionary law. *Evolutionary theory* 1:1-30.

19. Albertson RC, Markert J, Danley P, & Kocher T (1999) Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. *Proceedings of the National Academy of Sciences* 96(9):5107-5110.

20. Daltry JC, Wuester W, & Thorpe RS (1996) Diet and snake venom evolution. *Nature* 379(6565):537-540.

21. da Silva NJ & Aird SD (2001) Prey specificity, comparative lethality and compositional differences of coral snake venoms. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 128(3):425-456.

22. Barlow A, Pook CE, Harrison RA, & Wüster W (2009) Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. *Proceedings of the Royal Society of London B: Biological Sciences* 276(1666):2443-2449.

23. Richards D, Barlow A, & Wüster W (2012) Venom lethality and diet: differential responses of natural prey and model organisms to the venom of the saw-scaled vipers (Echis). *Toxicon* 59(1):110-116.

24. Starkov VG, Osipov AV, & Utkin YN (2007) Toxicity of venoms from vipers of Pelias group to crickets Gryllus assimilis and its relation to snake entomophagy. *Toxicon* 49(7):995-1001.

25. Williams V, White J, Schwaner T, & Sparrow A (1988) Variation in venom proteins from isolated populations of tiger snakes (Notechis ater niger, N. scutatus) in South Australia. *Toxicon* 26(11):1067-1075.

26. Voss RS (2013) Opossums (Mammalia: Didelphidae) in the diets of Neotropical pitvipers (Serpentes: Crotalinae): Evidence for alternative coevolutionary outcomes? *Toxicon* 66:1-6.

27. Heatwole H & Poran NS (1995) Resistances of sympatric and allopatric eels to sea snake venoms. *Copeia*:136-147.

28. Biardi JE & Coss RG (2011) Rock squirrel (Spermophilus variegatus) blood sera affects proteolytic and hemolytic activities of rattlesnake venoms. *Toxicon* 57(2):323-331.

29. Poran NS, Coss RG, & Benjamini E (1987) Resistance of California ground squirrels (Spermophilus beecheyi) to the venom of the northern Pacific rattlesnake (Crotalus viridis oreganus): a study of adaptive variation. *Toxicon* 25(7):767-777.

30. McCue MD & Mason R (2006) Cost of producing venom in three North American pitviper species. *Copeia* 2006(4):818-825.

31. Pintor AF, Krockenberger AK, & Seymour JE (2010) Costs of venom production in the common death adder (Acanthophis antarcticus). *Toxicon* 56(6):1035-1042.

32. Hayes WK (1995) Venom metering by juvenile prairie rattlesnakes, Crotalus v. viridis: effects of prey size and experience. *Animal Behaviour* 50(1):33-40.

33. Hayes WK, Herbert SS, Rehling GC, & Gennaro JF (2002) Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts. *Biology of the Vipers*:207-233.

34. Chippaux J-P, Williams V, & White J (1991) Snake venom variability: methods of study, results and interpretation. *Toxicon* 29(11):1279-1303.

35. Nestorov I (2003) Whole body pharmacokinetic models. *Clinical pharmacokinetics* 42(10):883-908.

36. Isaac NJ & Carbone C (2010) Why are metabolic scaling exponents so controversial? Quantifying variance and testing hypotheses. *Ecology Letters* 13(6):728-735.

37. Kodric-Brown A, Sibly RM, & Brown JH (2006) The allometry of ornaments and weapons. *Proceedings of the National Academy of Sciences* 103(23):8733-8738.

38. Bergmann PJ & Berk CP (2012) The evolution of positive allometry of weaponry in horned lizards (Phrynosoma). *Evolutionary Biology* 39(3):311-323.

39. Arbuckle K (2015) Evolutionary Context of Venom in Animals.

40. Heithaus MR, Wirsing AJ, Burkholder D, Thomson J, & Dill LM (2009) Towards a predictive framework for predator risk effects: the interaction of landscape features and prey escape tactics. *Journal of Animal Ecology* 78(3):556-562.

41. Møller A (2010) Up, up, and away: relative importance of horizontal and vertical escape from predators for survival and senescence. *Journal of evolutionary biology* 23(8):1689-1698.

42. Hedges SB, Dudley J, & Kumar S (2006) TimeTree: a public knowledge-base of divergence times among organisms. *Bioinformatics* 22(23):2971-2972.

43. Shine R & Schwaner T (1985) Prey constriction by venomous snakes: a review, and new data on Australian species. *Copeia* 1985(4):1067-1071.

44. Feldman A & Meiri S (2013) Length–mass allometry in snakes. *Biological Journal of the Linnean Society* 108(1):161-172.

45. de Queiroz A & Rodríguez‐Robles JA (2006) Historical contingency and animal diets: the origins of egg eating in snakes. *The American Naturalist* 167(5):684-694.

46. Júnior RM*, et al.* (1997) Snake bites by the jararacuçu (Bothrops jararacussu): clinicopathological studies of 29 proven cases in São Paulo State, Brazil. *QJM* 90(5):323-334.

47. Lira-da-Silva RM (2009) Bothrops leucurus Wagler, 1824 (Serpentes; Viperidae): natural history, venom and envenomation. *Gazeta Médica da Bahia* 79(1).

48. Leviton AE*, et al.* (2003) The Dangerously Venomous Snakes of Myanmar. *Proceedings of the California Academy of Sciences* 54(24).

49. Boback SM, Guyer C, & Wiens J (2003) Empirical evidence for an optimal body size in snakes. *Evolution* 57(2):345-351.

50. Shine R, Harlow PS, Branch WR, & Webb JK (1996) Life on the lowest branch: sexual dimorphism, diet, and reproductive biology of an African twig snake, Thelotornis capensis (Serpentes, Colubridae). *Copeia*:290-299.

51. Branch WR (1998) *Field guide to snakes and other reptiles of southern Africa* (Struik).

52. O'Shea M (2008) *Venomous snakes of the world* (New Holland Publishers).

53. Navy U (1991) Poisonous snakes of the world. *US Govt New York: Dover Publications Inc*:203-206.

54. Spawls S, Branch B, & Branch WR (1995) *The dangerous snakes of Africa: natural history, species directory, venoms, and snakebite* (Ralph Curtis Pub).

55. Ernst CH & Ernst EM (2003) *Snakes of the United States and Canada* (Smithsonian Books).

56. Campbell JAL (2004) *The venomous reptiles of the western hemisphere*.

57. Meiri S (2010) Length–weight allometries in lizards. *Journal of Zoology* 281(3):218-226.

58. Myhrvold NP*, et al.* (2015) An amniote life‐history database to perform comparative analyses with birds, mammals, and reptiles. *Ecology* 96(11):3109-3109.

59. Pough FH (1980) The advantages of ectothermy for tetrapods. *American Naturalist*:92-112.

60. Pyron RA & Burbrink FT (2014) Early origin of viviparity and multiple reversions to oviparity in squamate reptiles. *Ecology Letters* 17(1):13-21.

61. Hadfield JD (2010) MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. *Journal of Statistical Software* 33(2):1-22.

62. Team RC (2016) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.

63. Brooks SP & Gelman A (1998) General methods for monitoring convergence of iterative simulations. *Journal of computational and graphical statistics* 7(4):434-455.

64. Mackessy SP, Sixberry NM, Heyborne WH, & Fritts T (2006) Venom of the Brown Treesnake, Boiga irregularis: ontogenetic shifts and taxa-specific toxicity. *Toxicon* 47(5):537-548.

65. Pawlak J*, et al.* (2006) Denmotoxin, a three-finger toxin from the colubrid snake Boiga dendrophila (Mangrove Catsnake) with bird-specific activity. *Journal of Biological Chemistry* 281(39):29030-29041.

66. Vonk FJ*, et al.* (2013) The king cobra genome reveals dynamic gene evolution and adaptation in the snake venom system. *Proceedings of the National Academy of Sciences* 110(51):20651-20656.

67. Brown JH, Gillooly JF, Allen AP, Savage VM, & West GB (2004) Toward a metabolic theory of ecology. *Ecology* 85(7):1771-1789.

68. Currier RB*, et al.* (2012) Unusual stability of messenger RNA in snake venom reveals gene expression dynamics of venom replenishment. *PloS one* 7(8):e41888.

69. Rotenberg D, Bamberger E, & Kochva E (1971) Studies on ribonucleic acid synthesis in the venom glands of Vipera palaestinae (Ophidia, Reptilia). *Biochemical Journal* 121(4):609-612.

70. Hayes W (2008) The snake venom-metering controversy: levels of analysis, assumptions, and evidence. *The biology of rattlesnakes*:191-220.

71. Young BA (2008) Perspectives on the regulation of venom expulsion in snakes. *The Biology of the Rattlesnakes.*, ed Hayes WK, Beaman, K.R., Caldwell, M.D., Bush, S.P. (Loma Linda University Press, California), pp 79-90.

72. Harvey PH & Pagel MD (1991) *The comparative method in evolutionary biology* (Oxford university press Oxford).