Snake venom potency and yield are associated with prey-evolution, predator metabolism and habitat structure

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

[a] Department of Zoology, School of Natural Sciences, Trinity College Dublin, Dublin 2, Ireland. [b] School of Biology, University of St Andrews, St Andrews KY16 9TH, UK. [c] School of Natural Sciences, National University of Ireland Galway, Galway, Republic of Ireland. [d] Institute of Zoology, Zoological Society of London, London, UK

* Corresponding author: healyke@tcd.ie, School of Biology, University of St Andrews, St Andrews KY16 9TH, UK.

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Abstract

Snake venom is well known for its ability to incapacitate and kill prey. Yet, potency and the amount of venom available varies greatly across species, ranging from the seemingly harmless to those capable of killing vast numbers of potential prey. This variation is poorly understood, with comparative approaches confounded by the use of atypical prey species as models to measure venom potency. Here, we account for such confounding issues by incorporating the phylogenetic similarity between a snake's diet and the species used to measure its potency. In a comparative analysis of 102 species we show that snake venom potency is generally prey-specific. We also show that venom yields are lower in species occupying three dimensional environments and increases with body size corresponding to metabolic rate, but faster than predicted from increases in prey size. These results underline the importance of physiological and environmental factors in the evolution of predator traits.

Introduction

The ability of snake venom to incapacitate and disrupt the physiological systems of animals is one of its most defining features, with some species possessing enough venom to incapacitate tens of thousands of laboratory test animals (Figure 1a). From a human perspective this property of venom makes it both a source of novel biomedical compounds (Casewell *et al.* 2013) and a major health concern, with snake bites estimated to cause up to 94,000 deaths annually (Kasturiratne *et al.* 2008). Yet not all venomous snake species possess such lethal amounts of venom towards test animals (Chippaux *et al.* 1991; Weinstein *et al.* 2011), with the ability to subjugate potential prey ranging from the practically harmless egg-eating sea snake (*Emydocephalus annulatus*) to extremely potent species such as many-banded krait (*Bungarus multicinctus*) (Figure 1). While understanding this variation is important from both a medical (Kasturiratne *et al.* 2008) and evolutionary viewpoint (Casewell *et al.* 2013), much is still unknown regarding its ecological and evolutionary drivers.

Variation in traits associated with predation are expected to be closely linked to aspects of trophic ecology. This includes factors relating to encounter (Domenici 2001; Pawar *et al.* 2012; Kane *et al.* 2016); capture and ingestion rates (Kiltie 2000; Carbone *et al.* 2014); along with characteristics of the prey itself . Despite the central role of venom in predation for many snake species (Casewell *et al.* 2013), the role of ecological and evolutionary drivers of venom variation is yet to be tested in a large comparative framework. The lack of such a comparative framework has made it particularly difficult to resolve fundamental questions regarding venom evolution, such as whether the evolution of increased potency against frequently encountered prey is a general rule (Sasa 1999; Wüster *et al.* 1999; Mebs 2001).

One reason for the lack of such large-scale comparative analyses is the difficulty in conducting multi-species comparisons of venom across taxonomically diverse groups. This stems from the non-standardized choice of model species typically used to test venom potency, or using species which are not the natural diet for the snake (da Silva & Aird 2001). This can lead to the confounding case where potency measures are heavily influenced by how similar a snake's diet is to the potency test species. Here we incorporate the evolutionary distance between a snake's diet and the model species used to measure its potency in order to allow comparisons across the taxonomic diversity of venomous snakes. Using this framework, we test a series of hypotheses relating to the drivers of potency and venom yield in snakes (Figure 2).

Predator-prey arms race dynamics predicts the selection on venoms to be prey-specific, and conversely the evolution of venom tolerances in prey (Van Valen 1973). The alternative overkill hypothesis posits that once the level of lethality in a venom greatly exceeds typical feeding requirements, predator-prey dynamics play a minor role in the evolution of venom potency due to weak selection (Sasa 1999; Wüster *et al.* 1999; Mebs 2001). Evidence for both cases have been found, with prey-specificity previously demonstrated in several genera (Daltry *et al.* 1996; da Silva & Aird 2001; Mackessy *et al.* 2006; Starkov *et al.* 2007; Barlow *et al.* 2009; Richards *et al.* 2012; Vonk *et al.* 2013; Margres *et al.* 2017), while other examples have either found no such prey-specificity (Williams *et al.* 1988) or cases were the prey species have evolved tolerance towards their predators venoms (Heatwole & Poran 1995; Biardi *et al.* 2000; Voss 2013; Pomento *et al.* 2016; Arbuckle *et al.* 2017). However,

whether these cases are taxon specific or are the general rule across all venomous snakes has yet to be tested at a broad taxonomic scale. Similarly, while the amount of venom a species possess may influence its ability to capture prey (Morgenstern & King 2013), the role of snake and prey body size and foraging conditions has on venom yield is also poorly understood at macroecological scales.

Under the prey-specific hypothesis of venom evolution, potency is expected to be higher when measured on model species that more closely resemble the predator's diet. As closely related species are more likely to share physiological similarities compared to more distantly related species, the prey-specific hypothesis predicts a decrease in potency with increasing evolutionary distance between the snake's diet and the species used to measure its potency (Figure 2). In contrast, the overkill hypothesis predicts the absence of such a relationship. Alternatively, under a focal prey resistance hypothesis, species more distantly related to the snake's diet are predicted to be more susceptible to the snakes venom due to reduced selection for venom resistance, a hypothesis with experimental support in a *Crotalus*–*Sciurus* system (Pomento *et al.* 2016).

As venom production incurs some metabolic cost (McCue & Mason 2006), although the level of this cost is debated (Pintor *et al.* 2010; Smith *et al.* 2014), the yield of venom a snake possesses is also likely to be under selection. Like many other trophic traits, much of this variation is likely to vary with body size according to allometric relationships of the form $Y \sim Mass^{a}$ (Hayes *et al.* 2002; Brown *et al.* 2004), where the value of the scaling exponent a can offer insights into the potential drivers of venom yield variation across species (Figure 2). For example, if venom yields are constrained by rates of biological production they are predicted to increase with snake body mass according to the ¾ scaling relationship of metabolism (Brown *et al.* 2004; Isaac & Carbone 2010). In contrast, if yield is driven by predator-prey allometries a coefficient of 0.51 is predicted (Figure 2). At the other extreme, super-linear allometries (exponents >1) would suggest patterns associated with drivers such as sexual selection, such as proposed by the weapons hypothesis (Kodric-Brown *et al.* 2006), or, in the more likely case for snakes due to limited use of venom in sexual conflict (Casewell *et al.* 2013), for use in defence that may require increased effectiveness with size, such as in the allometry of horn growth in horned lizards (Bergmann & Berk 2012).

Other potential drivers of venom evolution relate to the prey itself. For example, due to a switch in diet to one that is almost completely comprised of fish eggs, the marbled sea snake's (*Aipysurus eydouxii*) venom system has almost completely atrophied (Li *et al.* 2005) (Figure 1). Due to the reduced need to incapacitate prey, species which display oophagy are predicted to have lower potencies and venom yields. Similar expectations can also be made for species which use other means of incapacitating prey, such as construction (Shine & Schwaner 1985), due to a similar selective release or conversely through the need to compensate for already having lower venom potencies and yields.

While the effects of body size on trophic traits has long been realised, the importance of factors influencing the rate of interaction between predators and their prey, such as habitat structure (Arbuckle 2015), has only more recently become realised. The structural complexity of a habitat, such as whether it is a 2-dimensional terrestrial surface or a complex 3-dimensional forest canopy, can influence both encounter rates (Pawar *et al.* 2012; Carbone *et al.* 2014) and the escape rates of prey, with higher dimensional spaces increasing both (Heithaus *et al.* 2009; Møller 2010). While high dimensional environments may increase the opportunity for prey escape (Møller 2010), and hence select for increased venom yields and potencies to compensate, the high encounter rates in these environments may conversely compensate for such increased potential prey escape rates.

By controlling for the model used to measure potency, we test these fundamental hypotheses of venom evolution (Figure 2) in a phylogenetic comparative analysis of 102 species.

Methods

Data

To test our hypotheses, we collated data on venom yield and toxicity from the literature. We used mean dry weight (mg) extracted as a measure of venom yield as it represents the amount of active ingredients available and is the most reported measure. For venom lethality, we used median lethal dose (LD_{50}). We only used intravenous (IV), subcutaneous

(SC), intraperitoneal (IP) or intramuscular routes (IM) of administering the venom. Only adult LD_{50} values were used due to ontogenetic variability in venom potency (Andrade & Abe 1999). As LD_{50} can show high intraspecific variability (Martinson *et al.* 2017) we also collated multiple measures of LD_{50} for each species and the reported measurements of variability associated with each LD_{50} value including, 95% credibility intervals, 5% Fiducial limits, standard deviation and ranges.

For snake body size, we used total length values from the literature and field guides as these were the most common measures available. All lengths were converted to mass using family-level allometric scaling as described in Feldman and Meiri (2013). We collated dietary data of quantitative estimates of prey proportions, mainly from studies of stomach contents. Only dietary analyses of adults were included in the analysis. Prey size data were included from these dietary studies when available. When prey size was not reported in the dietary studies and where prey species were identified to the species level, we used mean prey species body mass from available databases (Meiri 2010; Feldman & Meiri 2013; Myhrvold *et al.* 2015; Froese 2016). In cases where only body lengths were available for prey species, allometric scaling equations were used to convert to mass (Pough 1980; Feldman & Meiri 2013; Myhrvold *et al.* 2015). For species that were only identified to the genus level, the genus' mean body mass was used if available. For each snake species we calculated the weighted mean prey size (W_j) as $W_j = \sum_{i=1}^{n}(p_{ij}m_{ij})$, where the mass of each prey item (m_{ij}) was weighted by the proportion p_{ij} of the diet it comprised for a snake species j over the *n* prey items in its diet.

To test the prey-specific hypothesis we calculated the phylogenetic distance (Millions of years ago Mya) to the common ancestor of the LD_{50} model and the dietary prey items. For the phylogenetic distance between prey identified to species or genus level we used the recently published phylogenies for Mammalia (Bininda-Emonds *et al.* 2007), Aves (Jetz *et al.* 2012) and Squamata (Pyron & Burbrink 2014). For ancestral ages between major classes we used 272 Mya for the common ancestor between Lepidosauria and Archosauria (Jones *et al.* 2013), 316.35 Mya for the common ancestor of amniotes based on the fossil *Archerpeton anthracos* (Reisz & Müller 2004), 419 Mya for the common ancestor of Actinopterygians and

Sarcopterygians based on the fossil *Guiyu oneiros* (Zhu *et al.* 2009), and 556.5 Mya for the fossil *Kimberella quadrata* as the common ancestor of deuterostomes and protostomes (Fedonkin *et al.* 2007). For prey items only identified to family level or above we used phylogenetic distances calculated using TimeTree (Hedges *et al.* 2006). We then calculated the mean phylogenetic distance between the diet of each snake and each LD_{50} model used to measure its venom potency. We weighted this mean according to the proportion of each prey item in the diet. This was calculated as $D_{LD50\text{-}Diet\ (jk)} = \sum_{i=1}^{n}(p_{ij}d_{ik})$, were $D_{LD50\text{-}Diet\ (jk)}$ is the weighted phylogenetic distance between the diet of a focal snake species *j* and a LD_{50} model species k, p_{ij} is the proportion of the *j*th snake species' diet comprised by prey item i and d_{ik} is the evolutionary distance (mya) to the common ancestor of i and k.

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), 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 behaviour we collected data on any observation of constriction behaviour from the literature (Shine & Schwaner 1985).

Snake mass, prey mass, LD_{50}, and venom yield were all $\log_{10}$ transformed in order to test scaling allometry predictions. The phylogeny from Pyron and Burbrink (2014) was included in all analyses to account for non-independence in traits due to common descent. Only snake species which had data on the proportion of prey items in their diet, LD_{50}, venom yield and body size were included in the analysis. The Data is available in the supplementary information (S2-3).

Analysis

To test our hypotheses, we fitted Bayesian phylogenetic mixed models (BPMM) using the `MCMCglmm` package (Hadfield 2010) in R version 3.4.0 (Team 2016). We controlled for pseudoreplication due to shared ancestry between species by using the `animal` term in `MCMCglmm` (Hadfield 2010). This term uses a distance matrix of the phylogenetic distance between species to control for the expected similarity in trait values due to phylogenetic

relatedness. We calculated the term h^2 as the relative variance attributable to the animal term (Hadfield & Nakagawa 2010). This term can be interpreted in a similar fashion to the phylogenetic lambda value, with a h^2 value close to 1 indicating a Brownian model of trait evolution, and a value close to zero indicating independence between trait values (Hadfield & Nakagawa 2010). We fitted all models using parameter expanded priors, with standard non-informative priors also tested separately to ensure that choice of prior had no effect on model results (Hadfield 2010). Choice of 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 (Brooks & Gelman 1998).

LD_{50} models

To test the overkill, prey-specific potency and the focal prey resistance hypotheses we ran a BPMM with LD_{50} as the response with $D_{LD50\text{-}Diet}$ as a response variable (See Figure 2). We controlled for the effect of route of injection by including it as a fixed factor (SC, IM, IV, IP). To test the oophagy hypothesis we include a fixed factor for the presence of eggs in the diet (absent, present). To test the habitat complexity hypothesis, we included habitat dimensionality as a fixed factor (2D, 3D). As measures of LD_{50} can have large levels of intraspecific variation (Martinson *et al.* 2017), we include multiple measures of LD_{50} for each species when available and account for this in our model using a random effect term at the species level. We also ran a separate model for the subset of LD_{50} values which also had an associated measurement error using the `mev` term in `MCMCglmm` to incorporate this variation (Hadfield & Nakagawa 2010). As the dissimilarity between two species may not increase linearly with phylogenetic distance we also ran a model using the square-root transform of $D_{LD50\text{-}Diet}$ which reflects how dissimilar species are with phylogenetic distance under a Brownian model of trait evolution (Letten & Cornwell 2015).

Venom Yield model

To test the allometric hypothesis regarding venom yield (Figure 2) we ran a BPMM with mean venom yield as the response variable and snake body mass as an explanatory variable. To test the habitat complexity hypothesis, we included habitat dimensionality as a fixed factor (2D, 3D). To test the oophagy hypothesis we include a fixed factor for the presence of

eggs in the diet (absent, present). For the subset of species for which prey size could be estimated we also ran a BPMM with mean prey size included as an explanatory variable. Finally, we tested the relationship between prey and snake body size by running two additional BPMMs with maximum and mean prey size as response variables and snake body mass as the explanatory variables.

Models with response variables combining LD_{50} and yield

To test whether potential co-variance between LD_{50} and venom yield may affect the results of the main model we ran a multiple response BPMM with both factors included as response variables with snake body mass, habitat dimensionality (2D, 3D), the presence of eggs in the diet (absent, present), $D_{LD50\text{-}Diet}$ and the LD_{50} route of injection included as explanatory variables.

As the combination of potency, yield and the size of prey is likely to be an ecologically relevant trait we also ran a BPMM with a response variable of the incapacitating ability of each snake species towards its prey. We calculated incapacitating ability I for each LD_{50} measure i of a focal snake species j as $I_{ij} = \frac{Y_j}{LD_{50ij}}$, where Y is the snake species' mean venom yield. We then divide this by the mean weighted prey mass of its diet to estimate the number of prey items it can impart a 50% mortality rate on. We fitted the same explanatory factors as the multiple response BPMM.

Supplementary models

To test the constriction hypothesis, we ran the main LD_{50} and yield BPMMs with constriction behaviour included as an explanatory variable (absent, present). To test for potential family level taxonomic effects, we ran BPMMs with family level included as an explanatory variable in each of the main models. To test for the sensitivity relating to the environment species were categorised in, we re-ran the main yield model with the semi-aquatic species, *Agkistrodon piscivorus*, *Bungarus multicinctus*, *Hydrodynastes gigas*, recategorized to terrestrial environments. Finally, as *Didelphis virginiana* is known to be a predator of *Crotalus atrox* and has a level of resistance to its venom, we re-ran the main LD_{50} model excluding this species.

Results

Our dataset consisted of 538 measures of LD_{50} representing 102 snake species that span six families (Figure 1, Table S1). Venom yield ranged from 0.15 mg in the egg-eating sea snake (*Emydocephalus annulatus*) to 571 mg in the forest cobra (*Naja melanoleuca*). LD_{50} ranged from 1121 mg/kg for the Western diamondback rattlesnake (*Crotalus atrox*) when tested on the Virginia opossum (*Didelphis virginiana*), to 0.00031 mg/kg in the many-banded krait (*Bungarus multicinctus*) when measured on the White-rumped munia (*Lonchura striata*). Mammals comprised the majority of LD_{50} model species in the dataset (80% of all measures), followed by fish (6.7%), Reptiles (6.3%), Aves (5.7%), amphibians (0.9%) and arthropods (0.4%) (Table S1).

LD_{50} models

We found that the prey-specific hypothesis was supported with lower LD_{50} values, indicating higher potency, found when LD_{50} was measured on animal models phylogenetically closer to the species typically found in the snake's diet ($D_{LD50\text{-}Diet}$ slope = 0.12, lower 95% CI = 0.04, higher 95% CI = 0.19, n = 538 measures for 102 species; Figure 3, Figure 4A, Table S2). The phylogenetic regression identified a positive relationship between LD_{50} and $D_{LD50\text{-}Diet}$, with the increase of 5.5 in LD_{50} over the range of $D_{LD50\text{-}Diet}$ larger than the 3.9 difference in LD_{50} associated with the route venom was administered (Figure 3, Figure 4A). As expected IV (n = 168) and IP (n = 195) routes were associated with lower LD_{50} values when compared to a SC (n = 88) route (Figure 3, Table S2). While limited by sample size, our analysis also found support for the oophagy hypothesis with species that consume eggs (n = 7 species) found to be associated with higher LD_{50} values (Figure 3, Table S2). Variation associated with phylogeny was found to account for more variation than within-species variation, with a moderate phylogenetic signal between that of a full Brownian evolution and full independence of the trait (h^2 = 0.43, lower 95% CI = 0.18, higher 95% CI = 0.69, Figure 3, Table S2).

Similar results to the full model of potency were found in the sub-analysis which included measurement error for 146 measures of LD_{50} for 56 species (Table S3). This included

support for the prey-specific potency hypothesis, the oophagy hypothesis along with similar effects relating to the route venom was administered (Table S3). In the analysis using the square root transform of $D_{LD50\text{-}Diet}$ we found results qualitatively similar to those in the main LD_{50} analysis (Table S4).

Venom Yield Models

We found support for the metabolic rate hypothesis of venom yield scaling with an allometry of 0.74 between yield and snake body mass (slope = 0.74, lower 95% CI = 0.66, higher 95% CI = 0.82, n = 538 measures for 102 species; Figure 3, Figure 4B, Table S5). This exponent exceeded the scaling of 0.51 predicted if yield increased at a rate expected from changes in prey size (Figure 2, Eq. 5). In the model which included prey mass we also found an allometric increase of only 0.18 between venom yield and prey mass (n = 369 measures for 65 species, Table S6). Moreover, we found no significant statistical relationship between snake mass and the mean or maximum size of their prey indicating a weak relationship between venom yield and prey size when controlling for phylogeny (n = 65 species, Table S7). We also found that snake species which occupy three dimensional environments have lower venom yields by approximately half an order of magnitude in comparison to terrestrial species (B = -0.56, lower 95% CI = -0.83, higher 95% CI = -0.25, n = 538 measures for 102 species; Figure 3-4B, Table S5). The phylogenetic signal associated with venom yield was moderate throughout the analysis with a h^2 of 0.49 in the main analysis (Figure 3, Table S5).

Models with response variables combining LD_{50} and yield

In the analysis with both LD_{50} and yield included as response variables with co-variance between the terms included we found qualitatively similar results to those in the main LD_{50} and yield models (Table S8). We also found no relationship between LD_{50} and venom volume in an additional analysis with LD_{50} included as a response variable and venom volume included as an explanatory factor (Table S9).

In the incapacitating ability analysis, we found further support for the prey-specific hypothesis with incapacitating ability found to have a negative relationship with $D_{LD50\text{-}Diet}$ (396 measures, 71 species, S10). We also found a positive relationship between

incapacitating ability and body size, support for the oophagy hypotheses (7 oophagous species, S10), and to a lesser extent as the higher 95% CI of the posterior overlapped zero, for the habitat complexity hypothesis (15 species in high dimensional habitats S10).

Supplementary analysis

The results found in both the LD_{50} and yield models were qualitatively replicated in each of the sensitivity analysis. We found no support for the constriction hypothesis (S11-12) along with no qualitative change to the results in the models that included taxonomic family as a fixed effect (S11-12), the environmental designation sensitivity analysis (S13), or in the analysis which excluded *Didelphis virginiana* (S14).

Discussion

By incorporating the evolutionary difference between what a snake eats and the species on which its potency was measured, we show that venom is generally prey-specific and driven by snake size, oophagous behaviour, and dimensionality of the environment. Predator traits are predicted to be strongly shaped by both predator-prey co-evolution and macroecological forces such as body size and habitat structure. Traits such as jaw or beak morphology are tightly linked to diet (McGee *et al.* 2015; Cooney *et al.* 2017), while a predator's size and foraging environment also influences trophic interactions through limiting the size, encounter rate and escape rate of potential prey (Møller 2010; Pawar *et al.* 2012; Carbone *et al.* 2014). Here we show that, in contrast to predictions relating to the overkill hypothesis, snake venom is also driven by such ecological pressures. These results not only help us understand the drivers of variation of venom in snakes but are also likely to apply to other venomous animals. Moreover, as the ability of a venom to incapacitate a given prey item can be quantified and confounding factors appropriately controlled for, venom systems offer an ideal system to understand predator-prey interactions.

Historically, venom potency has been measured using laboratory species, in particular rodents as they allow for comparisons to human physiology due to our shared mammalian ancestry (Uhl & Warner 2015). While there has been a recent shift towards the use of natural prey models, which can account for the species specific effects of venoms found

here, these data are still unavailable for the majority of venomous snakes (da Silva & Aird 2001; Barlow *et al.* 2009). We demonstrate that, by accounting for how closely related a model species is to natural prey species, historical potency data can be used to test fundamental hypotheses regarding snake venom and predator-prey interactions at the macroecological scale. Similar to the use of medical model species that are more closely related to humans in order to mimic expected organismal responses (Barré-Sinoussi & Montagutelli 2015), model species that are more closely related to the species on which a snakes venom are selected towards show higher potencies.

Such prey-specific patterns in LD_{50} have previously been found when using natural prey species as potency models (da Silva & Aird 2001; Barlow *et al.* 2009). Moreover, we find that when the focal group of da Silva and Aird (2001) is highlighted in our analysis (blue circles in Figure 4a), they also display an increase in LD_{50} with $D_{LD50\text{-}Diet}$ similar to the pattern we find. However, while we find a consistent prey-specific pattern for LD_{50} in our analysis comparable to previous taxon specific studies there is still substantial variation associated with LD_{50}, accounting for 40% of the variance after accounting for fixed factors, much of which is likely to stem from context specific predator-prey interactions within species, such as demonstrated by phenotype matching (Holding *et al.* 2016). Such cases are likely to be the source of the large intraspecific variation of potency seen in some species in our analysis, such as the range in potencies in the Western diamondback rattlesnake (*Crotalus atrox*) due to being tested on both a typical prey species and one of its predators, the Virgina opossum, which has evolved resistance to its venom (Voss 2013). Another potential source of variation in LD_{50} with D_{LD50}-Diet is that venoms may be under natural selected for other aspects related to incapacitating prey, such as the speed of its effect (Barlow *et al.* 2009). However, even this measure shows large variation across studies. For example, in *Echis* species a prey-specific effect was found when using time to incapacitate as a metric in one study (Richards *et al.* 2012) but not in another (Barlow *et al.* 2009). The use of a linear or Brownian model of evolution to calculate $D_{LD50\text{-}Diet}$ values in our models is unlikely to capturing these and other genetic, biotic and abiotic sources (Holding *et al.* 2018) of variation in function and composition of venom. While our study shows that using large comparative approaches can identify general patterns in venom evolution, the inclusion of

different functional aspects of venom along with more complex models of its evolution are likely to further clarify the level of context specificity in venoms.

In terms of macroecological patterns, unsurprisingly we found that larger snakes had larger quantities of venom. However, these increases did not follow predictions based on the observed predator-prey mass relationships in snakes (Carbone *et al.* 2014), with yield increasing far more rapidly than expected if yield was mainly driven by prey size (Figure 2). Moreover, the non-significant relationship between snake and prey size found here further suggests the surprisingly minor role prey size may have on the scaling of venom yield in snakes. Instead yield was found to follow the 3/4 allometric scaling predicted from metabolic theory, assuming snakes invest a constant proportion of their metabolism to produce venom (Brown *et al.* 2004). This scaling signifies that the metabolic costs of venom (McCue & Mason 2006) may have a more significant role in the evolution of venom than previously supposed. Other drivers which may contribute to this scaling include the allometry of traits such as head size, however, head size has only been found to have minor effects when tested (Mirtschin *et al.* 2002).

Apart from size, habitat dimensionality was also found to influence venom yield. While we expected that species in high dimensional habitats may have larger venom yields to compensate for higher escape rates of prey (Møller 2010), we found they had smaller yields in comparison to species in low dimensional habitats. This may be associated with a potentially greater need for prey holding behaviours in high dimension environments, such as in arboreal habitats (Deufel & Cundall 2006), which in turn allows for the more accurate delivery of smaller volumes of venom. However, bite and release behaviours are known in arboreal species such as the eastern green mamba (*Dendroaspis angusticeps*) suggesting this behaviour is not fully restricted to low dimensional environments (Branch 1998). An alternative explanation of these results is that higher encounter rates in high dimensional environments (Pawar *et al.* 2012) may represent a case of foraging optimisation (Stephens & Krebs 1986). If expected foraging opportunities are high, the cost of losing a prey item by using less venom may not exceed the energetic costs associated with venom production. Furthermore, large reservoirs of venom may also be costly as venom replenishment times can be substantial, with estimates ranging from 7 days (Currier *et al.* 2012) to 30-50 days

(Hayes *et al.* 2002; Hayes 2008). Long periods of replenishment may select for larger venom reserves in species where prey encounter rates are low in order to minimise potential missed opportunity costs. While further research on the role of habitat dimensionality is required our results highlight that prey encounter rates may be an important factor driving venom yield evolution.

While our analysis demonstrates the importance of trophic and macroecological drivers in snake venom evolution these drivers are also expected to influence the evolution of venom in other taxa. For example, prey-specific venom is seen in cone snails and spiders (Casewell *et al.* 2013), while the energetic costs of producing venom is also suggested by venom metering in scorpions (Nisani *et al.* 2007). By extending the comparative approach used here to other venomous groups, the universality of these patterns in venom evolution can be tested. Furthermore, elements of prey-specificity and macroecological constraints are likely to apply to non-venomous predatory traits. By using venom as a system of predator trait evolution the importance of multiple evolutionary drivers can be tested and offer a window into both the evolution of venomous systems and predatory traits in general.

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References

1.
Andrade, D.V. & Abe, A.S. (1999). Relationship of venom ontogeny and diet in Bothrops. *Herpetologica*, 200-204.

2.
Arbuckle, K., de la Vega, R.C.R. & Casewell, N.R. (2017). Coevolution takes the sting out of it: Evolutionary biology and mechanisms of toxin resistance in animals. *Toxicon*.

3.
Barlow, A., Pook, C.E., Harrison, R.A. & 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, 2443-2449.

4.
Barré-Sinoussi, F. & Montagutelli, X. (2015). Animal models are essential to biological research: issues and perspectives. *Future science OA*, 1.

5.
Bergmann, P.J. & Berk, C.P. (2012). The evolution of positive allometry of weaponry in horned lizards (Phrynosoma). *Evolutionary Biology*, 39, 311-323.

6.
Biardi, J.E., Coss, R.G. & Smith, D.G. (2000). California ground squirrel (Spermophilus beecheyi) blood sera inhibits crotalid venom proteolytic activity. *Toxicon*, 38, 713-721.

7.
Bininda-Emonds, O.R., Cardillo, M., Jones, K.E., MacPhee, R.D., Beck, R.M., Grenyer, R. *et al.* (2007). The delayed rise of present-day mammals. *Nature*, 446, 507-512.

8.
Branch, W.R. (1998). *Field guide to snakes and other reptiles of southern Africa*. Struik.

9.
Brooks, S.P. & Gelman, A. (1998). General methods for monitoring convergence of iterative simulations. *Journal of computational and graphical statistics*, 7, 434-455.

10.
Brown, J.H., Gillooly, J.F., Allen, A.P., Savage, V.M. & West, G.B. (2004). Toward a metabolic theory of ecology. *Ecology*, 85, 1771-1789.

11.
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, 1553-1559.

12.
Casewell, N.R., Wüster, W., Vonk, F.J., Harrison, R.A. & Fry, B.G. (2013). Complex cocktails: the evolutionary novelty of venoms. *Trends in ecology & evolution*, 28, 219-229.

13.
Chippaux, J.-P., Williams, V. & White, J. (1991). Snake venom variability: methods of study, results and interpretation. *Toxicon*, 29, 1279-1303.

14.
Cooney, C.R., Bright, J.A., Capp, E.J., Chira, A.M., Hughes, E.C., Moody, C.J. *et al.* (2017). Mega-evolutionary dynamics of the adaptive radiation of birds. *Nature*.

15.

Currier, R.B., Calvete, J.J., Sanz, L., Harrison, R.A., Rowley, P.D. & Wagstaff, S.C. (2012). Unusual stability of messenger RNA in snake venom reveals gene expression dynamics of venom replenishment. *PloS one*, 7, e41888.

16.

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

17.

Daltry, J.C., Wuester, W. & Thorpe, R.S. (1996). Diet and snake venom evolution. *Nature*, 379, 537-540.

18.

Deufel, A. & Cundall, D. (2006). Functional plasticity of the venom delivery system in snakes with a focus on the poststrike prey release behavior. *Zoologischer Anzeiger - A Journal of Comparative Zoology*, 245, 249-267.

19.

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, 169-182.

20.

Fedonkin, M.A., Simonetta, A. & Ivantsov, A.Y. (2007). New data on Kimberella, the Vendian mollusc-like organism (White Sea region, Russia): palaeoecological and evolutionary implications. *Geological Society, London, Special Publications*, 286, 157-179.

21.

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

22.

Froese, R. (2016). Pauly & D. FishBase.(2016). *World wide web electronic publication. Available from:< http://www. fishbase. org>. Access on: Jun*, 13.

23.

Hadfield, J. & Nakagawa, S. (2010). General quantitative genetic methods for comparative biology: phylogenies, taxonomies and multi-trait models for continuous and categorical characters. *Journal of evolutionary biology*, 23, 494-508.

24.

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

25.

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

26.

Hayes, W.K., Herbert, S.S., Rehling, G.C. & Gennaro, J.F. (2002). Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts. *Biology of the Vipers*, 207-233.

27.

Heatwole, H. & Poran, N.S. (1995). Resistances of sympatric and allopatric eels to sea snake venoms. *Copeia*, 136-147.

28.

Hedges, S.B., Dudley, J. & Kumar, S. (2006). TimeTree: a public knowledge-base of divergence times among organisms. *Bioinformatics*, 22, 2971-2972.
29.
Heithaus, M.R., Wirsing, A.J., Burkholder, D., Thomson, J. & Dill, L.M. (2009). Towards a predictive framework for predator risk effects: the interaction of landscape features and prey escape tactics. *Journal of Animal Ecology*, 78, 556-562.
30.
Holding, M.L., Biardi, J.E. & Gibbs, H.L. (2016). Coevolution of venom function and venom resistance in a rattlesnake predator and its squirrel prey. *Proc. R. Soc. B*, 283, 20152841.
31.
Holding, M.L., Margres, M.J., Rokyta, D.R. & Gibbs, H.L. (2018). Local prey community composition and genetic distance predict venom divergence among populations of the northern Pacific rattlesnake (Crotalus oreganus). *Journal of evolutionary biology*.
32.
Isaac, N.J. & Carbone, C. (2010). Why are metabolic scaling exponents so controversial? Quantifying variance and testing hypotheses. *Ecology Letters*, 13, 728-735.
33.
Jetz, W., Thomas, G., Joy, J., Hartmann, K. & Mooers, A. (2012). The global diversity of birds in space and time. *Nature*, 491, 444-448.
34.
Jones, M.E., Anderson, C.L., Hipsley, C.A., Müller, J., Evans, S.E. & Schoch, R.R. (2013). Integration of molecules and new fossils supports a Triassic origin for Lepidosauria (lizards, snakes, and tuatara). *BMC evolutionary biology*, 13, 1.
35.
Kane, A., Healy, K., Ruxton, G.D., Jackson, A.L., Woods, H.A. & Winn, A.A. (2016). Body Size as a Driver of Scavenging in Theropod Dinosaurs. *The American Naturalist*, 187, 706-716.
36.
Kasturiratne, A., Wickremasinghe, A.R., de Silva, N., Gunawardena, N.K., Pathmeswaran, A., Premaratna, R. *et al.* (2008). The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. *PLoS Med*, 5, e218.
37.
Kiltie, R. (2000). Scaling of visual acuity with body size in mammals and birds. *Functional Ecology*, 14, 226-234.
38.
Kodric-Brown, A., Sibly, R.M. & Brown, J.H. (2006). The allometry of ornaments and weapons. *Proceedings of the National Academy of Sciences*, 103, 8733-8738.
39.
Letten, A.D. & Cornwell, W.K. (2015). Trees, branches and (square) roots: why evolutionary relatedness is not linearly related to functional distance. *Methods in Ecology and Evolution*, 6, 439-444.
40.
Li, M., Fry, B. & Kini, R.M. (2005). Eggs-only diet: its implications for the toxin profile changes and ecology of the marbled sea snake (Aipysurus eydouxii). *Journal of Molecular Evolution*, 60, 81-89.

41.
Mackessy, S.P., Sixberry, N.M., Heyborne, W.H. & Fritts, T. (2006). Venom of the Brown Treesnake, Boiga irregularis: ontogenetic shifts and taxa-specific toxicity. *Toxicon*, 47, 537-548.
42.
Margres, M.J., Wray, K.P., Hassinger, A.T., Ward, M.J., McGivern, J.J., Moriarty Lemmon, E. *et al.* (2017). Quantity, not quality: rapid adaptation in a polygenic trait proceeded exclusively through expression differentiation. *Molecular biology and evolution*, 34, 3099-3110.
43.
Martinson, E.O., Mrinalini, Kelkar, Y.D., Chang, C.-H. & Werren, J.H. (2017). The Evolution of Venom by Co-option of Single-Copy Genes. *Current Biology*, 27, 2007-2013.e2008.
44.
McCue, M.D. & Mason, R. (2006). Cost of producing venom in three North American pitviper species. *Copeia*, 2006, 818-825.
45.
McGee, M.D., Borstein, S.R., Neches, R.Y., Buescher, H.H., Seehausen, O. & Wainwright, P.C. (2015). A pharyngeal jaw evolutionary innovation facilitated extinction in Lake Victoria cichlids. *Science*, 350, 1077-1079.
46.
Mebs, D. (2001). Toxicity in animals. Trends in evolution? *Toxicon*, 39, 87-96.
47.
Meiri, S. (2010). Length–weight allometries in lizards. *Journal of Zoology*, 281, 218-226.
48.
Mirtschin, P., Shine, R., Nias, T., Dunstan, N., Hough, B. & Mirtschin, M. (2002). Influences on venom yield in Australian tigersnakes (Notechis scutatus) and brownsnakes (Pseudonaja textilis: Elapidae, Serpentes). *Toxicon*, 40, 1581-1592.
49.
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, 1689-1698.
50.
Morgenstern, D. & King, G.F. (2013). The venom optimization hypothesis revisited. *Toxicon*, 63, 120-128.
51.
Myhrvold, N.P., Baldridge, E., Chan, B., Sivam, D., Freeman, D.L. & Ernest, S. (2015). An amniote life-history database to perform comparative analyses with birds, mammals, and reptiles. *Ecology*, 96, 3109-3109.
52.
Nisani, Z., Dunbar, S.G. & Hayes, W.K. (2007). Cost of venom regeneration in Parabuthus transvaalicus (Arachnida: Buthidae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 147, 509-513.
53.
Pawar, S., Dell, A.I. & Savage, V.M. (2012). Dimensionality of consumer search space drives trophic interaction strengths. *Nature*, 486, 485-489.
54.

Pintor, A.F., Krockenberger, A.K. & Seymour, J.E. (2010). Costs of venom production in the common death adder (Acanthophis antarcticus). *Toxicon*, 56, 1035-1042.
55.
Pomento, A.M., Perry, B.W., Denton, R.D., Gibbs, H.L. & Holding, M.L. (2016). No safety in the trees: local and species-level adaptation of an arboreal squirrel to the venom of sympatric rattlesnakes. *Toxicon*, 118, 149-155.
56.
Pough, F.H. (1980). The advantages of ectothermy for tetrapods. *American Naturalist*, 92-112.
57.
Pyron, R.A. & Burbrink, F.T. (2014). Early origin of viviparity and multiple reversions to oviparity in squamate reptiles. *Ecology Letters*, 17, 13-21.
58.
Reisz, R.R. & Müller, J. (2004). Molecular timescales and the fossil record: a paleontological perspective. *TRENDS in Genetics*, 20, 237-241.
59.
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, 110-116.
60.
Sasa, M. (1999). Diet and snake venom evolution: can local selection alone explain intraspecific venom variation? *TOXICON-OXFORD-*, 37, 249-252.
61.
Shine, R. & Schwaner, T. (1985). Prey constriction by venomous snakes: a review, and new data on Australian species. *Copeia*, 1985, 1067-1071.
62.
Smith, M.T., Ortega, J. & Beaupre, S.J. (2014). Metabolic cost of venom replenishment by Prairie Rattlesnakes (Crotalus viridis viridis). *Toxicon*, 86, 1-7.
63.
Starkov, V.G., Osipov, A.V. & Utkin, Y.N. (2007). Toxicity of venoms from vipers of Pelias group to crickets Gryllus assimilis and its relation to snake entomophagy. *Toxicon*, 49, 995-1001.
64.
Stephens, D.W. & Krebs, J.R. (1986). *Foraging theory*. Princeton University Press.
65.
Team, R.C. (2016). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.
66.
Uhl, E.W. & Warner, N.J. (2015). Mouse models as predictors of human responses: evolutionary medicine. *Current pathobiology reports*, 3, 219-223.
67.
Van Valen, L. (1973). A new evolutionary law. *Evolutionary theory*, 1, 1-30.
68.
Vonk, F.J., Casewell, N.R., Henkel, C.V., Heimberg, A.M., Jansen, H.J., McCleary, R.J. *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, 20651-20656.

69.
Voss, R.S. (2013). Opossums (Mammalia: Didelphidae) in the diets of Neotropical pitvipers (Serpentes: Crotalinae): Evidence for alternative coevolutionary outcomes? *Toxicon*, 66, 1-6.
70.
Weinstein, S.A., Warrell, D.A., White, J. & Keyler, D. (2011). Venomous" bites from non-venomous snakes. In: *A Critical Analysis of Risk and Management of "Colubrid" Snake Bites*. Elsevier London.
71.
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, 1067-1075.
72.
Wüster, W., Daltry, J.C. & Thorpe, R.S. (1999). Can diet explain intraspecific venom variation? Reply to Sasa. *TOXICON-OXFORD-*, 37, 253-258.
73.
Zhu, M., Zhao, W., Jia, L., Lu, J., Qiao, T. & Qu, Q. (2009). The oldest articulated osteichthyan reveals mosaic gnathostome characters. *Nature*, 458, 469-474.

Figures

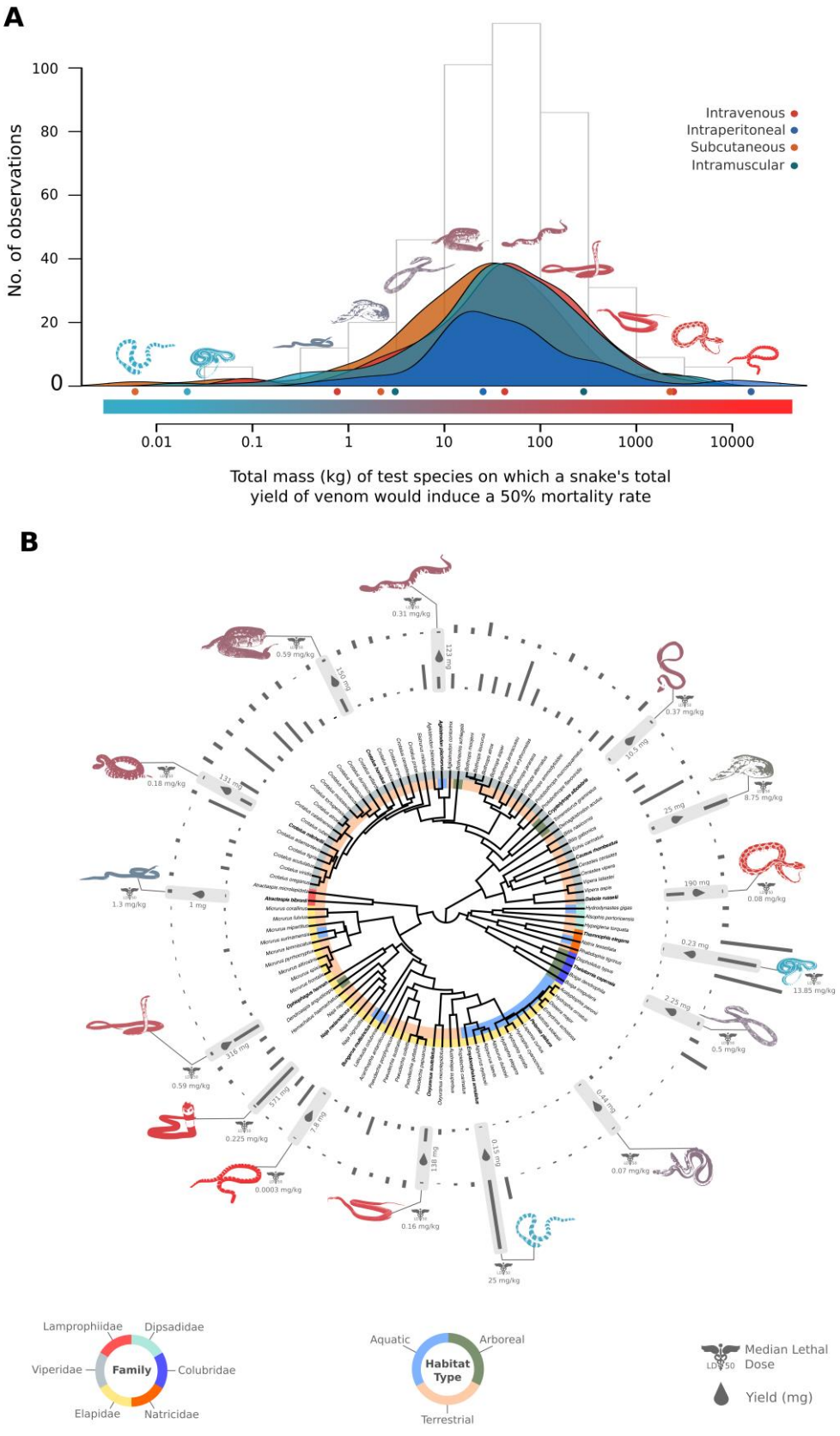
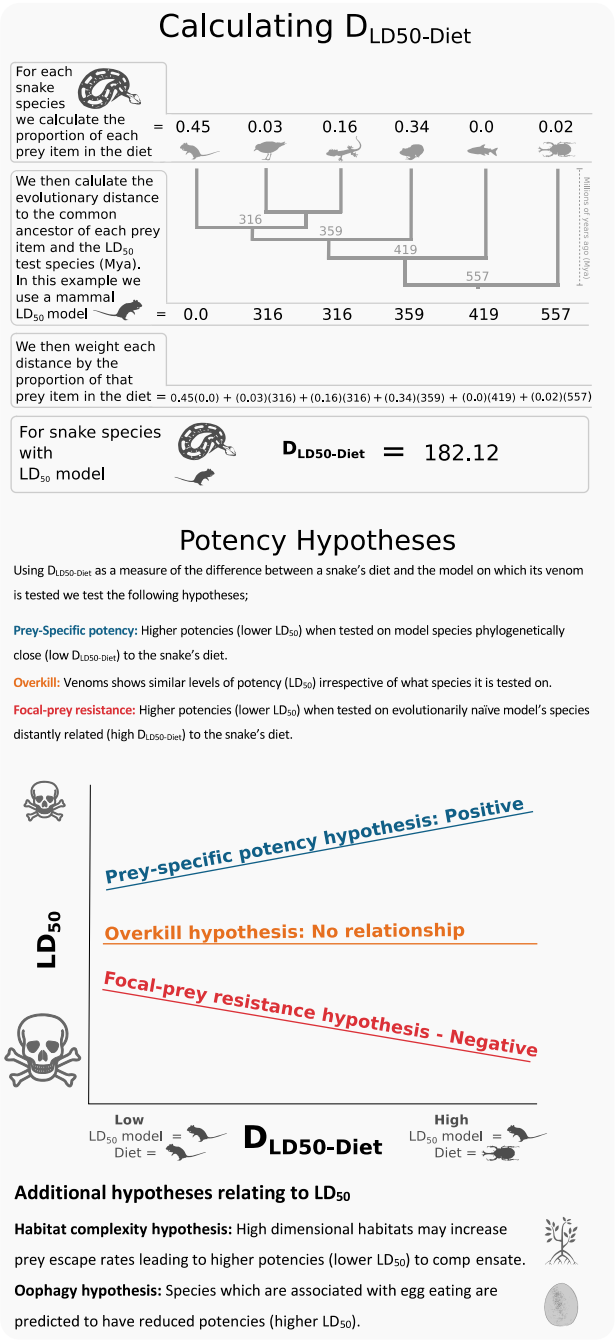

A
No. of observations
100
80
60
40
20
0
Intravenous
Intraperitoneal
Subcutaneous
Intramuscular
0.01
0.1
1
10
100
1000
10000
Total mass (kg) of test species on which a snake's total yield of venom would induce a 50% mortality rate
B
Lamprophiidae
Dipsadidae
Viperidae
Family
Colubridae
Elapidae
Natricidae
Aquatic
Arboreal
Habitat Type
Terrestrial
Median Lethal Dose
Yield (mg)

Figure 1. A. Histogram representing the distribution of the incapacitating potential of species in our dataset of 538 potency measures for 102 species. Incapacitating potential is the animal mass (kg) which a snake's total yield of venom can impart a 50% mortality rate on. This was calculated as the mean volume of dried venom for a species divided by its LD_{50} (mg/kg). The colour bar ranges from low incapacitating ability (blue) to high (red) with the corresponding colour used for the highlighted species silhouettes in both **A** and **B**. The density curves represent the distribution of incapacitating ability for each of the routes LD_{50} was administered to the test model (red (IV, n = 168), blue (IP, n = 195), orange (SC, n = 88) and green (IM, n = 87)). **(B)** Phylogenetic relationship between the 102 species included in the analysis. Outwards from the centre of the phylogeny the first colour band describes each species habitat followed by a band indicating the taxonomic family. The first circular bar-plot represents the mean yield for each species, with the outermost bar-plot describing the lowest median lethal dose (LD_{50}) for a given species. Species are highlighted as silhouettes with colours matching the incapacitation ability scale from **1.A**.



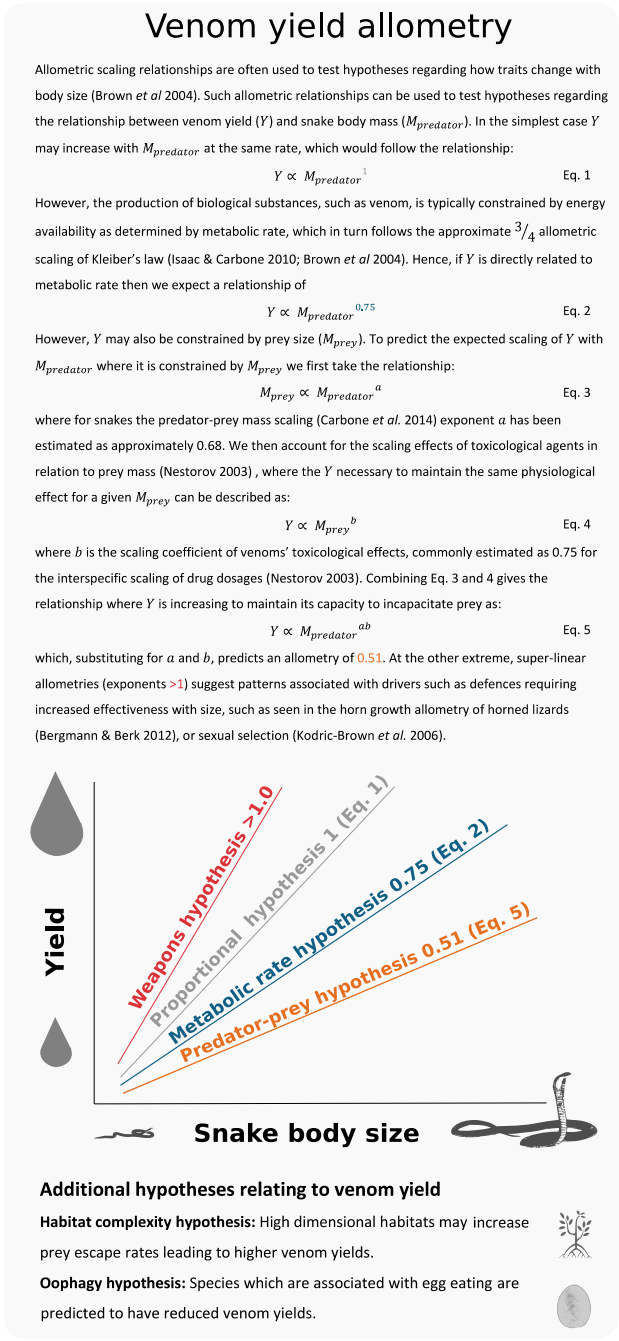


Figure 2. Summary of the predicted drivers of venom potency and yield.

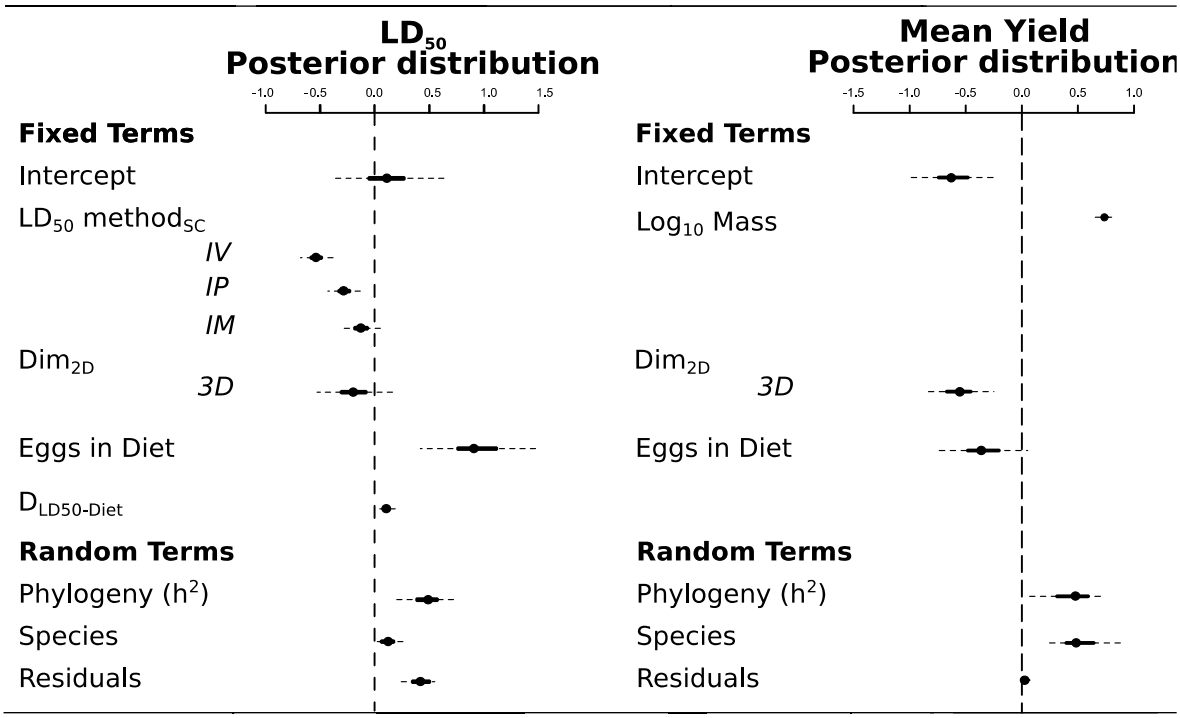


Figure 3. Posterior distributions from the LD_{50} and mean venom yield models, with modes represented by dots and higher and lower 95% credibility intervals represented by dotted horizontal bar. Fixed factors include mass; LD_{50} method (subcutaneous (SC), intravenous (IV), intraperitoneal (IP) and 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 LD_{50} model ($D_{LD50\text{-}Diet}$). The random terms are also presented. Significance is determined when 95% of the posterior estimate is above or below zero.

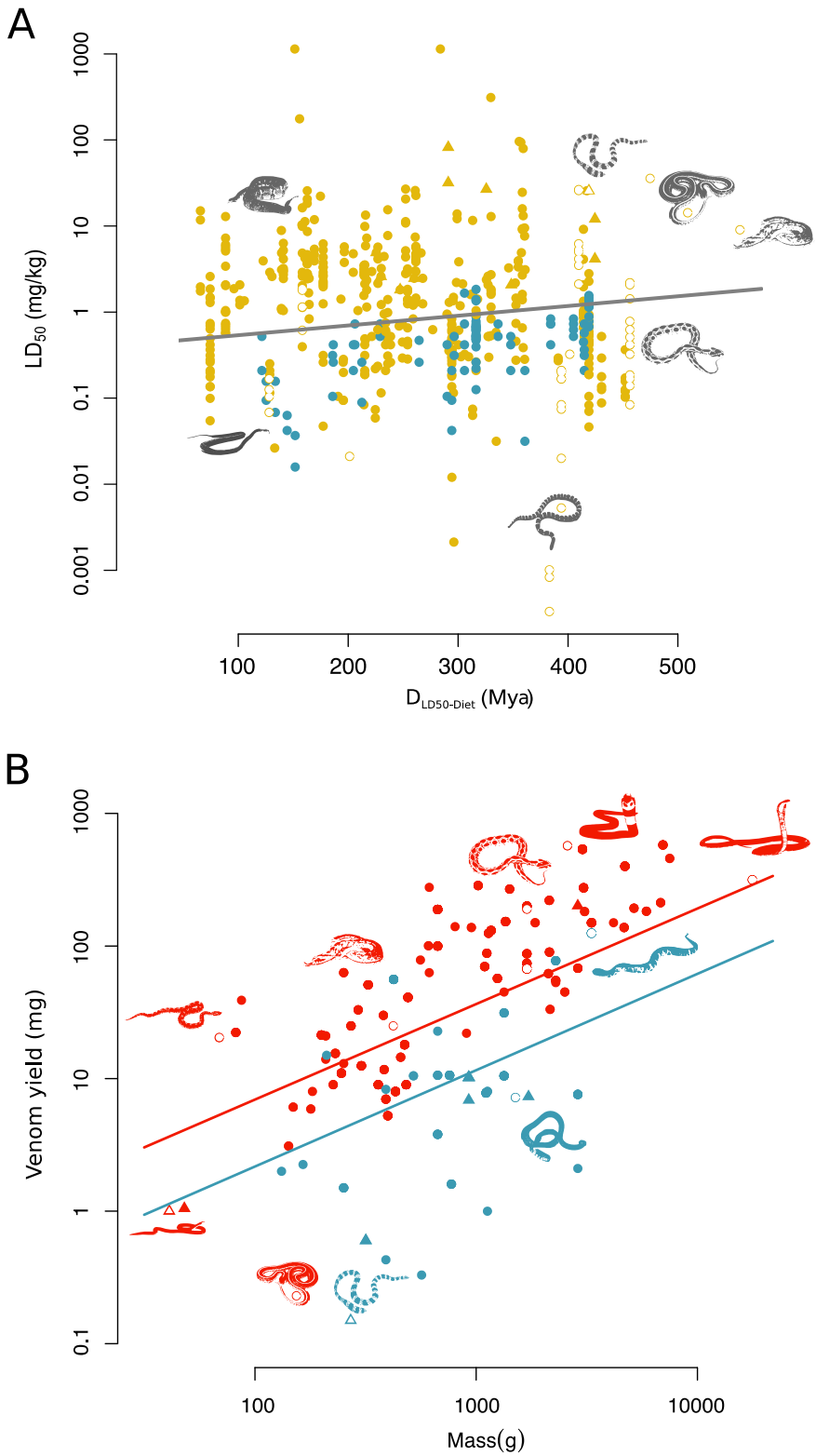


Figure 4. (A) Mean phylogenetic distance between diet species and LD_{50} model ($D_{LD50\text{-}Diet}$) against $\log_{10}$ LD_{50}. The positive relationship supports the prey-specific hypothesis (intercept (IV) = -0.4, slope = 0.12). Hollow points represent silhouette species which are from left to right; *Oxyuranus scutellatus*; *Crotalus horridus; Bungarus multicinctus*; *Emydocephalus annulatus*; *Daboia russelii*; *Thamnophis elegans*; *Causus rhombeatus*. (B) Relationship

between $\log_{10}$ mass (g) against $\log_{10}$ venom yield (mg). The higher intercept for species 2D habitats (Red points and fitted line intercept = -0.65, slope = 0.74) compared to species in 3D habitats (blue points and fitted line: intercept = -1.13, slope = 0.74) supports the differential habitat hypothesis. The oophagy hypothesis was supported by the low yields observed in oophagous species (triangles). Hollow points represent silhouette species which are from left to right *Atractaspis bibronii*; *Bothrops ammodytoides*; *Thamnophis elegans; Causus rhombeatus*; *Emydocephalus annulatus*; *Daboia russelii; Hydrophis elegans*; *Naja_melanoleuca*; *Agkistrodon piscivorus*; *Ophiophagus hannah*. All intercepts and slopes are from the values in Figure 3, with model fit incorporating random effects and other marginal effects as outlined in the main model (See Methods). The *Micrurus* genus is highlighted by blue circles in (A) as an example of the importance of accounting for such marginal effects and as a comparison to a previous study on LD_{50} in the group by da Silva and Aird (2001).