

RESEARCH ARTICLE

How venom pore placement may influence puncture performance in snake fangs

Stephanie B. Crofts^{1,*} and Philip S. L. Anderson^{2,*,†}

ABSTRACT

When designing experimental studies, it is important to understand the biological context of the question being asked. For example, many biological puncture experiments embed the puncture tool to a standardized depth based on a percentage of the total tool length, to compare the performance between tools. However, this may not always be biologically relevant to the question being asked. To understand how definitions of penetration depth may influence comparative results, we performed puncture experiments on a series of venomous snake fangs using the venom pore location as a functionally relevant depth standard. After exploring variation in pore placement across snake phylogeny, we compared the work expended during puncture experiments across a set of snake fangs using various depth standards: puncture initiation, penetration to a series of depths defined by the venom pore and penetration to 15% of fang length. Contrary to our hypothesis, we found almost no pattern in pore placement between clades, dietary groups or venom toxicity. Rank correlation statistics of our experimental energetics results showed no difference in the broad comparison of fangs when different puncture depth standards were used. However, pairwise comparisons between fangs showed major shifts in significance patterns between the different depth standards used. These results imply that the interpretation of experimental puncture data will heavily depend upon which depth standard is used during the experiments. Our results illustrate the importance of understanding the biological context of the question being addressed when designing comparative experiments.

KEY WORDS: Biomechanics, Comparative biology, Experimental design, Venom, Fang penetration

INTRODUCTION

Experimentation has long been a cornerstone of biomechanical studies. However, a key part of any comparative experimental study is understanding the aspects of performance that are important to a given system and can be compared across individuals/taxa, which is not always straightforward. Take the example of biological puncture; an elongate, sharpened tool is used to fracture and penetrate a target material (Zhang and Anderson, 2022). This seemingly straightforward mechanism is widely diverse in terms of scale (from rhinoceros horns down to stinging cells in jellyfish), taxonomic representation (vertebrates, invertebrates, plants, fungi)

and biological purpose (damage, injection, defense) (Anderson, 2018). This wide diversity means that designing experiments that allow for comparisons between taxa can be challenging. Here, we explored how assumptions of biological function/purpose influence experimental comparisons between taxa in a biological puncture system: venomous snake fangs.

Biological puncture can be broken down into different stages: initiation, deformation, fracture, penetration and withdrawal (Anderson, 2018; Kundanati et al., 2020; Zhang and Anderson, 2022). Organisms using puncture to perform different functions may emphasize different stages, requiring different types of puncture experiments to test the relationship between morphology and performance. In our previous study looking at the functional relevance of different tip sharpness measures in snake fangs, we focused entirely on the energy required to initiate puncture (Crofts et al., 2019). In contrast, for our study focused on the influence that surface ornamentation has on the penetration of cactus spines, we made a point to measure energy all the way to a puncture depth of 50% of the spine length, ensuring that the ornamentation in question penetrated the target deeply enough to have an effect (Crofts and Anderson, 2018). A similar study, exploring the effect of barbs on puncture and penetration of porcupine spines, compared how changing the distribution of barbs along the length of the spine tip would change the work of penetration and removal from tissue (Cho et al., 2012). Another study on the influence of the angle of puncture seen in wasps and bees specifically varied the approach angles during puncture to establish how that variable affected performance (Zhao et al., 2015). Sometimes, puncture itself is being compared with other actions, requiring multiple experiments with different set-ups such as work on puncture–draw mechanics in shark teeth (Whitenack and Motta, 2010; Whitenack et al., 2011). Finally, interactions between puncture and the puncture tool's own resistance to buckling require tests that accommodate measurements of both (Galloway and Porter, 2019, 2021). These studies illustrate how the design of a puncture experiment can be varied to address questions related to the specific biological functions, such as prey-capture or defense.

When running a puncture test, the depth to which a tool penetrates the target material is a particularly important aspect of both the experimental design of the test and the biological context in which the test is run. In order to make meaningful comparisons between the individuals or taxa being tested, the puncture depth needs to be standardized. How should such a depth-standard be determined for a given set of tests? This is not a trivial question, as different potential ‘puncture depths’ may be required for different functions, such as damage creation, toxin injection or even defense. Is it enough to simply initiate fracture or is there a certain depth of penetration that should be achieved? If the latter, how deep? The question of depth will be of particular importance, as there may be a minimum depth required for the puncture to be considered ‘successful’ for a given function and only such successful events

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can lead to increased fitness for the organism. The challenge is deciphering what the biologically relevant depth might be for a given situation and ensuring that the resulting experimental design is still comparable across taxa.

We explored this question by examining puncture mechanics in venomous snake fangs, using venom pore placement as an easily identifiable biologically relevant depth. While Duvernoy's gland, homologous to the venom glands in venomous snakes, seems likely to have developed early on in the colubrids, the fangs of viperids, elapids and atractaspis (all monophyletic and independent clades) and venomous colubrids (a paraphyletic group) appear to have evolved independently (Jackson, 2007). Venomous snake fangs generally have a venom pore or groove that directs venom during a bite/strike. Given the variable nature of the placement of these pores and grooves, an experiment using pore placement as the standard puncture depth would likely be more biologically relevant than an experiment that uses a more arbitrary percentage fang length as the standard puncture depth. In theory, we would expect a puncture with these fangs to only be truly successful if the venom pore makes it into the open wound, allowing the venom to be injected. This then leads to the question of how variation in pore placement across taxa influences puncture performance across venomous snakes and whether we can capture that signal in experimental tests.

To determine whether we can identify a biologically relevant signal in the relative placement of the venom pore along a snake fang, we first explored morphological patterns across phylogeny. We compared pore placement variation across 60 species of venomous snakes (including vipers, elapids and colubrids) and tested whether this morphological feature correlates with phylogeny, diet or toxicology. Our hypothesis was that if pore placement is a functionally relevant feature, we would expect it to show significant variation across at least one of these *a priori* groupings.

In order to test how the identified variation in puncture placement may influence puncture performance, we evaluated the extent to which using a biologically relevant puncture depth (related to pore placement) alters comparisons of puncture performance across snake fangs compared with less biologically defined depths (such as percentage of overall fang length). We use the term 'depth standard' in this paper to denote the depth to which a puncture experiment is performed. So a percentage fang length or depth to a morphological landmark, such as the venom pore, would be considered alternative depth standards. To achieve this, we experimentally measured the work to penetrate across a subset of snake fangs; each fang was used to puncture a homogeneous material to a series of depth standards, some based on venom pore placement, some on a percentage of overall fang length (e.g. Crofts and Anderson, 2018; Galloway and Porter, 2021) and others on work to puncture initiation alone as several previous studies have done (e.g. Evans and Sanson, 1998; Schofield et al., 2016). We hypothesized that the pattern of relative performance between fangs would differ depending on the penetration metric used.

MATERIALS AND METHODS

Snake fang specimens

Snake fangs used for the comparative analysis of pore morphology were collected from several sources (see Table S1, Comparative specimens, for full details). Physical fang specimens, both isolated and attached to the jaw, were obtained from museum collections including: the Field Museum of Natural History (FMNH; Chicago, IL, USA), the University of Michigan Museum of Zoology (UMMZ; Ann Arbor, MI, USA) and the Smithsonian Museum

(USNM; Washington, DC, USA). Additional isolated fangs were provided by Latoxan (Portes-lès-Valence, France) and the Atlanta Zoo (Atlanta, GA, USA). To supplement physical specimens and expand the diversity included in the study, particularly elapid and colubrid fangs, we measured fangs isolated from computed tomography (CT) scans of an additional 45 specimens pulled from Morphosource (www.morphosource.org/). Only specimens with complete fangs and scale information were used for pore measures. In total, 75 fangs representing 57 species were used. The 57 species were placed in three groups based on phylogeny: viperids (19 taxa), elapids (26 taxa) and colubrids (12). For the phylogenetic analyses, we used the species-level, maximum-likelihood phylogeny of Figueroa et al. (2016).

Isolated fangs used in puncture experiments were obtained physically from museums (FMNH, UMMZ, Atlanta Zoo) (see Table S1, Comparative data, for full details), with express permission to use in non-destructive tests. In total, 26 fangs were tested representing 18 taxa across viperids (8), elapids (5) and colubrids (5).

Morphological data

For each fang included in our morphological analysis, we collected two morphological measures: total fang length (TL) (Fig. 1; top row) and the distance from the tip of the fang to the distal edge of the venom pore or groove (DDP) (Fig. 1; bottom row). We used the distal point as a conservative estimate for puncture depth as it is not clear how much of the pore needs to actually be in the prey for venom injection to be effective, and this may vary with venom toxicity. For viperid and elapid fangs, which have a fully enclosed venom tube and complete pores, we also measured the distance from the tip of the fang to the proximal edge of the venom pore (DPP) (Fig. 1; bottom row) and calculated the length of the pore (PL) by taking the difference of the two pore measures (DDP–DPP).

For isolated fang specimens, we use a stereomicroscope (Leica M205C) to collect lateral images of each fang to measure the total length of the leading edge, and rostral images of the fang tip and pore to measure the linear dimensions of the pore. For specimens too large to work with the stereomicroscope, we took lateral and rostral images using a Nikon D70 SLR with a Sigma 105 mm Macro Lens. Both sets of physical specimen images were measured in ImageJ (Schneider et al., 2012).

To collect equivalent measurements from the CT data, individual fangs were isolated and segmented in Avizo lite (v.2020.1). Because some scans had multiple erupted fangs, we focused our measurements on the longest fang on the right side of the jaw. Linear pore measurements (DDP and DPP) were collected via the built-in measuring tools. We were unable to collect the TL measurement of the leading edge of the fang in the same manner, as the surface is often strongly curved. To collect these data, we oriented the fangs to a lateral view and captured images with scale indicators, and then used ImageJ to measure the leading edge of the fangs.

Experimental design and data

To experimentally measure the work required to achieve biological puncture, we attached each of the 26 fangs to a custom-built holder which was hooked up to the 500 N load cell (0.25% accuracy at 1/1000 of load capacity) of an Instron 5944 (Norwood, MA, USA). Fangs were lowered at a rate of 30 mm min⁻¹ for quasi-static puncture, based on previous fang puncture papers (Crofts et al., 2019). Fangs were positioned such that the terminal portion of the fang was perpendicular to the surface of the target medium,

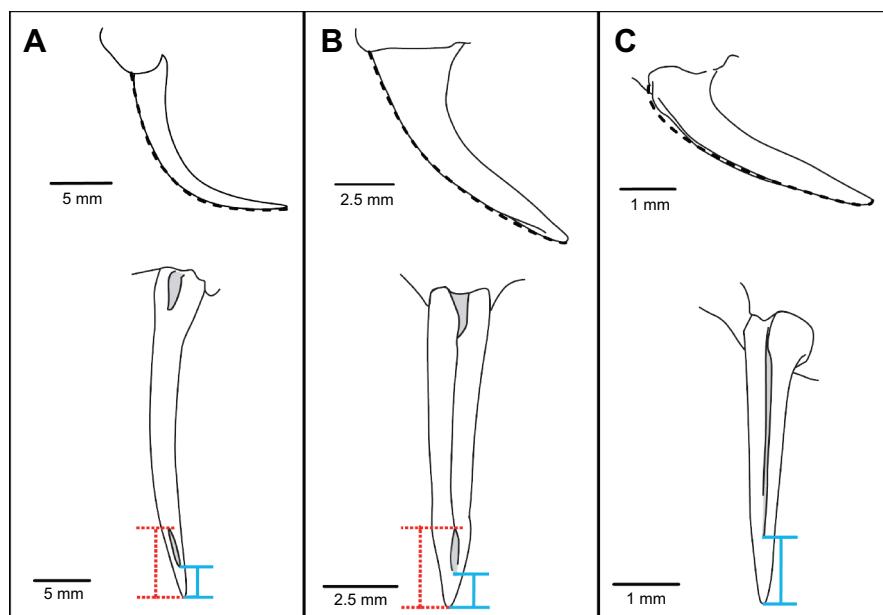


Fig. 1. Venomous fang morphology highlighting venom pore/tube location in the three groups studied. (A) Viperids: lateral (top) and anterior (bottom) view of fang from *Bitis arietans* (FMNH 31316). (B) Elapids: lateral (top) and anterior (bottom) view of fang from *Ophiophagus hannah* (FMNH 22275). (C) Colubrids: lateral (top) and anterior (bottom) view of fang from *Boiga irregularis* (USNM 301418). Black dashed lines (top panels) indicate measures of total fang length along the rostral face of the fang. Solid blue lines (bottom panels) indicate the minimum puncture depth required for the leading edge of the venom pore/tube to enter the target. Dotted red lines (top and bottom panels) indicate the depth to which the venom pore will be fully inserted into the target.

touching but exerting no force. This was done by lowering each fang slowly to the point where force began to register as greater than zero, then raising the fang until force once again read zero. To control for the material properties of the target material, we drove all fangs into cubes of 10:1 polydimethylsiloxane (PDMS) elastomer. Prey materials in nature will vary between taxa and will likely have an influence on puncture mechanics; however, we were specifically interested in the influence of different morphological characterizations here. We address the potential effects of varying materials in the Discussion. The 10:1 PDMS was prepared, following the manufacturer's instructions, with 10 parts Sylgard 184 silicone elastomer base to 1 part Sylgard 184 silicone elastomer curing agent by weight, which, when fully cured, has an elastic modulus of 2.66 MPa (SD 0.0797 MPa) (Wang, 2011).

We measured the force and displacement required to (i) initiate fracture, (ii) penetrate beyond the displacement at fracture to the depth of the leading pore edge (DDP), (iii) penetrate past fracture until the entire pore has been driven into the target (DPP) and (iv) penetrate to a depth of 15% of the total fang length past fracture. To account for deflection of the PDMS prior to fracture, we assumed all displacement until fracture occurs is deformation of the gel, confirmed via experimental observations of the gel surface (Movies 1 and 2). Measures of penetration to different functional depths were assumed to start at fracture, with minimal change in elastomer deflection. For most colubrids, with a venom tube rather than a venom pore, we estimated DPP to be approximately 32% of the total fang length, based on the average DPP measured in elapid and viperid specimens for the morphological analysis. The venom groove of the boomslang fang (*Dispholidus typus*) used in this study has a noticeable flare at the distal end, similar to a venom pore, which was used as the DPP instead (Fig. S1).

These force displacement data were used to calculate the work required to achieve each step (Fig. 2; Fig. S2): (i) the work to initiate fracture (WI), (ii) the work to penetrate to the leading edge of the pore (WPD), (iii) the work to penetrate to the trailing edge of the pore (WPP) and (iv) the work to penetrate to the arbitrary measure of 15% of the total fang length (W15). The work measured here is only one portion of the overall energy involved during puncture (Kundanati et al., 2020; Zhang and Anderson, 2022). Snake strikes in particular

will also have energy expenditures related to the ballistic head strike, venom production, venom injection, etc. However, the purpose of this study was specifically to evaluate how aspects of tooth form may influence the energetics of tooth penetration, so we focused specifically on the mechanical work measured over the course of a puncture event.

Morphological data analyses

To account for differences in size between fangs, the raw values of DDP, DPP and PL across specimens were regressed against TL and residuals were calculated, resulting in three new variables: DDP_r, DPP_r and PL_r. Regressions were performed using R statistical software (v.4.0.3; <http://www.R-project.org/>). These residuals were then averaged for each species. For all the following analyses, two datasets were created. For DDP_r, all 60 taxa were used; however, for DPP_r and PL_r, colubrids were removed from the dataset. Note that when comparing DDP_r directly with the other two measures [phylogenetic least-squares regression (PGLS) analyses, see below], the viper-elapid dataset was used.

To assess whether pore morphology is associated with taxonomy, diet or toxicology, we tested for differences in mean value for all three measures across different categories using phylogenetic ANOVA (Garland et al., 1993) as implemented in phytools (<https://CRAN.R-project.org/package=phytools>). For the ANOVA test, 1000 simulations were run, and *post hoc* tests were conducted to calculate pairwise differences between categories. For taxonomic grouping, the 67 species were placed in three groups: viperids, elapids and colubrids.

For dietary categories, we used a simplified version of the dietary categories defined in Cleuren et al. (2021). That study defined six distinct dietary categories based on general resistance of the food items. Based on how our taxon list overlapped with theirs, we combined their categories into the three used here: category 1 includes snakes that feed almost exclusively on prey with scales, representing more resistant integument (combines Cleuren's categories 2 and 4). Category 2 are generalists, with diets including both scale-covered prey and prey with soft integument (mammals and birds) (Cleuren category 3). Category 3 are snakes that eat primarily soft-skin prey items such as mammals, but also includes one taxon that eats fish eggs and another

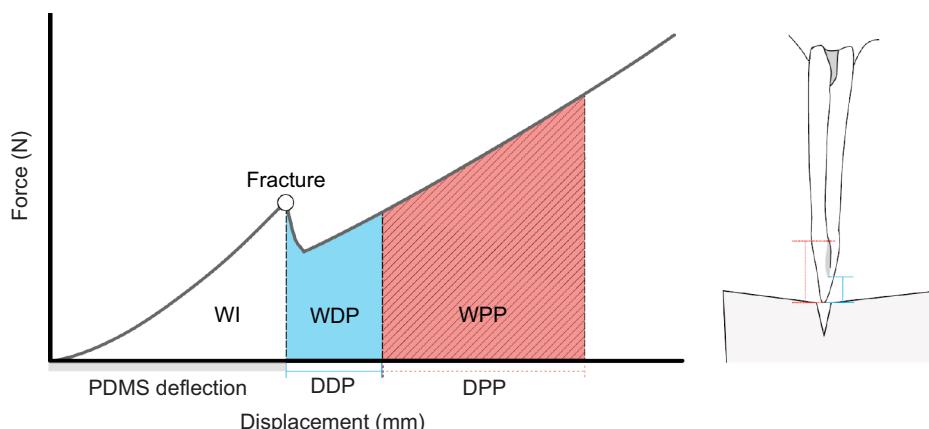


Fig. 2. Force displacement curve for an idealized puncture event, used to calculate four measures of work to fracture to penetrate the target. Work to initiate fracture (WI) was calculated as the white area under the force–displacement curve from test initiation to displacement at fracture (white circle), identified as a sharp drop in force. Work to penetrate to the distal edge of the venom pore (WDP; blue area) was calculated as the area under the curve from the displacement at fracture and extending the distance from the tip of the fang to the leading edge of the venom pore (DDP; solid blue line). Work to continue fang penetration until the entire pore was engulfed (WPP; red hatched area) was calculated as the area under the curve from the point where the pore first enters the target (DDP) to the point where the trailing edge of the pore enters the target (DPP; dotted red line). The final work calculated was work to penetrate 15% of the total fang length beyond fracture (W15), which has not been included in this figure as this displacement may occur before or after DPP, depending on the specimen. PDMS, polydimethylsiloxane elastomer (see Materials and Methods).

than eats primarily annelids (combines Cleuren categories 5 and 6). Some of the taxa in this study were not analyzed in Cleuren et al. (2021). For those groups, dietary data were sourced from the literature (Table S1, Comparative data, shows the dietary assignments for each taxon with references).

When considering what might be influencing venom pore morphology, we speculated that aspects of the venom itself may be important and predicted that snakes with less-toxic venom may need to ensure more of the pore enters the wound created to better deliver a lethal dose. Although snake venom toxicity is a major area of research, we were not able to identify a standard measure that has been applied to a wide enough range of taxa to be used for comparative work. Most studies report the dosage required to kill 50% of mice administered to (LD50); however, the methodology used to measure this varies between studies. For this study, we made use of an online database (www.snakeDB.org) which has collected published toxicity data with references. As the toxicity data collected from this website will include measures from different methods, we used broad categories based on order of magnitude of the LD50 values: category 1 is less than 1 mg kg^{-1} ; category 2 is between 1 and 5 mg kg^{-1} ; and category 3 is greater than 5 mg kg^{-1} . As the venom data are a mix of different experimental methods themselves, we do not claim that this analysis captures the actual relationship between toxicity and pore morphology but see it as a preliminary test to ascertain whether this avenue is worth pursuing with more rigor.

To assess whether the pore measures were correlated with overall fang size across all taxa or within specific groups, we performed PGLS using the R package CAPER v.0.2 (<http://cran.r-project.org/web/packages/caper/index.html>). PGLS is a common method for comparing evolutionary covariation between variables (Pagel, 1997). For these analyses, delta (change in rate of evolution), kappa (gradual versus punctuated evolution) and lambda (phylogenetic signal) were left at default values. PGLS analyses were run for all three measures both with and without taxonomic group as a factor.

Experimental data analyses

To account for differences in size between fangs, the raw values of WI, WDP, WPP and W15 were regressed against TL and residuals were

calculated as for the morphological data. However, the performance values being measured were work required to penetrate, which will be directly related not to just the length of the fang but also to the fracture surface created by the penetration. Therefore, the work should scale not with length but with area. To account for this, regressions were done against TL^2 resulting in four new variables: WI_r, WPDr, WPPr and W15r. Regressions were performed using R statistical software (v.4.0.3; <http://www.R-project.org/>). Residual values were then averaged across the individual runs for each specimen (Table S1, Experimental data). To assess differences in the performance metrics between the three major taxonomic groups, we utilized ANOVA tests to compare mean values.

To assess how relative functional performance between snake fangs can be influenced by the use of different penetration standards, we performed two specific tests. First, to explore broad scale patterns, we ran Spearman rank correlations between each pair of work values; these test for monotonic correlation between data, describing whether the rank order of energetic performance across fangs remains constant regardless of the metric used. Then, we performed Bonferroni pairwise comparisons across all 26 fangs, to see whether the specific patterns of mean differences remained constant across the different work measures.

RESULTS

Comparative pore morphology results

Phylogenetic ANOVA tests across our snake fang dataset showed no significant differences in any of the pore measures between taxonomic groups, dietary categories or toxicity categories (Table 1). The only *P*-value that was somewhat close ($P<0.15$) was for PLr across toxicity categories. However, given the vast uncertainty in the toxicity data used, we can only say that no systematic differences in pore morphology were shown across taxonomic group, diet or toxicity level.

The PGLS results showed some relationship between pore measures with each other and size (Table 2). DDPr was the only pore measure that showed significant correlation with fang length (with both full and viper–elapid only datasets); however, the r^2 values were around 0.1. In none of these comparisons with size did taxonomic group act as a significant factor. While DDPr did not

Table 1. Phylogenetic ANOVA results for pore structure measures against the categorical variables clade, diet and toxicity

Measure	Category	Dataset	F-value	P-value
DDPr	Taxonomic group	Full	1.583	0.600
DDPr	Taxonomic group	Viper and elapids	0.867	0.599
DPPr	Taxonomic group	Viper and elapids	0.286	0.776
PLr	Taxonomic group	Viper and elapids	0.1782	0.843
DDPr	Diet	Viper and elapids	0.791	0.536
DPPr	Diet	Viper and elapids	0.0319	0.977
PLr	Diet	Viper and elapids	0.0367	0.967
DDPr	Toxicity	Venom list	0.285	0.753
DPPr	Toxicity	Venom list	1.767	0.226
PLr	Toxicity	Venom list	2.292	0.127

None of the analyses showed significant differences between categories for any of the measures. DDPr, distance to distal edge of the pore; DPPr, distance to proximal edge of the pore; PLr, total pore length; r, residual.

show significant correlation with PLr, there was a very tight correlation between DPPr and PLr (Table 2, Fig. 3). The scatterplots in Fig. 3 show these relationships graphically, making it clear that the only close correlation was between DPPr and PLr.

Experimental results

ANOVA tests showed no significant difference between taxonomic groups for any of the four work metrics (WI, W15, WPD and WPP; Table 3).

Spearman rank tests between each pair of work metrics showed significant monotonic correlations between each pair (Table 4), indicating that the rank order of work efficiency across fang tests was similar for all metrics. However, the actual rho values showed a range of variation from $\rho=0.84$ between WPP and W15 down to only $\rho=0.47$ between WPD and WI. The fact that for none of these did $\rho=1$ means that, in all comparisons, there were differences in the rank order. This is made even more apparent when examining the pairwise Bonferroni tests between the 26 specimens for each work metric (Fig. 4). Fig. 4 shows the pattern of significant differences between fangs for all four metrics. The gray cells represent comparisons between two fangs that were significantly different. It can be seen that the pattern across fang comparisons was vastly different for each work metric.

Table 2. Phylogenetic least-squares regression (PGLS) results for various pore structure measures

Comparison	Dataset	F-statistic	r^2	P-value
DDPr~TL	Full	5.977	0.098	0.018*
DDPr~TL	Viper and elapid only	8.474	0.1646	0.0057*
DPPr~TL	Viper and elapid only	0.296	0.00683	0.59
PLr~TL	Viper and elapid only	0.0382	0.0008	0.846
PLr~DPPr	Viper and elapid only	2.18	0.048	0.1471
PLr~DPPr	Viper and elapid only	252.7	0.855	<0.00001*

An asterisk denotes a significant phylogenetic correlation between measures. DDPr, distal edge of the pore; PLr, total pore length; TL, total length; r, residual.

DISCUSSION

The combined results from the morphological and experimental data paint a potentially surprising picture of how pore placement influences function. While the morphological data showed almost no pattern across phylogeny, whether it be differences between clades, dietary groups of venom toxicity, the experimental data did illustrate that pore placement is variable enough to influence experimental puncture results, at least at the level of individual comparisons. The experimental data present a forest versus trees scenario: a broad comparison across all fangs showed no significant differences between experimental results derived using different depth standards; however, pairwise comparisons did show major shifts in experimental results depending on which depth standard was used. Below, we unpack what these results mean for comparative biomechanical studies using experimental data as well as the functional morphology of venom pores in snakes.

Venom pore morphology and function

Despite our initial hypothesis, pore placement and morphology showed no significant pattern with regards to taxonomic group, diet or venom toxicity. One potential explanation is that the variation seen in pore placement is simply not functionally significant. Our four experimental depth standards did not result in significantly different rank orders between the taxa, which could mean that the difference in work to puncture cost is simply not enough to drive evolutionary adaptation towards a particular pore morphology. Alternatively, perhaps the variation is simply too low, possibly due

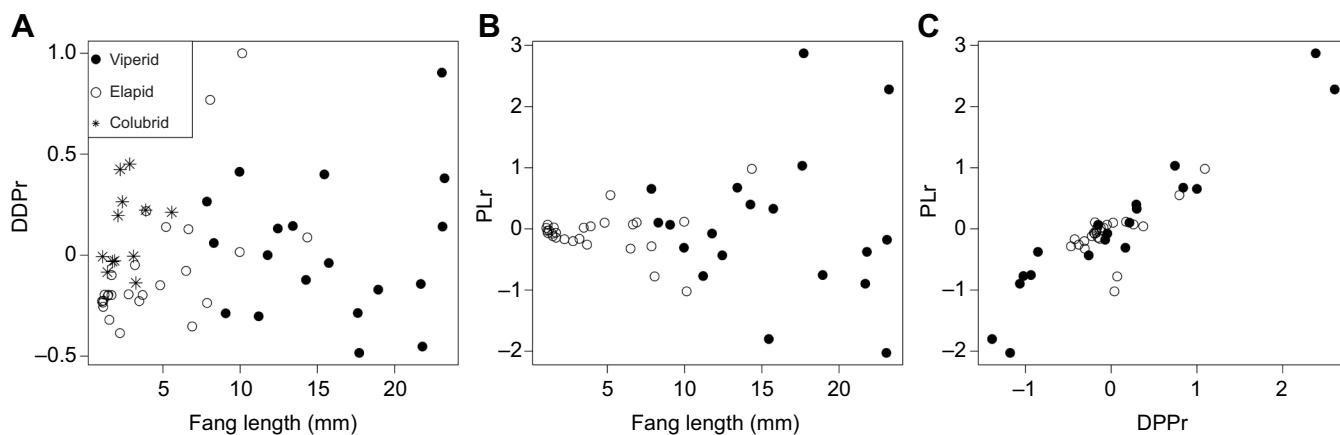


Fig. 3. Scatter plots showing general patterns of morphological covariance between measures across 57 snake taxa. (A) Comparison between the distal edge of the pore (DDPr) and fang length shows no scaling, but does show how limited in size range and variation colubrid specimens ($n=12$) are compared with viperid ($n=19$) and elapid ($n=26$) specimens. (B) Comparison between total pore length (PLr) and fang length shows no scaling, but illustrates a dramatic difference in variation in pore length between short and long fangs in viperids ($n=19$) and elapids ($n=26$). (C) The total pore length (PLr) correlates strongly with the distance from the tip of the fang to the proximal pore edge (DPPr) in both viperids ($n=19$) and elapids ($n=26$). For results of statistical phylogenetic least-squares regression (PGLS), see Results.

Table 3. ANOVA results for work metrics based on four different depth standards

Work measure	Category	F-value	P-value
WI	Three clades	2.973	0.071
W15	Three clades	1.894	0.173
WPD	Three clades	0.655	0.529
WPP	Three clades	0.086	0.918

None of the analyses showed significant differences between clades for any of the measures. WI, work to initiate fracture; W15, work to penetrate to 15% total fang length; WPD, work to penetrate to the distal pore edge; WPP, work to penetrate to the proximal pore edge.

to genetic drift on a feature otherwise not under selection. This seems particularly to be the case for smaller sizes, with minimal variation in PLr for the shortest fangs examined in this study (Fig. 3B). Even at larger fang sizes, which the variation in PLr increases, the overall pattern showed no noticeable trend.

It is of note that one of the few PGLS analyses that resulted in a significant correlation between measures was between pore length and

Table 4. Spearman rank correlation results for work measures based on the four different depth standards

Comparison	p	P-value
WPDr versus W15r	0.693	0.00013
WPPr versus W15r	0.838	0.000002
WPDr versus WPPr	0.668	0.00027
Wlr versus WPDr	0.473	0.016
Wlr versus WPPr	0.581	0.0022
Wlr versus W15r	0.819	0.000002

All pairwise comparisons show significant monotonic correlations. WPD, work to penetrate to the distal pore edge; W15, work to penetrate to 15% total fang length; WPP, work to penetrate to the proximal pore edge; WI, work to initiate fracture; r, residual.

distance from the tip to the proximal end of the pore. Why these two correlated while neither correlated with distance to the distal end of the pore is not clear. Fang development begins at the tip of the fang and extends up to the base, following a wave of differentiation (Jackson, 2007; Zahradnicek et al., 2008). The development of the venom tube

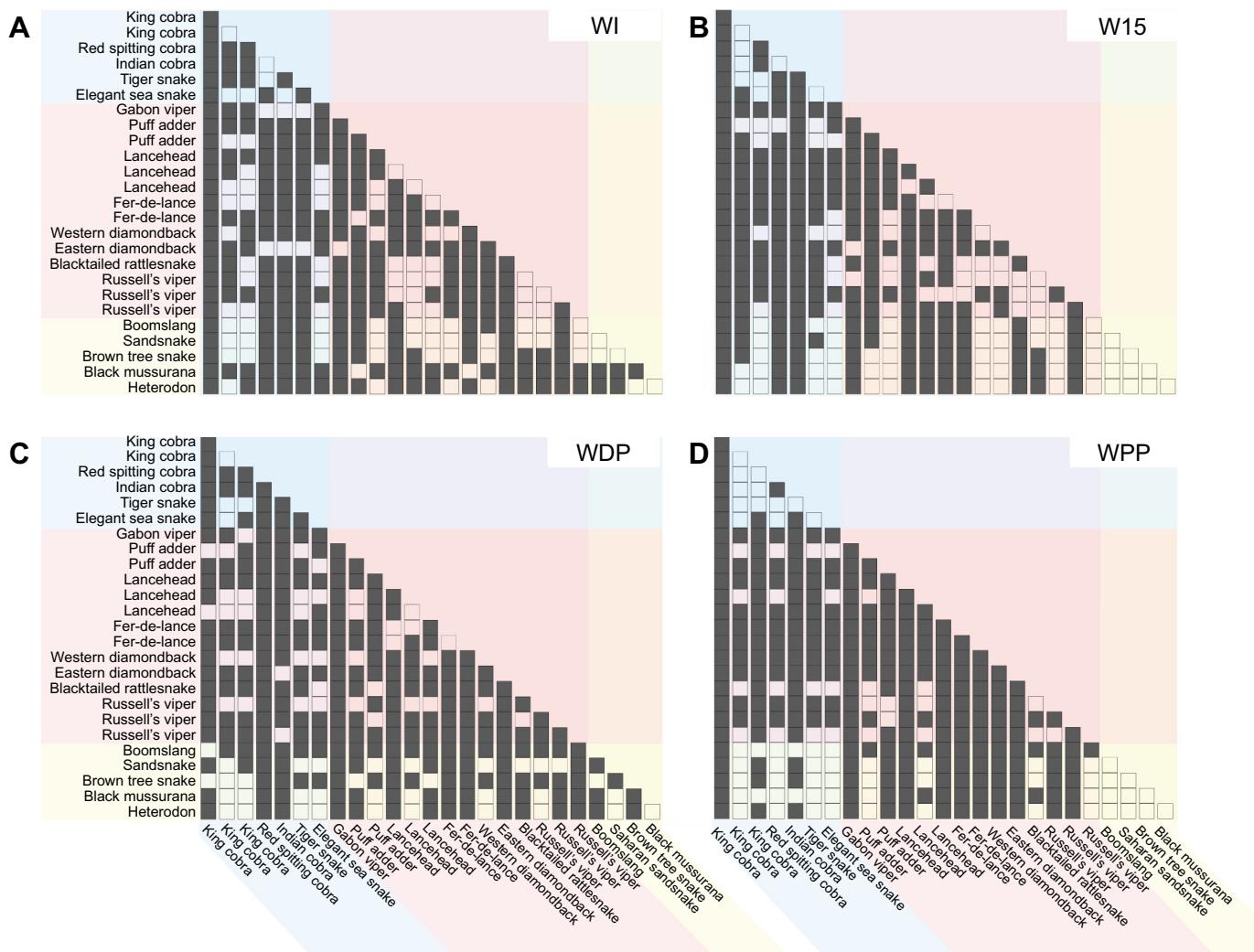


Fig. 4. Bonferroni pairwise comparisons of puncture work data using four different depth standards. (A) Work to initiate fracture (WI). (B) Work to penetrate to 15% total fang length (W15). (C) Work to penetrate to the distal pore edge (WDP). (D) Work to penetrate to the proximal pore edge (WPP). A total of 26 fangs were each tested 10 times per depth standard. To aid in visualization, comparisons have been color coded: elapid fangs are shaded blue, viperid fangs are shaded red and colubrid fangs are shaded yellow. Gray squares indicate significance ($P<0.05$). While the Spearman rank analysis showed significant overall correlations between depth standards, more fine-scale patterns of pairwise significance varied dramatically.

(or groove), basal orifice and pore arises from varying degrees of infolding of the developing tooth epithelium (Zahradnicek et al., 2008). The timing of this tip to base signal in conjunction with the degree of cell proliferation may be responsible for the variation we observed in pore placement and may simply not be that tightly controlled. There are a limited number of papers on venom canal development, and the mode of regulation underpinning pore location remains unknown. It may be that there is variation between taxa, as one study found that the pore of *Naja melanoleuca* (elapid) fangs remained the same length between the working fang and all replacement fangs, whereas the pore of *Bitis arietans* (viperid) appeared to become smaller in successively more mature replacement fangs (Jackson, 2002). Alternatively, epigenetic influences could also play a role in the end result of this development.

Another possibility, and one that is hinted at by our experimental results, is that we simply have not asked the right questions. We specifically tested whether pore morphology is different between three taxonomic groups (two monophyletic and one not), dietary groups or clusters of taxa based on the toxicity of their venom. While these seem like reasonable groupings to test against pore morphology, they may not be hitting the mark. If we believe pore morphology to be functional, we may not expect it to be associated with taxonomic groups which contain a diversity of snakes with variable diets and life histories. While diet has been shown to potentially correlate with overall fang shape (Cleuren et al., 2021), this may be more related to initial fracture than to the penetration needed to inject venom.

Venom toxicity

Venom toxicity would seem to have a direct connection to the venom pore; however, any such patterns are more difficult to tease out than one might expect. Snake venom toxicity is a major area of research because of the consistently high mortality rate from snake bites every year. Unfortunately, this has led to multiple measures of toxicity being developed with no apparent standard to better allow for quantitative comparison, and we were not able to find a standard measure for comparative work. The closest we came was an online database (www.snakeDB.org) in the form of a table collecting published toxicity data with references. Most toxicity measures in snake venom use LD₅₀, the dosage of venom that kills 50% of the mice it is administered to. However, the method of delivery varies highly, with some studies injecting the venom under the skin (subcutaneously) while others inject directly into the bloodstream (intravenously). Data taken with the intravenous method likely give a good minimum for the amount of the toxin necessary to kill. However, unless a snake fang is lucky enough to hit a vein during a bite, it is likely not as biologically relevant as the subcutaneous method, which better mimics a fang breaking the skin and injecting into the body. Thus, it is inappropriate to directly compare values based on these different methods, but in order to get a broad enough coverage of taxa, that is all that can be done. If future work is going to utilize toxicity data for comparative work, some standardization will need to be implemented.

While the nature of venom toxicity data may be one confounding factor when trying to tease out patterns in venom pore functional morphology (as elaborated above), another is that it may not be the toxicity as much as the volume of toxin delivered that could correlate with pore morphology. Volume of delivery, while sometimes recorded in studies on toxicity, is still rarely reported, making it not possible to compare with the pore data. Moreover, there is evidence that viperids, with their enclosed tubular fangs, can control the amount of venom delivered via pressure-driven injection as well as suction (Young and Zahn, 2001). In contrast, colubrids are unable to

generate a pressurized bolus of venom and depend on the non-Newtonian fluid dynamics of the venom to ensure that it flows into wounds, with little to no control over the volume of venom transferred (Young et al., 2011; Kardong and Lavin-Murcio, 1993).

The importance of experimental design

The Spearman rank correlation results make it clear that, at a broad scale, the choice of puncture depth standard may not have much influence on a comparison of experimental results. Spearman correlations are monotopic, meaning they test whether the rank order of the data is maintained, and this seems to be broadly true between the different depth standards. However, in none of the cases did $p=1$, meaning there is some shifting in the rank order. This is made abundantly clear in the pairwise tests, which show large changes in patterns of significant differences between pairs of fangs (Fig. 4). As an example, the red spitting cobra shows a significant difference in work from all other fangs when distal pore depth is used as the standard; however, when 15% fang length is used as the standard, its performance does not differ significantly from that of other cobras or even a puff adder (Fig. 4). Similarly, the work to puncture used by the boomslang fang tested to 15% fang length is significantly different from that of 12 of the 20 viper and elapid fangs tested, but not from that of any other colubrid fangs included in the analysis. When tested to the distal pore depth standard, however, the boomslang performance is significantly different to that of all fangs save those of one king cobra and the brown tree snake fang (Fig. 4).

Our experimental data illustrate the importance of understanding the biological context and question being addressed when designing an experiment. Looking at the venom pore data, it is not surprising that altering the depth standard during puncture tests would alter the comparative results of the study. What is less intuitive is which standard is best for a given project. WI, WPD and W15 are all valid depth standards, just for different questions at different levels. WPD makes the most sense when comparing work related to successful puncture-for-injection in venomous snakes, but both WI and W15 could be important for other questions: a lot of previous work on tooth shape has correlated the sharpness of cone-like teeth with their ability to initiate puncture in different types of biomaterials (Evans and Sanson, 1998; Lucas, 2004; Conith et al., 2016; Crofts et al., 2019) and even the study from which we derived our dietary categories suggests that snake fang shape may correlate with diet based on the properties of the integument (Cleuren et al., 2021). In all of these studies, work to initial fracture (WI) would be the most relevant metric for comparative experimental studies. The difference between these metrics is simply whether your interest is in the ability to break the skin or to successfully inject venom, which are strongly connected to each other, but do represent distinct questions. Even the '15% of fang length' standard depth (W15) is useful if the question is less about the function of specific taxa and more about tooth form in general. Several recent studies concerning the shape of puncture tools have treated tool taper as an important metric (Bar-On, 2019; Kundanati et al., 2020; Evans et al., 2021; Zhang and Anderson, 2022). For such comparative studies, a 15% depth standard may be appropriate as it removes details of the different systems and allows for a comparison purely based on the shape across systems.

Experimental design and biological reality

In this study, we were interested in how altering experimental design to account for an aspect of biology may influence the experimental results. We were specifically focused on a morphological feature: venom pore position. As such, we held several other variables

constant which will be potentially important in a snake strike: prey materials and strike dynamics. While it was necessary to remove these variables from the present study to allow us to isolate the variable of interest, they are important enough to demand discussion of how they may further alter the results found here.

The material properties of the prey substrate being punctured by the snake fangs will likely have a large influence on the relationship between puncture depth and puncture energy (Anderson, 2018). At the broadest scale, brittle or stiff materials (ceramics such as mollusk shells) and ductile materials (vertebrate integument) will show very different behaviors when punctured (Anderson, 2018; Zhang and Anderson, 2022). While ductile materials subject to a load will deform and dissipate energy before failure, brittle materials do not deform and instead store energy until catastrophic failure occurs. For the most part, these snake fangs are puncturing more deformable/ductile materials such as vertebrate integument (Cleuren et al., 2021).

However, not all integument (skin) will act the same, as vertebrate integument is a fiber-reinforced composite composed of multiple layers often with varying properties (Lanir and Fung, 1974; Wainwright et al., 1978; Greal et al., 2018). Unfortunately, while several studies have examined the material properties of different types of vertebrate skin (Belkoff and Haut, 1991; Dirdolou et al., 2000; Shergold et al., 2006; Pailler-Mattei et al., 2008), little has been done on how variation in biological materials or thickness of materials in multi-layered integument influences puncture directly (Carra and Avila-Vilchis, 2010; van Gerwen et al., 2012).

Moreover, the speed at which strikes occur, another biological variable we have not considered in this study, could also affect the functional material properties of the prey substrate during a strike. Biological tissues, such as skin, are viscoelastic, meaning that their response to applied stresses is dependent on the rate of application (Vogel, 2013). A general rule of thumb for materials is that when the rate of deformation increases, materials become more resistant to deformation, essentially becoming stiffer (Anderson, 2005; Karunaratne et al., 2018). While many studies have shown this pattern of rate-dependent strain stiffening in bovine and porcine integument and connective tissues (McElhaney, 1966; Van Sligtenhorst et al., 2006; Shergold et al., 2006; Song et al., 2007; Cheng et al., 2009; Nie et al., 2011; Comley and Fleck, 2012), few studies have explicitly investigated how this rate dependency might influence puncture. Materials do respond to puncture forces differently at variable speeds (Anderson et al., 2016, 2019; Clark et al., 2021), including differences between the puncture speeds used in this study and the speed of the few snake strikes that have been measured in the literature, all of which are around $1\text{--}3\text{ m s}^{-1}$ (see Penning et al., 2016, for a review). If we think about this in relation to our viper fangs, striking at higher rates would cause the prey material to deform less, potentially causing different integument materials to act more similarly than they would at static speeds. However, it is unclear how this would affect puncture depth in relation to pore placement.

While the lack of good data on puncture resistance in biological material precludes incorporating such data directly into our study, we did attempt to account for prey material in a broad sense through the comparative analysis. The dietary categories we used were taken from a previous study, which based their categories on the assumed puncture resistance of the prey items (Cleuren et al., 2021). These categories generally separated prey covered in scales versus integument, with some much softer prey included as well. Interestingly, our analysis did not show diet as having much correlation with pore placement, implying that at least one aspect of the properties of the prey material may have little influence on venom pore position. However, dietary categories often require the

clustering of disparate prey that likely obscure any real biological signal. While coarse categories such as these are ill suited to understanding biomechanical systems as investigated here, they remain an unfortunate necessity when trying to explore ecology.

Overall, there are a lot of factors that can influence the depth of puncture during snake strikes that may help explain the variation in venom pore placement we observed. However, a great deal more work must be done on understanding puncture resistance across biological tissues and the influence of dynamic strain rate on tissue behavior during puncture before we can fully address these complications.

Conclusions

Snake strikes and envenomation have long been subjects of interest for comparative biologists, with recent studies increasingly focused on the underlying mechanics at play. The goal of this study was to see how the choice of experimental design, represented here by the depth to which a puncture experiment is performed, in a comparative experimental study of snake fangs can influence the results. We found that for the puncture depth standards we tested, while rank analysis shows significant overall correlations, more fine-scale patterns of pairwise significance vary dramatically. This highlights the importance of considering how to best compare experimental data across disparate taxa. While this study focused entirely on puncture experiments, the lessons learned are invaluable to any experimental study in biology.

Experimental design has always been a vitally important part of biological studies and as more and more biologists attempt to incorporate experimentally derived data into large-scale comparative analyses, it will only become more important. Regardless of the system, care must be taken when determining the specific biological traits to be used in comparative studies. Depending on the taxonomic breadth being considered, comparisons may be difficult as some traits simply do not exist in all groups. For example, comparing respiratory systems across all vertebrates would require finding functionally relevant traits that are measurable in both gills and lungs, such as flow rate past the respiratory organ. As experimental biomechanics becomes more incorporated into comparative studies, experimental biomechanists need to take similar care in designing experiments that capture the question being asked across the level of taxonomy being explored. While these considerations can be challenging to experimental biologists trying to design their methodologies, the benefits are significant. Incorporating experimental data into comparative studies will deepen our understanding of these fundamental systems, so long as we can see the trees within the forest.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.B.C., P.S.L.A.; Methodology: S.B.C., P.S.L.A.; Formal analysis: S.B.C., P.S.L.A.; Investigation: S.B.C., P.S.L.A.; Data curation: S.B.C., P.S.L.A.; Writing - original draft: S.B.C., P.S.L.A.; Writing - review & editing: S.B.C., P.S.L.A.; Visualization: S.B.C., P.S.L.A.; Supervision: P.S.L.A.; Project administration: P.S.L.A.; Funding acquisition: P.S.L.A.

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Data availability

All relevant data can be found within the article and its supplementary information.

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