Curiouser and curiouser: Meta-analysis of venom toxicity of 160 lethal ophidian species shows extraordinary variance, and falsifies common assumptions, providing a basis for estimating human lethal doses.

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Research

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Abstract

Background: This is the first meta-analysis to characterize intra-ophidian-species variation in whole venom. The largest meta-analysis possible at this time, it encompasses all known publicly available records of animal lethality studies over the past 100 years. These results are not artifacts of resistant test-animal-species, and show orders of magnitude beyond the 1.6 logs (40 fold change) range of lethal dose documented in literature between amphibians, lizards and mice.

Methods: 1005 lethal dose study results for 160 of the most lethal venomous ophidian species in the world are analyzed.

Results: LDLo does not differentiate from LD50 across studies, indicating the true range of toxicity is probably larger. The belief that for route of inoculation, IC<IV<IP<IM<SC has good support (R^2 = 0.86). However, 8% of SC inoculations were the lowest (most toxic) dose. Within the mouse test species, for one route of inoculation, the mean LD range is 0.94 logs (8.89 fold change), and widest LD range is 2.15 logs (141 fold change, N = 26). Within 1 test species, for multiple routes of inoculation, mean LD range is 2.0 logs (98.97 fold change), and the widest LD range is 3.6 logs (4,150 fold change). For all test species and all routes of inoculation, the mean LD range is 2.97 logs (936.59 fold change) and widest LD range is 4.76 logs (57,471 fold change). The strongest correlate for range of lethal dose results is the number of studies (R^2 = 0.56). The average variance appears to follow a power law from single test species and route, to single test species and multiple routes, to all test species and all routes, as a factor of 10 multiple for each.

Conclusions: Scientists working with humans should use combined LDLo and LD50 meta-datasets for all data and calculate: mean, median, minimum, range, and standard deviations. Standard deviation multiples, or at minimum, standard error, will provide desired coverage. For estimating LD50 range and minimum lethal dose for species with little data, I recommend curating a meta-dataset of related snakes, and computational research to strengthen this.

Preamble

Klauber noted that it was difficult to reproduce results of lethal dose experiments in 1956, saying a factor of ten was common, but without presenting data [1]. That 70 year old observation may have permeated the field of venomics, and some have filed this under a “do not investigate” sign. In recent time, others have confirmed this observation and this work attempts to begin examining it [2]. The results presented here were not only utterly unexpected, they have created complications for a research clinical trial that sparked it. An afternoon’s curiosity creating a first graph of the 18 venoms studied in the human project showed clearly that variance was far beyond anything I had imagined or found literature about. Within the venomics scientific community some have shown extraordinary resistance to accepting the data and results presented here. This quote typifies this:

“*...including in the same equation data subject to individual, geographical, and ontogenetic intra- and inter- variability across all levels of ophidian taxonomy and taxa with different dietary habits, in addition of variable route of inoculation and experimental animal used, is like drawing conclusion from the analysis of the variability of the parts of a complex machine, without differentiating the type of piece or the type of machine!*” – *Anonymous*

Curiously, this statement intended to oppose, actually supports the results presented here. If one accepts that individual ophidians have so much intraspecific variation in venom toxicity that venoms should not be compared, then the variability shown herein should not surprise, nor should this first attempt to quantify and characterize it be objectionable. Likewise, if there is such geographical and ontogenic variability across all levels due to dietary habits,

then again, these results should not shock, but simply quantify. However, the contention that one cannot do such comparison meaningfully is obviously not accepted by scientists in the field, because quite a few studies published by luminaries seek to determine variability of venom [3-17].

What this work does not do, is to throw all into one bin thoughtlessly. Each ophidian species is evaluated within itself. When the data is compared across species, these comparisons are done with what is called normalized data in order to make a valid comparison. That is the purpose of the basic algebra that is used. Route of inoculation and test animal species are major foci of this paper. Again, this was not done haphazardly, it was done using methods carefully described to attempt to determine whether common beliefs within venomics science are correct. The use of R^2 as a measure of regression fit, and the basic algebra herein are not controversial methods.

Biology in general has embraced mathematical methods such as those used here. There are, biostatistics, bioinformatics, a long history of mathematics in biology, and an exciting future [21]. And sometimes the results of such analysis may contradict common beliefs. For those interested, I recommend Gullberg's, Mathematics From the Birth of Numbers [22].

It should be noted that this analysis provides justification for considerable future research that could lead to new understandings regarding venomous ophidians.

Introduction

Humans are not prey for any venomous ophidian species, and bites are defensive [23]. Consequently, what the prey species taxa is for an ophidian species does not matter for estimations of human lethal dose. Nor is it common practice in testing to use test species that are not mice, pigeons, or rats, and this dataset is no exception. Thus, human lethal dose-response to ophidian venom is a thorny problem, as it is impossible to perform ethical lethal dose studies on humans[24]. This forces the use of animal studies, despite the problem that human dose-response may be different than other animals, including monkeys. When LD50 for a different species is used to guess at the human lethal dose, this is typically calculated by choosing a single study and multiplying a value by the mass of a typical human [2]. This procedure is based on common assumptions that will be discussed below. Anecdotally, scientists and physicians have a bias toward more recent studies. Only recently have scientists begun to formally study the high intraspecific variance in ophidians [2].

While surface area methodology for venom dose response could be used, there is no identified instance in literature, and none in this database, where venom dose is stated allometrically. No physician who works with venom said they use this alternate method to estimate venom dose effect. Allometric scaling is further discussed in the confounders section.

When selecting a study, some variation is expected between test species for the same ophidian species, but not a great deal within the same test species.

A common assumption is that lethal dose toxicity varies by method of inoculation from most toxic to least toxic as: intracerebral (IC), intravenous (IV), intraperitoneal (IP), intramuscular (IM) and subcutaneous (SC). Regarding inoculation methods, there is also a view that comparison of results between inoculation methods varies radically, like "apples and rocks"[25], but no formal meta-analysis backs up either assumption, nor does any seek to quantify. These assumptions imply that as long as the route of inoculation is constant within the same test species, then toxicity reports across studies should be reasonably close, which makes selection of a study to use for estimating human effects fairly arbitrary.

Every scientist in the field whom I asked (data not shown), believed that the LDLo value should be lower than LD50, and that this should hold across studies. This assumption regarding LDLo is also implicit in the publication of LDLo values at all, because if they were not meaningful then they would not be defined as something worthy to capture and record. By definition, LDLo should be lower than or equal to LD50.

Variation in venom and prey (test animal) susceptibility

There has been literature suggesting the common assumptions of lethal dose should be reconsidered for some time, with recent authors documenting variation in venom components. Variation of venom by season, and between related species and subspecies has been a consideration for antivenom preparation [5, 6, 10, 14]. Geographic area and predator-prey evolutionary relationships are another factor in venom variation [4, 7, 9, 12, 13, 15-17, 26]. In addition to geographic differences, ontogeny can affect venom component variance [3, 14], which is presumed to be an adaptation to differing prey species during development. Differences may also be linked to the sex of the ophidian [8].

Recently, some degree of plasticity of venom in *S. m. barbouri* in response to prey species was documented, which should create some intra-test-species variance in LD50 [11]. It was also shown that *C. simus* modifies its venom with miRNA to achieve small variation based on prey species, which implies some unquantified degree of intra-test-species variation in LD50 for this ophidian species [27]. And recently, intraspecific variation in rattlesnake venom neurotoxin was identified as significant within a geographic area, ranging from near zero neurotoxin, to a large fraction. This implies an unquantified degree of intra-test-species LD50 variation by an unspecified mechanism [28, 29].

Generally, as long as the test animals are not species that have adaptations to venom such as the western ground squirrel (*Spermophilus beecheyi*)[30], some opossums (*Didelphidae*), hedgehogs (*Erinaceidae*), mongooses (*Herpestidae*), weasels (*Mustelidae*), some skunks (*Mephitidae*)[31], or the honey-badger (*Mellivora capensis*)[32], then test species is not documented to make major a difference. Except for the ground squirrel, these are predator species that prey on venomous snakes. I note, however, that in this metadataset there are indications that other resistant species exist besides the western ground squirrel; for instance the cotton rat's results were an extreme outlier. For this reason the cotton rat data was removed in curation as it is not a standard laboratory rat.

Among prey species, literature shows that lizards probably require roughly 4X the venom dose that mice do as demonstrated by the relative dose delivered by *C. concolor* [33]. Frogs are an order of magnitude (10X) more resistant to *Sistrurus* venom than lizards, while in mice, the variation in toxicity of *Sistrurus* venoms correlates with mammals in the snake's diet [34]. (Mouse variance was unspecified, but assumed less than an order of magnitude since it was not specified in the same paper discussing frog resistance.)

Multiplying the single order of magnitude of frogs by the 4X of lizards gives us a total 40 fold change currently documented between test-animal-species, which is 1.6 logs. Thus, differences between test-animal-species are expected, but based on current literature, with the exception of frogs, these differences should be less than one order of magnitude.

Characterization Of Data

This meta-analysis includes every known study for the top 264 venomous ophidian species winnowed to the 160 species with 2 or more studies. These data were primarily extracted from the Steinhoff database, and secondarily from the Drugfuture database[35, 36]. The Drugfuture database is cited because more than half of the Steinhoff database entries were obtained from it.

Operation of databases

The Drugfuture database (“□□□□□□□□□ Chemical Toxicity Database”) is maintained in China. The operation panels are bilingual in English and Chinese. There is one data entry field. To start using it, enter ‘venom’ in this field and press the enter key. This will bring up a list of the first 200 venoms in the database. Click on an entry and a record is displayed, including the citation for each. The search can be refined by Latin name. All data records are in English. The Drugfuture site is supported by advertising, which is not intrusive.

The Steinhoff database is also easy to use. One can scroll down through the list which is ordered by nominal toxicity, or use the Ctrl+F feature of your web browser to find a particular species. Click on one of the “ref” links in the toxicity columns, and the entire set of citations will be presented. The Steinhoff database has the Drugfuture references pulled in so they are directly visible there. There is no need to click through to the Drugfuture database.

Curation

The dataset was extracted in May of 2017 and curated according to the following algorithm:

Attempt to obtain each cited source. For each entry, find it in the paper, and document in the comment column. If the data in the paper requires conversion, describe the conversion.

If the cited book or paper could not be obtained, then apply the following rules:

1. If the data has verified cites above and below it, then accept it as reasonable.
2. If the data is a high or low value that is less than a factor of 3 from the value next to it, accept it.

In the curation process, 206 records were removed (table 1A), and 70 corrections made, (table 1B). However, as expected, this had a mild effect on the meta-analysis results. The dataset is large enough that errors should tend to be randomly distributed. Few of the venom fraction records removed changed the range, which suggests that venom components tend to be more toxic when combined.

Table 1A: Deletions from metadataset.

23	33	13	5	6	53	40	8	25	5	206
Venom fraction	Not found in paper.	Bad citation	Wrong species	Arithmetic error	Duplicate	Neither LDLo or LD50	Dubious	Unable to validate	Only 1 entry is left.	Total

Table 1B: Corrections to metadataset.

18	38	2	4	2	6	70
Additional data record added	Calculation error	Moved to correct species	Numeric transcription error	Found an alternate citation for a value	LDLo, not LD50	Total

Table 1C: Partial table of data source quality. Complete table available in dataset supplement. This table of 20 out of 95 citation sources comprises 82% of data sources.

% of sources	Cite count	Cited source
37.11%	373	Toxicon.
6.27%	63	American Journal of Physiology.
5.07%	51	Bulletin of the World Health Organization, 61(6): 949-956 (1983)
3.88%	39	"Neuropoisons: Their Pathophysiological Actions, 1971-1974," 974
3.48%	35	Journal of Immunology.
3.28%	33	Clinical Pharmacology & Therapeutics.
2.79%	28	PLoS Neglected Tropical Diseases.
2.69%	27	Toxins (Basel).
2.59%	26	"Rattlesnakes: Their Habits, Life Histories, and Influence on Mankind," Klauber, 1956
1.89%	19	Indian Journal of Experimental Biology.
1.69%	17	JVATITD Journal of Venomous Animal Toxins Including Tropical Diseases.
1.39%	14	"Animal Toxins, a Collection of Papers Presented at the International Symposium on Animal Toxins, 1st, 1966", 1967
1.39%	14	Tai-wan I Hsueh Hui Tsa Chih. Journal of the Formosan Medical Association.
1.29%	13	Comparative Biochemistry and Physiology C.
1.19%	12	American Journal of the Medical Sciences.
1.19%	12	Journal of Proteome Research.
1.09%	11	"Venomous and Poisonous Animals and Noxious Plants of the Pacific Region," 1963
1.09%	11	“Sea snakes of New Caledonia.” Ivan Ineich, Pierre Laboute. (2002)
0.90%	9	"Natural Toxins, Proceedings of the International Symposium on Animal, Plant Microbial Toxins, 6th, 1979," 1980.
0.90%	9	"Collected Papers on Snake Venoms: Contributions from the Pharmacological Institute, National Taiwan University, Taipei, Taiwan, China, 1948-1973," 974
0.90%	9	Journal of Toxicology: Toxin Reviews.

Of the 264 venomous ophidian species, 160 species with 2 or more DB entries were accepted, for a total of 1005 lethal dose (LD) DB entries from 95 sources comprising: books, reports, government documents, and journal articles. The data for this meta-analysis had several factors that could be examined relative to lethal dose: Ophidian species, test-animal-species, and route of inoculation.

There are 17 test species in the dataset plus 2 that are generic “species,” mammal and bird, for a total of 19 test-animal-species categories. Most of the data is mouse (75%).

The maximum number of test-animal-species for one ophidian species is 10. Test-animal-species is sometimes a loose term in this context, as there are many species of monkey, duck or frog, and there might be some venom component sensitivity variances. However, literature did not indicate variances of more than 1 order of magnitude between test-animal-species, and one would not normally expect exotic monkeys, ducks or frogs to be tested. For part of this analysis, “mammal” and “bird” are treated as separate species. In most cases the designation of mammal would be a rat or a mouse. And most birds would be pigeons or chickens. As will be seen, using only mouse data did not meaningfully change results.

These studies were mostly performed in the 20th century, with the peak from 1970 to 1975 (Fig. 1). This suggests that venom science may not believe that further studies are necessary or useful. As will be seen, this does not appear to be the case.

Methods

The analysis had several questions. First, does ophidian venom variance fall within the literature documented parameter of 1.6 logs (40X fold change) between mice and frogs test-animal-species? Second, to what extent did the assumption that IC < IV < IP < IM < SC hold? Third, what correlates are there to dose range, and how much of a contribution could be assigned to each factor?

For this analysis, null hypotheses conform to what is available in published literature. To make data comparable between ophidian species, normalization was performed based on the range of values present. These normalization steps created fractions of the total range, or the fold change of the highest value over the lowest value.

Null hypotheses

The minimum lethal dose for single ophidian species has a factor of 40 or less fold change variation (log of 1.6). This statement captures the idea that across test-animal-species and inoculation methods, while there is variance, it should not exceed what has been reported in literature. (e.g. 4X and 10X, for a total of 40X)

Within a single test-animal-species for a single ophidian species, there is a factor of 2 or less range of lethal doses between studies (log approximately 0.3).

LDLo values cluster below LD50 values such that the mean of the respective fractions of the range differ from each other by more than one standard deviation, or, barring this, by standard error.

LDLo values will not be maximum lethal doses reported across studies.

The lethal dose varies by route of inoculation such that IC < IV < IP < IM < SC for 90% or more of venomous ophidian species across studies.

Primary views of data

This meta-analysis examined 3 primary views of the data with subset views: 1.) Minimum lethal dose (LDmin) and maximum lethal dose (LDmax) for each ophidian species; 2.) The range fold changes for: all data (LD-Adrf), single test species (LD-SSrf), single test species – single route of administration (LD-SRrf), for each ophidian species; 3.) The fraction of the range within one ophidian species that each DB entry represented, expressed as a percentage, is explained below.

Range fold change is Rmax ÷ Rmin where Rmax is the highest LD for the species, and Rmin is the lowest LD for that ophidian species. (LD can be LDLo or LD50.)

LDmin is the lowest lethal dose reported for an ophidian species. LDmin can be either an LD50 or an LDLo. LDmax is the highest lethal dose reported across ophidian species, and similarly, can be either an LD50 or an LDLo.

The lethal dose range fold change is the LDmax divided by the LDmin (LDmax ÷ LDmin) for an ophidian species. The fraction of the range for an entry is LDentry ÷ LDrange, where LDrange = LDmax – LDmin.

There are three LD range fold changes: All data (LD-Adrf), single species (LD-SSrf), and single species, single route (LD-SRrf). LD-Adrf means DB entries for all test-animal-species were used. LD-SSrf means that the widest range fold change within one test-animal-species for an ophidian species was used. LD-SRrf means that the widest range fold change within one test-animal-species that was administered by the same route was used. (e.g. use the highest and lowest LD for IC, IV, IP, IM, and SC, and take the largest range multiple for one of the routes of inoculation.)

After curation, the data was sorted by species and then by lethal dose. It was further segregated by species family, finding 1 Atractaspid, , 5 Colubrids, 69 Elapids, and 85 Viperids. Having only 5 Colubrid species in the dataset made those results questionable to indicate a significant difference, although it may exist. (Data not shown.) The LD-SRrf difference between Elapids and Viperids did not appear to be significant. (Not shown.)

The coefficient of determination, R^2, is the primary measure of significance used in this meta-analysis. Best fit regressions were exponential curves of the form $y = C \bullet x^m$ (shown in the form y = $C \bullet x \wedge m$), or $y = C \bullet e^{mx}$ (shown in the form y $= C \bullet exp(m \bullet x)$.

Analysis

LDLo does not differentiate from LD50 across studies.

65 out of 160 ophidian species had at least one LDLo value. These 65 ophidian species had 196 LDLo values, and 809 LD50 values. In the meta-dataset, 28 of these 65 ophidian species (~43%) had an LDmin that was, indeed, an LDLo. However, 25 of the 65 ophidian species (~38%) had LDLo values that were the meta-study LDmax, which was against expectations. (see Equation 1 in the Supplementary Files)

For figure 2 and 3, the range fraction is determined by equation 1. This way, relative toxicity between ophidian species is normalized, and comparison of variation can be done between ophidian species. In Figure 2, the mean of the LDLo fractions is 31.1%, and the mean of the LD50 fractions is 30.0%. The standard deviation of the LDLo fractions mean is 36.6%, and standard error is 2.9%. The standard deviation of the LD50 fractions mean is 33.0%, and standard error is 1.6%. Even by the less stringent method of standard error, the difference between them is insignificant. Thus, the mean of the LDLo and LD50 range fractions for the 65 snake species out of 160 are not meaningfully differentiated.

One might argue that significant difference might be seen in LDLo values for single test-animal-species, so this was examined in figure 3. Here, as above, the data does not show this. Instead it shows that for 6 out of 7 test-animal-species, mean LDLo is higher than LD50, and for 3 of them, this exceeds standard error. (Rat, mouse and dog.) Note that this also occurs for the highest *N* dataset (mouse). Only the sparse data for cat has mean LDLo below meanLD50, which is probably not significant. However, using median, 3 test animal species have higher LD50 than LDLo (dog, cat, and rabbit),

Strengthening this point, for 10 ophidian species, both LDmin and LDmax were LDLo's. The mean number of DB entries for an ophidian species with one or more LDLo values was 2.9.

Consequently, because LDLo did not differentiate significantly from LD50 when examined across studies, LDLo designations were categorized together with LD50 for the rest of this meta-analysis.

Route of inoculation minimum and maximum lethal dose

In figure 4, there is good support for the concept that IC (intra-cerebral) < IV (intra-venous) < IP (intra-peritoneal) < IM (intra-muscular) < SC (subcutaneous). This is the only hypothesis not falsified in this analysis. However there are contradictory instances.

Out of 22 intracerebral injections (IC), 15 were LDmin values, which is as expected. So, approximately 2/3rds the time, an IC injection was the minimum, and the *N* should be meaningful at 22. Using a synthetic x-axis the LDmin 0.859 R^2 coefficient of determination suggests that approximately 86% of the distribution fits the assumption that route of

inoculation varies as IC < IV < IP < IM < SC. The curve fit for LDmax shows the opposite trend, with a good R^2 suggesting approximately 86% of results can be attributed to route of inoculation distributed in this manner.

However, 18 out of 228 of the subcutaneous injections were LDmin values, which makes SC, unexpectedly the route of highest toxicity for 11% of the 160 ophidian species. Out of these 18, there were 2 venoms with strong hemotoxic or nephrotoxic effects, and 13 were neurotoxic.

Venom toxicity range fold change

The range of venom toxicity per ophidian species within the mouse test species has a mean average of 2.00 logs (98.97 fold change) within a single test-animal-species, as shown in figure 5. This is 2.47 times the 1.6 logs (40 fold change) documented in literature for toxicity difference between test-animal-species, as discussed above. For all test-animal-species together, the mean range is 2.97 logs (936.59 fold change), which is approximately 23 times what current literature indicates.

The largest range fold change seen for an ophidian species tested in mouse for one route of inoculation is *Crotalus oreganus*, 2.15 logs (141.3 fold change), *N(studies)* = 26. The largest range fold change for an ophidian species tested only in mouse for all routes of inoculation is *Crotalus horridus*, 3.6 logs (4,150 fold change), *N(studies)* = 16, *routes of inoculation:* SC/IM. For one ophidian species data for all test-animal-species, including all routes of inoculation, the largest range fold change is *Naja naja*, 4.76 logs (57,471 fold change), *N (studies)* = 27, *N (test-animal-species)* = 9, *routes of inoculation:* unknown/IC between rabbit (LDmax) and rat (LDmin). These are among the highest *N* studies counts for ophidian species. Note that a specific ophidian species mentioned does not mean this species has been determined to be the most venomous, or the widest range of all.

One might ask whether the range fold change increasing holds up when a single species and single route of inoculation is examined. We see this in figure 6, compared to all data, where the range fold change is plotted against the number of studies. By inspection, one can see that the range fold change does appear to increase as the number of different studies rises, and the curve fit appears to be likely an exponential.

Figure 7, which looks at single test species for multiple routes of inoculation, shows a linear regression trend that reaches significance for the range fold change increasing as the *N* for number of studies gets larger. This graph appears to signal the same thing that a set of ecological diversity transects continuing to increase would. It indicates that to fully characterize ophidian venom lethal doses, probably requires more than 50 different studies.

Regressions of LDmin

Null hypotheses for minimum lethal dose: A.) Minimum lethal dose within each ophidian species varies by less than a factor of 2. B.) The minimum lethal dose does not correlate with the number of times the lethal dose has been tested (e.g. the number of LD DB entries).

Alternate hypothesis: Minimum lethal dose varies by more than a factor of 2, and minimum lethal dose correlates with the number of times dose has been tested.

Table 2: Regressions curve fit summary for LDmin, rounded.

Correlation examined	R^2
Number of routes of inoculation	0.21
Number of test species tested	0.31
Number of LD DB entries (Number of studies)	0.396

Number of routes of inoculation correlation to LDmin

In figure 8, as the number of routes of inoculation increases, the likelihood of having more test-animal-species for the ophidian species also increases. The fitted curve is probably determined by the probability of inclusion of a lower lethal dose value rising as the number of inoculation routes goes to 5, because, as was seen above, IC < IV < IP < IM < SC does tend to hold true.

Here in figure 9 the apparent drop visible in the fitted curve is 1.5 logs, a fold change of 32X. Similarly to the above, it should be expected that lethal dose would drop some with larger numbers of test-animal-species, because literature indicates that some animals are up to 1.6 logs (fold change of 40X) more susceptible to certain venoms than others, and there is some frog data in the dataset.

Additionally, the more test-animal-species there are for one ophidian species, the more likely it is that there will be more routes of inoculation. However, in the dataset, there are multiple instances of the same test-animal-species occupying LDmin and LDmax, and quite a few are very close to this state, which suggests that, indeed, the number of times an ophidian species is tested is a major factor.

Number of DB entries (studies reported) per ophidian species correlation to LDmin

In figure 10 the “All data LDmin” fitted curve does not control for different test species. To address this criticism, “Mouse LDmin” shows the same graph filtered for inoculation of mice only. The R^2 value of 0.245 is not as good as the 0.39 R^2 value is for all data, however, the N is lower, and by inspection, there is a fairly good match for the curves. If there were no correlate by number of studies per species, then the fitted curves should be flat, whereas, both fitted curves span over 1.75 logs, and have fairly close exponents and constants. Consequently, these data suggest that the primary correlate for lethality is the number of studies that have been performed.

Regressions on range fold change

The range fold change is Rmax ÷ Rmin. The R^2 values for these regressions are larger than what we see above. These data indicate that there is a correlation for all measures with number of DB entries for lethal dose (number of studies reported). The number of routes of inoculation has a meaningful correlation for all data, and for single test species. Fit equations in this section are of the form $y = C \bullet e^{mx}$.

Table 3: Regressions on all data for range fold changes of highest over lowest dose. All data (LD-ADrf), single species (LD-SSrf), single species, single route (LD-SRrf). Number of species tested is not applicable for LS-SSrf, of LDSRrf. Number of routes is not applicable forLD-SRrf.
R^2 below 0.20 not shown. N = 160 ophidian species.

Data, by species	LD-ADrf R^2	LD-SSrf R^2	LD-SRrf R^2
N	N = 160	N = 160	N = 113
Number of routes of inoculation	0.43	0.41	NA
Number of species tested	0.46	NA	NA
Number of LD DB entries	0.56	0.41	0.26

Table 3 confirms that the number of studies done is probably the primary correlate of toxicity for whole venom, and just above the correlation of number of test-animal-species used. The number of routes of inoculation roughly ties with the number of species tested.

Discussion Of Possible Confounders

Data errors

Some papers or books may have gotten the numbers wrong. However, my own complete audit of the records I used found that while values change somewhat after the audit, the thesis presented here did not. The audit removed 205 records, added 18, and corrected 52 records. Prior to my audit, Sascha Steinhoff made efforts to validate the data entries as evidenced by the sourcing of each one, and his comments, which I discussed with him in personal communications. I do not believe that this is a significant source of error. If anything, due to inability to obtain some sources to validate them, there may be some conservative bias, and I note that there is a more data to be harvested from publications.

Allometric scaling hypothesis

To evaluate the allometric scaling hypothesis, assume that a 25 gram mouse has a surface area of 70.79 cm^2 [37]. A 70 kg human is 1.95 m^2. However, to calculate this way, one must first correct the mg/kg dose for the mouse. To do that, I used a published equation for rabbit surface area which have normal mass in the 1 kg range (BSA = (9.9 × (body weight in grams)$^{2/3}$)/10,000.) [38]. The cited equation gives 0.099 m^2 for a 1 kg animal. So we divide 1.95 by 0.099 = 19.7, which is the multiplier for the mouse LD50 to convert to human dose, instead of multiplying by 70 kg. There is an approximately 3.6X difference between allometric and body mass calculation. If one scales dose this way, then the larger animals should die from doses 3.6 times lower on the mg/kg scale. Consequently, they should concentrate in the lowest dose region when graphed by body mass. They do not. For the relative few larger animals in the dataset such as cattle, dog and monkey, LD50 is centrally located in the distributions. Note that while it is correct that a mouse scaled up to 1 kg probably would have lower surface area than a rabbit due to ears, this might change the factor from 3.6X to 3.2X or so. The problem will remain. Thus, allometric scaling of venom dose does not appear to be a correct method for whole venom.

The biomedical reproducibility problem

Biomedical science in general has a reproducibility problem[39]. However, venom LD50 and LDLo studies are straightforward to perform and the reproducibility issues in bioscience tend to be in more complex work. Against that, after multiple discussions with animal handlers and scientists practiced at injections, some plausible sources of error emerged. It is conceivable that an intracerebral injection was performed incorrectly sometimes, as this procedure is arguably more difficult than the others. It is plausible that injections into animals are more difficult to standardize than believed, particularly small animals. For instance, subcutaneous injections may be done in different locations on the animal, and some of these may be more effective spots than others. It could happen that a subcutaneous injection hits a vein more often than thought. Similarly, veins may be missed and either become subcutaneous injections or intramuscular injections. In animals such as mice, muscles can be missed.

However, for this study, there is no way to know what issues there may be, and rejecting data post hoc because it doesn't fit preconceptions, particularly when no injection method appears to fit those preconceptions better, is hard to support. I do not have a basis for quantifying the degree to which these data might represent a window into the rate of bench error in venom lethality studies nor the rate of such error.

Testing is concentrated in snakes most dangerous to humans Another plausible influence on the dataset could be that the ophidian species that kill humans get tested more, and so those species that do get tested more have a wider range fold change of LDmax ÷ LDmin. To test this hypothesis, the top 40 venomous snakes listed as a threat to humans by Steinhoff were analyzed relative to their LDmin and the number of studies, by quartile. (Table 4) What we see is that the mean and median number of studies declines by quartile, as the mean venomous dose increases. And we see that half of the top 40 cluster in the 1st quartile, and the number declines in each succeeding quartile. So there might be some effect from studies being directed at snakes dangerous to humans. However, we won't know until far more studies have been done by many different laboratories.

Table 4. Quartile analysis of distribution of top 40 snake species.

		Quartiles			
	All	1st	2nd	3rd	4th
Studies mean	6.34	10.48	6.98	4.88	3.03
Studies median	4	9.5	5	4	2
Venom mean	0.901	0.029	0.179	0.425	2.910
Top 40 count		20	15	3	2

Subspecies collated together

Another confounder could be that the venom database contains subspecies lumped together and that this might affect the range fold change and minimum lethal dose. This hypothesis was tested by finding ophidian species that had called out multiple subspecies.

I found 22 out of the 160 species had subspecies listed. Only 7 out of those 22 could have had impact because the LDmin and LDmax were possibly different subspecies. Just 2 of these 7 ophidian species had a range fold change that changed by a factor of 2 or more, and just one changed by a factor of 20. The mean range fold change for the 7 that changed out of the 22 with listed subspecies was 0.88.

The mean LDmin fold change for the 7 species out of 22 was 5.29. For all 22 species, the mean LDmin fold change was 2.36. The impact of segregating named subspecies and treating them as different species was tiny, out in the 3rd and 4th decimal place of the fitted curve exponent. This suggests that unidentified subspecies might sometimes matter, however, overall it is not defensible as a meaningful contributor in this dataset.

Stress and captivity effects

Stress and captivity induced changes to venom are not usually significant, however, there is question regarding *Lachesis muta*, and the disparity between laboratory testing and the high mortality rate in humans [40]. While this may be a factor, literature indicates it is rare.

Ecological Transects And Venom Variation

An ecological transect is a survey line of some length laid out in an area. Along that line, to some distance on each side, a survey is conducted to count the number of species. The transect is divided into segments, and each segment is a sampling of the species along the transect. As one progresses along the transect, the new species discovery rate will decline. Based on that slowing discovery rate, one can fit a curve, and using this, estimate the number of species in the area of the transect [41, 42].

These venom lethal dose data represent a kind of transect of the variation in potency of venom, where the transect is everywhere that scientists collect venomous snakes, and the discovery rate is some time period. However, I could not analyze this because there is simply not enough data on a per-test-species, per-inoculation-route basis to make it meaningful. The fact that LDLo and LD50 do not differentiate indicates that we are so far from a proper sample size that we cannot currently make an estimate of where the limits are.

This venom diversity transect is filtered through the interactions with both the route of administration and the different test species used. This has implications for medicine, because human envenomation is a similar transect, where humans are the target species interacting with venom variance and dose delivered.

Conclusion

The import of this meta-analysis is several. First, the correlation between number of times a venomous ophidian's lethal dose is studied and the range of lethal dose, as well as the minimum lethal dose, indicates there is quite a bit of room for exploring lethal dose range, and that to properly characterize toxicity of whole ophidian venom is a large meta-project.

Second, the inability to differentiate between LDLo and LD50, and the preponderance of subsets where LDLo is higher than LD50, indicates that the *N* required to fully characterize venom is beyond what current studies have collected. This indicates, in turn, that the range of toxicity results for whole venom should be less reliable than they appear to be here, even for those ophidian species with the highest number of studies reported. In ecological parlance, the transect is at the beginning of its discoveries.

Third, while venomics has put a stake in the ground for venom protein components [43], these data show that there cannot be a single relative level for components. The relative composition is a most likely explanation, as may be synergistic effects, although the latter may depend on non-protein components, perhaps nucleic acids, or other molecules. If this hypothesis proves to be true, then one should ask why such variation would exist, and what drives it. To date, we have but scratched the surface of both degree of variance and why it exists.

Beyond this, there are several areas that this analysis has bearing on: How to best estimate LD50 given current limitations; confirmations and caution relative to existing medical practice; and further research.

LD50 estimations

The correct way to define LD50 at this time is to use a meta-dataset. Because it does not differentiate in meta-analysis, treat LDLo the same as LD50, and provide LD mean, median, minimum, range, and standard deviation, along with the *N* for number of studies per test species used. This is supported by recent recognition that intraspecific variability is high, so others have started to us similar methods when processing LD50 data [2]. Doing this should not be controversial. Safety could be estimated by specifying from 1 up to 6 standard deviations (6 sigma) depending on desired safety margin. If an LDLo value is desired, this should simply be the minimum lethal dose in the meta-dataset, and should be referred to as the LDmin (e.g. the minimum) to avoid confusion. This can be done separately for each route of inoculation.

Human LD50 estimations

The ethical problems of determining human dose-response force us to develop methods of estimation based on animal data. Yet, the human dose-response may be different than other animals, including monkeys. Humans are, for instance, around 3,000 times more susceptible to sepsis than any animal, including our closest primate relatives.

It may be reasonable to consider exclusion of amphibian, bird and reptile data if there is sufficient *N* from mammals, where *N* is the number of studies conducted. However, from this meta-analysis it appears that a sufficient *N* is more than 50 different studies, and may be as high as several hundred studies, and this is unlikely to happen soon. Also, there are multiple instances in larger ophidian datasets within this meta-analysis data where non-mammal data is bracketed within mammalian data. Consequently, the most reasonable general course is to use aggregate data for all species, and compute as discussed above for the general case. Be cautious about overconfidence if some particular ophidian species has a set of studies that appear to conform to preconceptions. Until that dataset is quite large, using data from a divers set of contributors, such a pattern is probably explained by chance.

It has been argued that humans cannot receive IP or IC inoculations, except possibly in the case of infants. However, there are cases of bites to the thorax in adults that appear to progress more quickly which may be similar enough to include it as similar to IP.

Should there be sufficient *N* for specific routes of inoculation, or if the IC inoculations appear to conform to the IC<IP<IV<IM<SC model, then for human estimations, IC could be excluded.

LD50 estimations with little data, and computational research to support it

I recommend for estimating LD50 for an ophidian with little data, to use a customized meta-dataset for one or more related species, and factor proportionally from the mean of the minimal known data. To do this, curate a related ophidian meta-dataset based on what is known of venom makeup and/or genetic distance, plus the prey species. For that meta-dataset, determine the LD mean, minimum, range, and standard deviation. This could be a significant computational research project.

Estimation of margin of error for this proposed algorithm will require non-trivial development and validation against existing datasets such as the one used for this meta-analysis, and represents an area of computational research.

Human bite treatment: confirmations and caution

This meta-dataset tells us that, controlling for dose, envenomation effect can vary by over 28,000 times within one species. Adding uncontrolled venom dose into the equation indicates that medicine is probably dealing with effective dose ranges spanning up to 1 million times. Consequently, physicians treating patients with snakebite cannot presume that because they saw 10, or even 50 cases for one species, that this will necessarily tell them what will happen on the next bite. This is true even if they have gotten good at estimating the size of the animal from the distance between the fang puncture marks. This analysis confirms that snakebite treatment should always be treated symptomatically, and that this should be done aggressively, because sooner or later an outlier should appear.

These results also confirm that antivenom manufacturers should use mixtures from a variety of snakes of the same species for immunization of animals. Most already do this.

Research for venom toxicology

Ophidian venoms are a cocktail of many components. For a toxicologist working on individual venom components, whether there is significant variation in the lethality of venom components between snakes within the same species is an open question. There are tantalizing reports from India, for instance the practice of recreational cobra bites, which are suggestive of inbred strains [44, 45]. Researching this question will require many samples from wild as well as inbred, quasi-domestic, snakes, optimally, with geolocation, size, estimated age, and sex recorded, with attention to sample sizes and control of test species. Gene sequencing of the whole genome and/or exome could also provide

meaningful insight. Given that snakes migrate slowly relative to many other animals, genetic drift could plausibly generate some variation. However, given the complexity of mRNA factors and gene regulation, there may be reasons we don't understand yet. This is a question that could take many years to characterize.

Abbreviations

DB: database. Here it refers to the Steinhoff database.

IC: intra-cerebral

IV: intravenous

IP: intraperitoneal

IM: intramuscular

LD: Lethal dose.

LD50: Lethal dose that kills 50% of the test animals

LDLo: Lowest lethal dose in a set.

LDmean: the mean average of the LD's

LDmin: lowest lethal dose within a set of lethal doses

LDmax: highest lethal dose within a set of lethal doses

LD-ADrf: lethal dose range fold change using all data

LD-SRrf: lethal dose range fold change using a single test species and a single route of inoculation.

LD-SSrf: the widest range fold change within one test-animal-species for an ophidian species was used. (e.g. within each test species use the highest and lowest LD, and take the largest range of the set.)

Range fold change: Rmax ÷ Rmin

SC: subcutaneous

Declarations

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Availability of data and materials

All data analyzed in this study are primarily sourced from the Steinhoff database[35]. The secondary source is the Drug Future database, which is referenced by the Steinhoff database. The curated version of this data is provided as supplemental material.

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Author contributions

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Competing interests

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Consent for Publication

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Not applicable.

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Figures

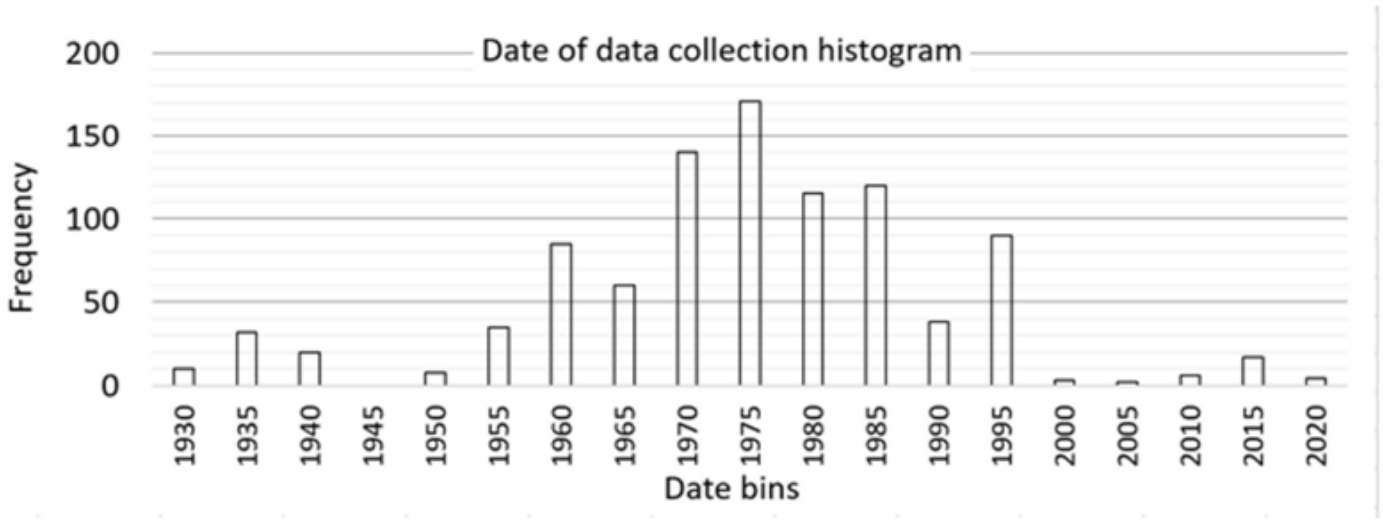

Figure 1

Time distribution of studies. Bins for date of report are the termination year for the bin. The generic mammal DB entries have dates from 1953 to 1985, with 24 of them in 1967. The unrecorded method of inoculation DB entries spanned 1958 to 1987, with 29 in 1967. This indicates a period when some researchers appeared to believe that the test-animal-species and route of inoculation was insignificant.

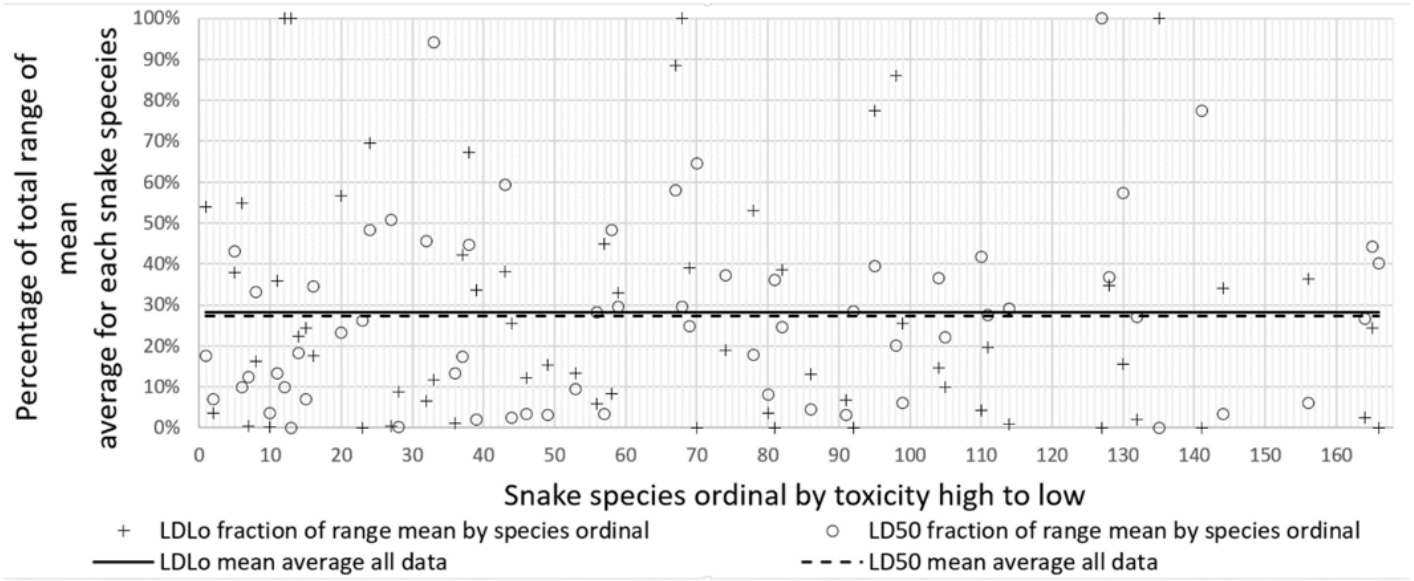

Figure 2

LDLo fraction of range compared to LD50 fractions of range. 64 out of 167 ophidian species had at least one LDLo value and at least one LD50 value. Average of the LDLo fractions is 28.3%, and the average of the LD50 fractions is 27.2%. They differ by 1.1%, or roughly 1/3rd of the standard error. One would expect LDLo to average considerably below LD50.

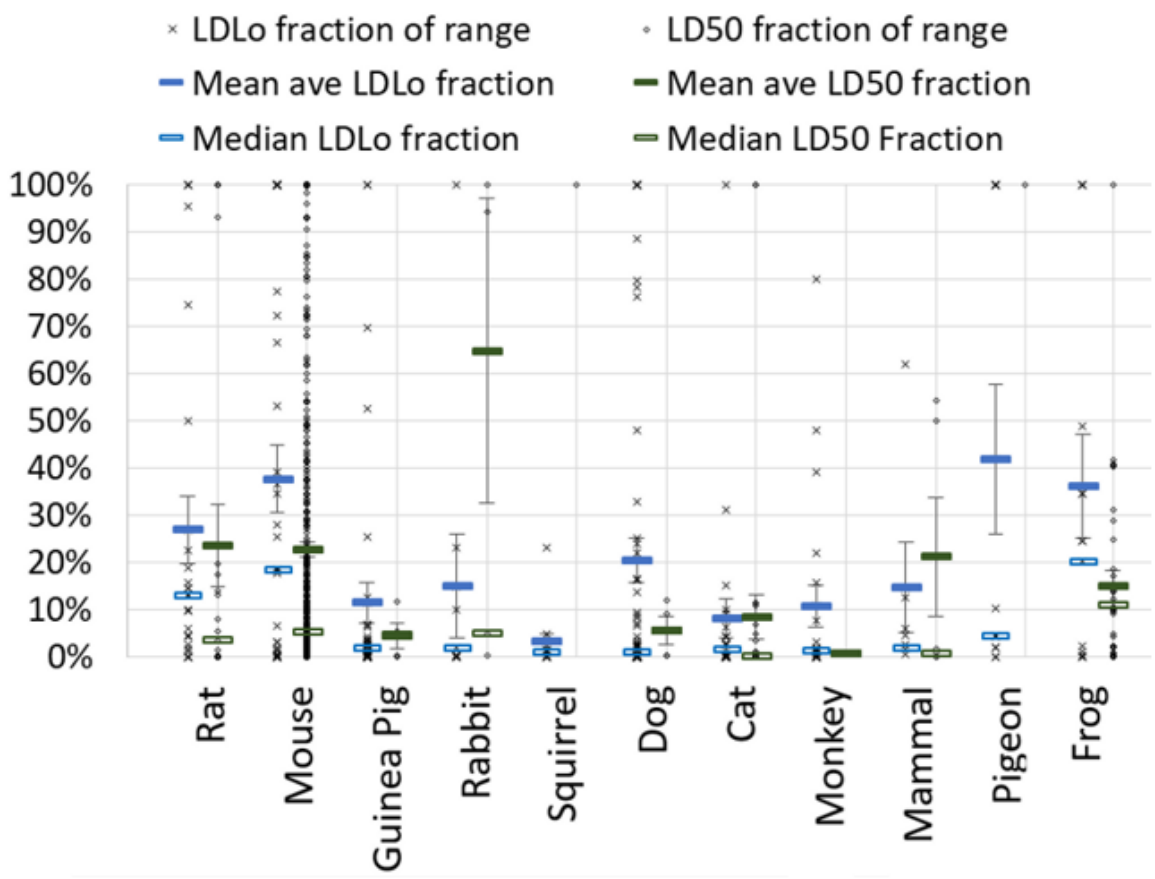

Figure 3

Fractions of range by test animal. Error bars show standard error. Only in rabbit is LD50 above LDLo with significance, however this disappears if we use median instead of mean. (hollow bars are median values) Higher N animal datasets like mouse, continue to show LDLo above LD50, and that this is significant.

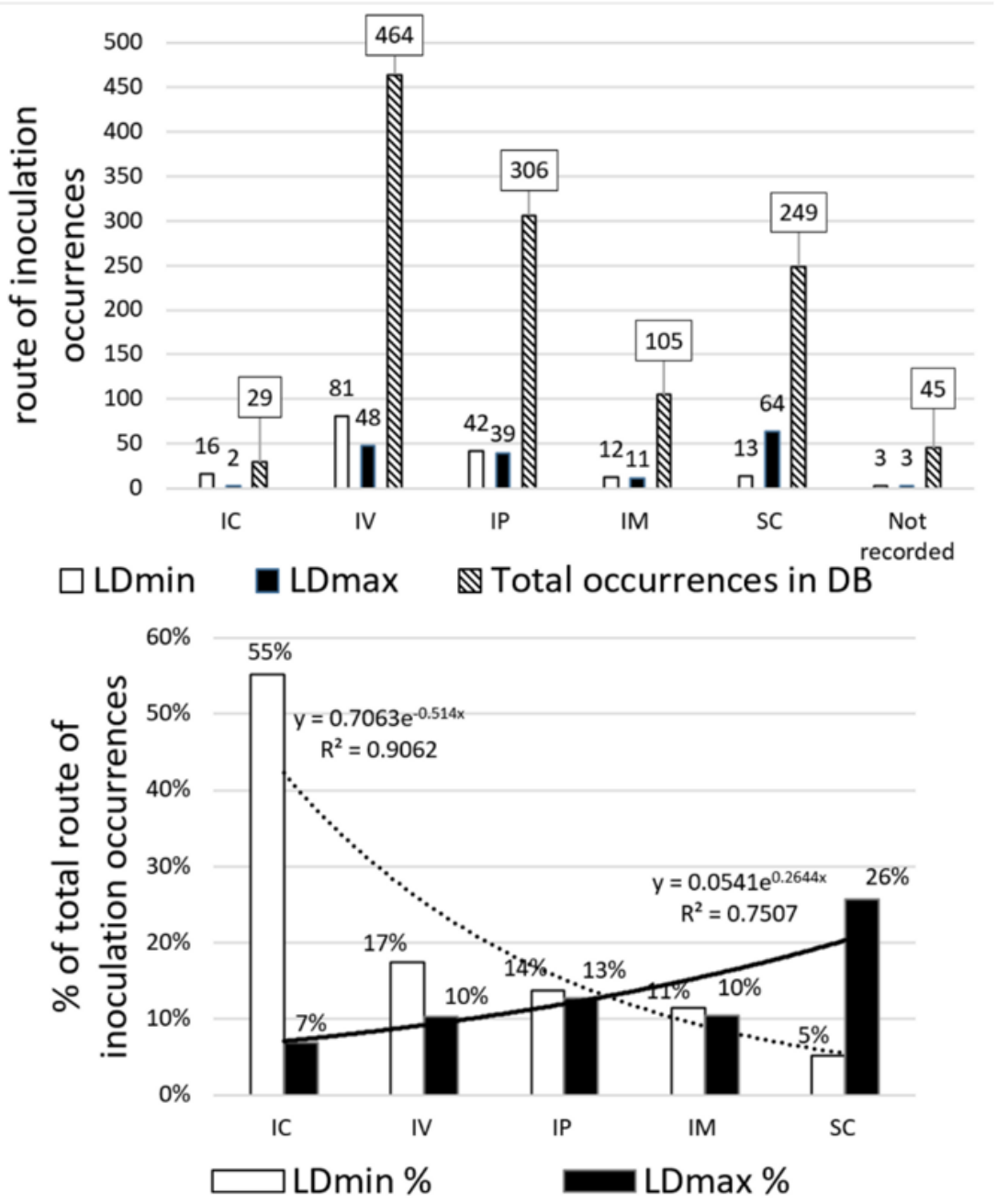

Figure 4

Route of inoculation distribution: minimum and maximum lethal doses.

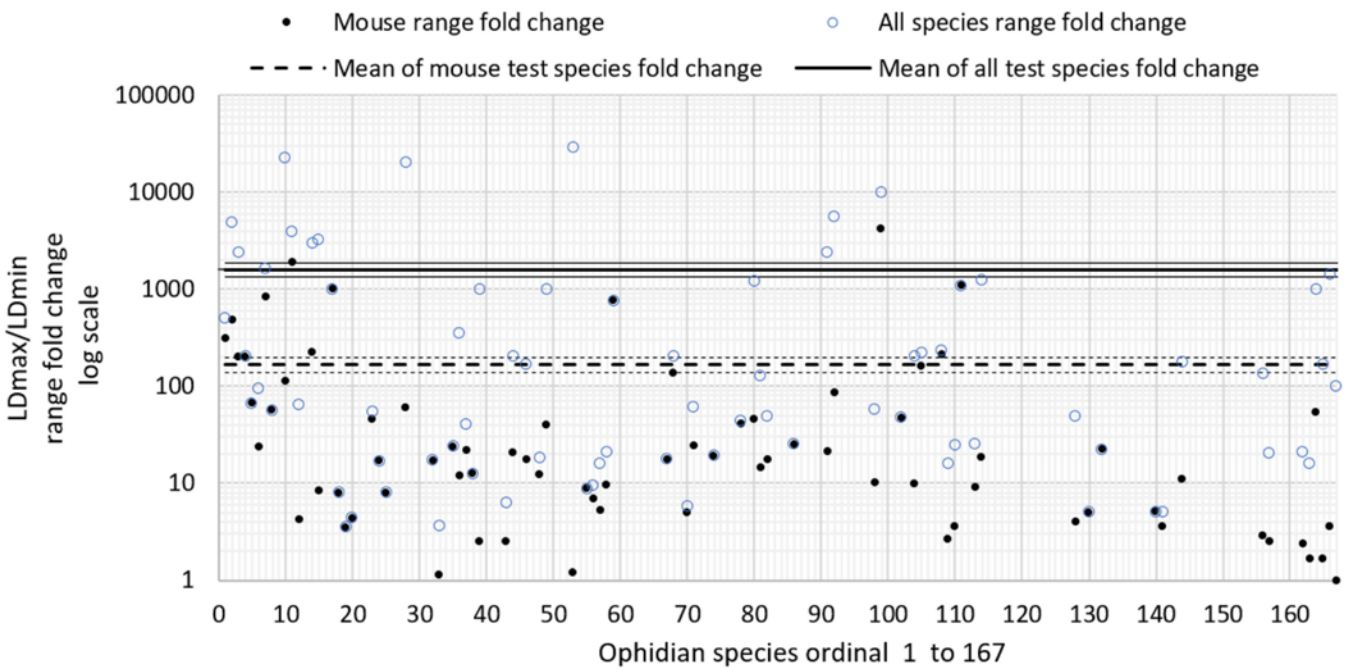

Figure 5

Mouse vs all test species LDmin and averages. Standard error of the means is shown as solid or dashed bars above and below mean average lines.

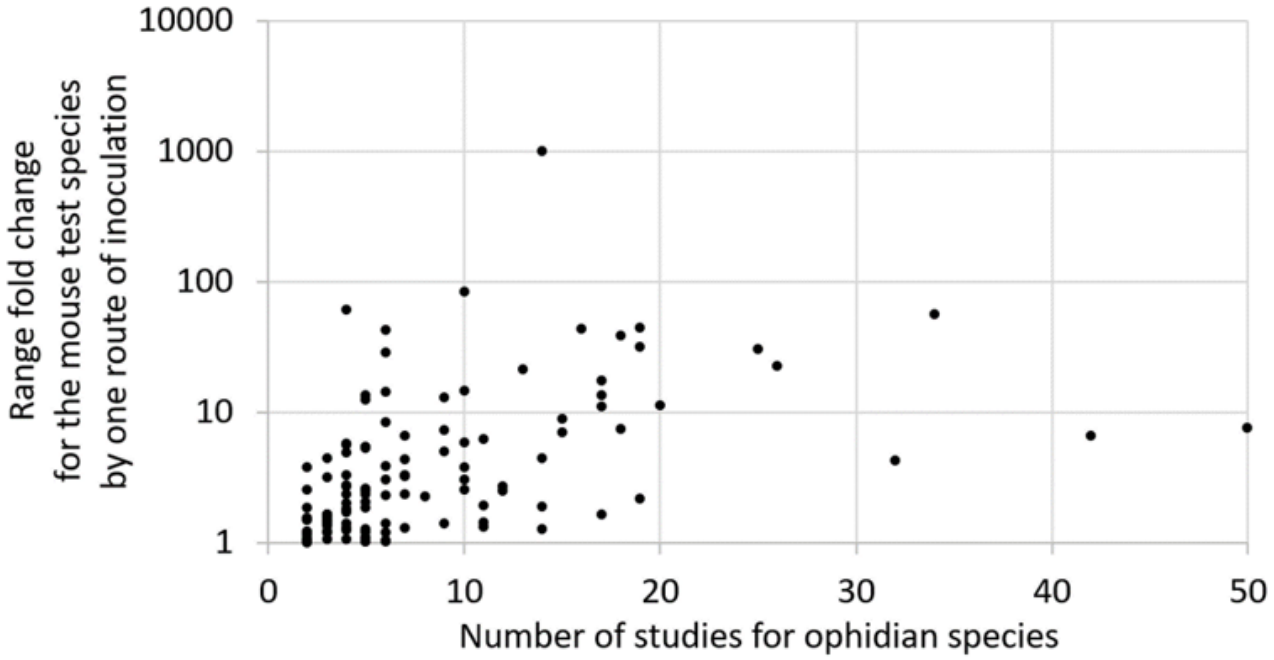

Figure 6

Range fold change (log scale) vs. number of studies for one test-animal-species and one route of inoculation. N = 116 ophidian species that have two or more studies for the same route of inoculation.

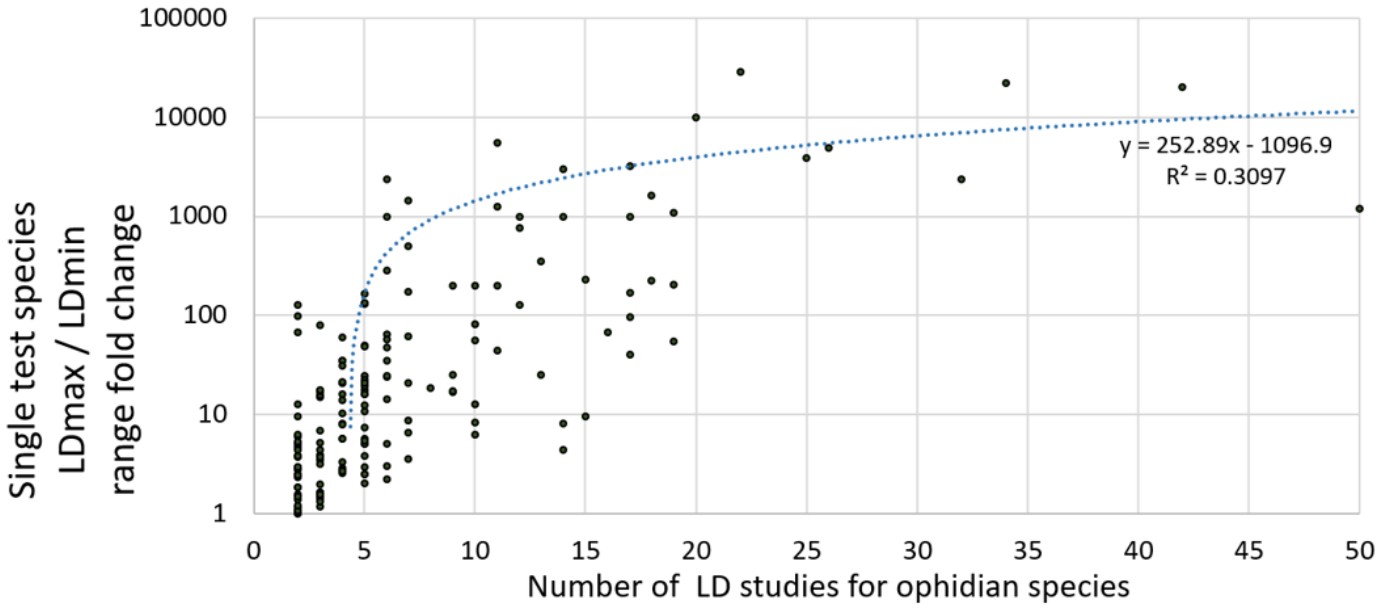

Figure 7

Venom range fold change for single test-animal-species and multiple routes of inoculation. N = 167 ophidian species with 2 or more reports for the same test species. There is a strong linear trend of increase in the range as the number of studies rises. In this graph, within each ophidian species, for test species with 2 or more entries, the test species with the largest fold change is shown.

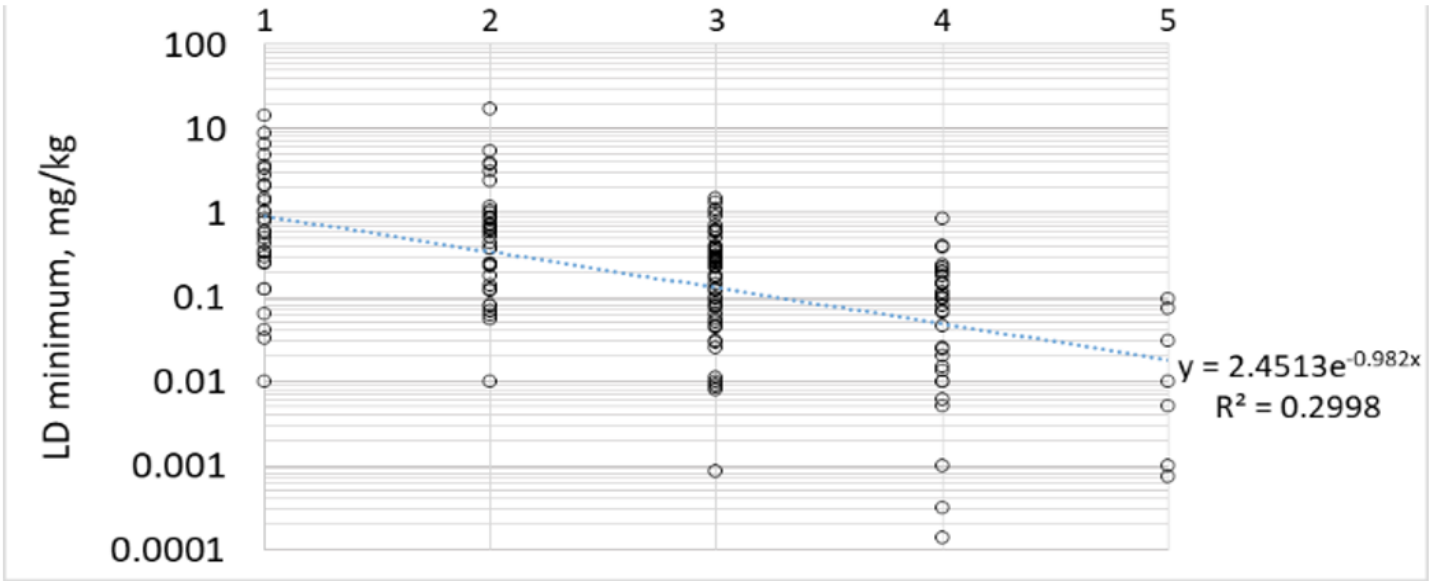

Figure 8

Minimum lethal dose vs number of routes of inoculation. (Table 1, first entry,). The N for the number of routes of inoculation 1 to 5 are, respectively: 30, 36, 61, 33, and 7.

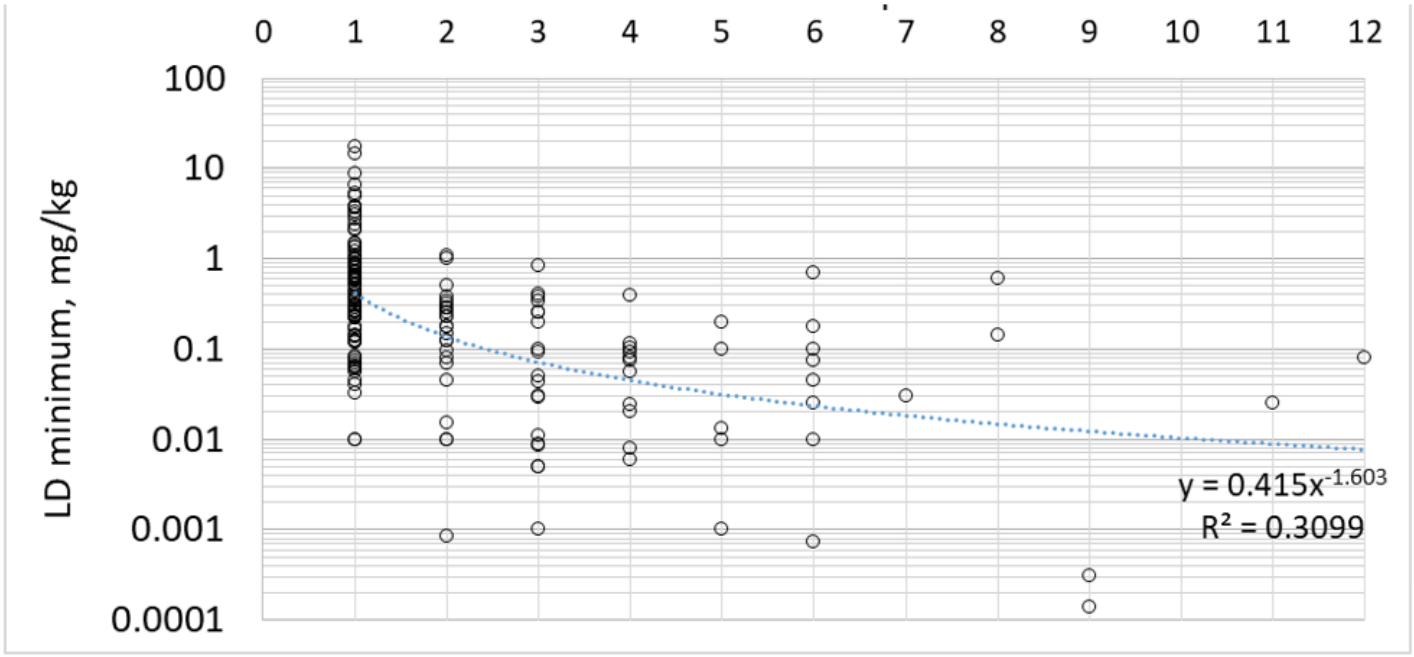

Figure 9

Minimum lethal dose vs number of test-animal-species. (Table 1, second entry.) X axis is number of test-animal-species.

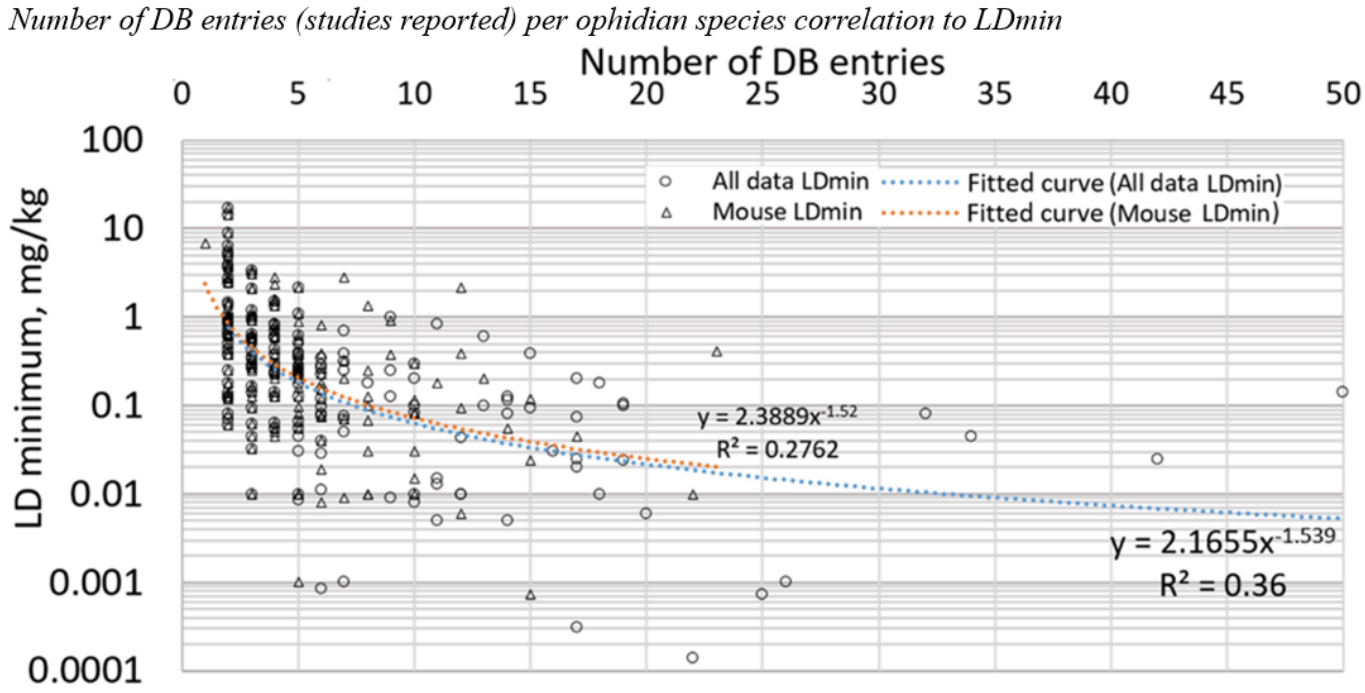

Figure 10

Comparison of LDmin for all data vs. mouse only data. Minimum lethal dose vs count of database entries per ophidian species. For all data, the fitted curve spans ~2.15 logs. For mouse data only, the fitted curve spans ~2.05 logs. (Ophidian species N = 167). What is visible by inspection is that when data is filtered to only include mouse

studies, the curve fit for mouse data is a near exact match, it is just truncated because of fewer ophidian species with higher number of DB entries.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SteinhoffsDBSelectedVenomscurateddatasetv10Sub.xlsx
- Equations.pdf