Research Article

Extraordinary variance in meta-analysis of venom toxicity of 160 most lethal ophidians and guidelines for estimating human lethal dose range

Brian P. Hanley[1]*, Gustavo Gross[2]

[1]Butterfly Sciences PO Box 2363, Davis, CA 95617 USA
[2]University of Texas Rio Grande Valley School of Medicine, 1201 West University Drive, ITT 1.210, Edinburg, TX 78539 USA

Abstract

Background: This is the first meta-analysis to characterize intra-ophidian-species variation in whole venom. Being the largest possible meta-analysis at this time, it encompasses all known records of animal lethality studies over the past 100 years. These results were not artifacts of resistant test-animal species and showed orders of magnitude beyond the 1.6 logs (40-fold change) range of lethal dose documented in the literature between amphibians, lizards, and mice. **Methods:** A total of 1003 lethal dose study results for 160 of the most lethal venomous ophidian species in the world were analyzed. **Results:** LDLo was not different from LD50 across studies, indicating the true range of toxicity is probably larger. The belief that, for the route of inoculation, IC < IV < IP < IM < SC was well supported ($R^2 = 0.90$). However, 5% of ICs were the highest dose, and 7% of SC inoculations were the lowest dose. Within the mouse test species, for one route of inoculation, the widest LD range was 2.96 logs (917-fold change, $N = 20$). Within mouse species, for multiple routes of inoculation, the widest LD range was 3.6 logs (4,150-fold change, $N = 20$). The strongest correlation for the range of lethal dose results was the number of studies ($R^2 = 0.56$), followed by the number of test-animal species ($R^2 = 0.55$) and then the number of routes of inoculation ($R^2 = 0.43$). **Conclusion:** Scientists working with humans should use combined LDLo and LD50 meta-datasets for all data and calculate mean, median, minimum, range, and standard deviation as shown in the supplement spreadsheet, and the equations we provide. Standard deviation multiples may provide the desired safety for experimenters. For estimating the LD50 range and minimum lethal dose for species with little data, we recommend curating a meta-dataset of related snakes, and computational research to strengthen this estimation.

Keywords: Venom toxicity, LD50, LDLo, Venom meta-analysis, Venom lethality

1. INTRODUCTION

The global snakebite problem comprises 1.8–2.7 million envenomations, resulting in 81,000–130,000 deaths/year in addition to permanent disability. Snakebite is the leading cause of death among neglected tropical diseases [1]. Thus, it is of great importance to understand the lethal dose.

When scientists select a study and use its results as the lethal dose standard, some variations are expected between test species for the same ophidian species but not a great amount within the same test species. Selecting single studies or a very small number of them and averaging the findings have been the standard. Here, we show that this appears to be incorrect and provide guidelines for approaching the problem of lethal dose estimation.

Human lethal dose response to ophidian venom is a thorny problem since it is impossible to perform ethical lethal dose studies on humans [2]. This forces the researchers to use animals for such studies, despite the possibility that human dose response may be different from that of other animals, including monkeys. When LD50 for a different species is used to guess the human lethal dose, the dose is typically calculated by choosing a study and multiplying a value by the mass of a typical human. This procedure is based on common

***Corresponding author:**
Brian P. Hanley (brian.hanley@bf-sci.com)

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assumptions that will be discussed below. Anecdotally, scientists and physicians have a bias toward more recent studies.

A common assumption is that the lethal dose of toxicity varies with the method of inoculation, and, from the most toxic to the least toxic, it is as follows: Intracerebral (IC), intravenous (IV), intraperitoneal (IP), intramuscular (IM), and subcutaneous (SC) inoculation. Regarding inoculation methods, there is also a view that the comparison of results between inoculation methods varies radically, such as "apples and rocks" [3], implying inoculation methods may not be comparable. However, no formal meta-analysis backed up either the IC < IV < IP < IM < SC or the "apples and rocks" assumption. The first assumption is that IC < IV < IP < IM < SC, whereas the second assumption is that inoculation methods vary radically such as "apples and rocks." 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 the selection of a study to use fairly arbitrary.

Every scientist in the field whom we 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 of capturing and recording. By definition, LDLo should be lower than or equal to LD50, and we find that it is not.

1.1. Variation in venom and prey (test animal) susceptibility

For some time, there has been literature suggesting the common assumptions about lethal doses should be reconsidered, with recent authors documenting variations in venom components. Variation of venom by season and between related species and subspecies has been a consideration for antivenom preparation [4-7]. Geographic area and predator– prey evolutionary relationships are other factors in venom variation [8-16]. In addition to geographic differences, ontogeny can affect venom component variance [7,17], which is presumed to be an adaptation to differing prey species during development. Differences may also be linked to the sex of the ophidian [18].

Quite recently, some degree of plasticity of venom in *Sistrurus miliarius barbouri* in response to prey species was documented, which should create some intra-test species modification to LD50 [19]. It was also shown that *Crotalus simus* modified 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 [20]. Moreover, 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 [21,22].

In general, as long as the test animals are not species that have adaptations to venom, such as the western ground squirrel (*Spermophilus beecheyi*) [23], some opossums (*Didelphidae*), hedgehogs (*Erinaceidae*), mongooses (*Herpestidae*), weasels (*Mustelidae*), some skunks (*Mephitidae*) [24], or the honey-badger (*Mellivora capensis*)[25], then test species was not documented to make a major difference. Except for the ground squirrel, these are predator species that prey on venomous snakes.

Among prey species, the literature showed that lizards probably required roughly ×4 the venom dose that mice did as demonstrated by the relative dose delivered by *C. concolor* [26]. Frogs are an order of magnitude (×10) more resistant to *Sistrurus* venom than lizards, whereas in mice, the variation in toxicity of *Sistrurus* venoms correlated with mammals in the snake's diet [27] (mouse variance was unspecified, but was 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 ×4 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.

2. CHARACTERIZATION OF DATA

This meta-analysis included every known study for the top 264 venomous ophidian species winnowed to the 160 species covered by two or more studies, with accepted test species. Some test species were judged to be outliers. These data were primarily extracted from the Steinhoff database and secondarily from the Drugfuture database [28,29]. The data for this meta-analysis had several factors that could be examined: ophidian species, test-animal species, and routes of inoculation.

2.1. Curation of data

The dataset was extracted in May of 2017 and curated. Curation included identification of each reference and tabulation, as more than half of the Steinhoff database entries were pointers to the Drugfuture database which contained the literature reference. Apparent duplicates were removed. Of these 264 venomous ophidian species, 160 species having two or more DB entries were accepted, for a total of 1198

lethal dose (LD) DB entries. Of those 1198 DB entries, 1003 survived the curation process.

Of those 160 species records, 94 ophidian species had more than one DB entry for the same inoculation route in the same test-animal species. There were 17 test species in the dataset plus two that were generic "species," mammal and bird, for a total of 19 test-animal species categories. Most of the data were either mouse (70%) or rat (5%).

The maximum number of test-animal species for one ophidian species was 12. Test-animal species was sometimes a loose term in this context, as there are many species of monkey, duck, or frog, and there might be some variances in venom component sensitivity. However, no previous literature indicated variances of more than 1.6 orders of magnitude between these test-animal species, and one would not expect exotic monkeys, ducks, or frogs to be tested. In parts of this analysis, "mammal" and "bird" were treated as separate species. In most cases, the designation of mammals would be a rat or a mouse. Moreover, most birds would be pigeons or chickens. As will be seen, using only mouse data did not nullify the extraordinary variance results.

These studies were mostly performed in the 20th century, with the peak spanning from 1975 to 1979 (Figure 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.

3. METHODS

The analysis raised several questions. First, does ophidian venom variance fall within the literature-documented parameters of 1.6 logs (40-fold change) between mice, lizards, 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 the dose range, and how much of a contribution could be assigned to each factor?

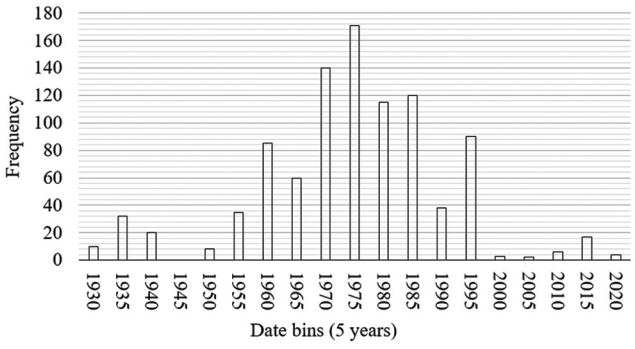

Figure 1. Time distribution of studies. Bins for date of the report are the termination years. The generic mammal DB entries had dates from 1953 to 1985, with 24 of them conducted in 1967. The unrecorded method of inoculation DB entries spanned from 1958 to 1987, with 29 in 1967. This was a period when some researchers appeared to believe that the test-animal species and route of inoculation was insignificant.

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.

3.1. Null hypotheses

The minimum lethal dose for a 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 the literature (e.g., ×4 and ×10, for a total of ×40).

Within a single test-animal species and for a single ophidian species, there should be a factor of two 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 differs from each other by more than one standard deviation, or, barring this, by standard error. LDLo values would not be the maximum lethal doses reported across studies. The lethal dose should vary by route of inoculation such that IC < IV < IP < IM < SC for 90% or more of venomous ophidian species across studies.

3.2. Primary views of data

This meta-analysis examined three primary views of the data with subset views: (1) Minimum lethal dose (LD_{min}) and maximum lethal dose (LD_{max}) 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), and for each ophidian species; (3) the fraction of the range within one ophidian species that each DB entry represented, expressed as a percentage, as explained below.

Range fold change is $R_{max} \div R_{min}$, where R_{max} is the highest LD for the species and R_{min} is the lowest LD for that ophidian species (LD can be LDLo or LD50.)

LD_{min} is the lowest lethal dose reported for an ophidian species. LD_{min} can be either an LD50 or an LDLo. LD_{max} 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 LD_{max} divided by the LD_{min} ($LD_{max} \div LD_{min}$) for an ophidian species. The fraction of the range for an entry is $LD_{entry} \div LD_{range}$, where $LD_{range} = LD_{max} - Ld_{min}$.

We defined three LD range fold changes: all data (LD-ADrf), single species (LD-SSrf), and single species, single route (LD-SRrf). LD-ADrf means that 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 takes the largest range multiple for one of the routes of inoculation).

After curation, the data were sorted by species and then by lethal dose. Further segregation by species family found one Atractaspis, one Homalopsidae, eight Colubrids, 69 Elapids, and 88 Viperids. Having only eight Colubrid species in the dataset made those results questionable to indicate a significant difference, although it might exist (data not shown). The LD-SRrf difference between Elapids and Viperids did not appear to be significant (data not shown).

For this analysis, in addition to what we saw by inspection of graphs, we used a mix of statistical measures: mean, median, standard error, and R^2. The coefficient of determination, R^2, was the measure of significance used in regressions for this meta-analysis. An $R^2 < 1$ indicated a degree of unexplained residual noise present. In a dataset with sufficient n and high variance, R^2 tends to be low, which is the case with our dataset. Econometric models are known for high variance and n; for instance, wages versus education level normally had an R^2 of approximately 0.3 [30]. This does not mean that the model is false, and it suggests that a lot of the variation is not explained by a single variable. Here, we used R^2 as an estimator of what variation may be related to each variable examined, and there was some conditional overlap to the variables. Our best-fit regressions were exponential curves of the form: $y = C \cdot x^m$ with negative exponents, with one exception, which was a linear fit. Because LD_{min} and LD_{range} should settle down, a curve fit was expected and that would suggest a limit. One of the equations with the best fit was linear and we believed this indicated that there were insufficient data to suggest a fold-change maximum.

4. ANALYSIS

4.1. LDLo did not differentiate from LD50 across studies

Sixty four out of 160 ophidian species had at least one LDLo value. These 64 ophidian species had 188 LDLo values and 532 LD50 values. In the meta-dataset, 24 of these 64 ophidian species (~40%) had an LD_{min} that was, indeed, an LDLo. However, 20 of the 64 ophidian species (~31%) had LDLo values that were the meta-study LD_{max}, which was against expectations.

$$\frac{\overline{LD}}{R_{max} - R_{min}} \quad (1)$$

Where $\overline{LD}$ is the mean comprised of the LDLo or LD50 values within one ophidian species.

For Figures 2 and 3, the range fraction was determined by equation 1. This way, relative toxicity between ophidian species was normalized, and a comparison of variation could be done between ophidian species. In Figure 2, the mean of the LDLo fractions was 28%, and the mean of the LD50 fractions was 32%. The standard deviation of the LDLo fractions mean was 32%, and the standard error was 4%. The standard deviation of the LD50 fractions mean was 20%, and the standard error was 2%. Even by the less stringent method of standard error, the difference was insignificant. Thus, the mean of the LDLo and LD50 range fractions for 64 out of 160 was not meaningfully different.

However, one might argue that a significant difference might be seen in LDLo values for single test-animal species, as shown in Figure 3. Here, as aforementioned, the data did not show this. Instead, it showed that for three out of eight test-animal species that had both LD50 and LDLo, mean LDLo was higher than LD50 and exceeded standard error. Note that this occurred with the highest N dataset (mouse). There were no test-animal species where mean LD50 was higher than LDLo and exceeded standard error as expected. The mouse test-animal species exceeded standard error if the median was used, and it still showed that LDLo was higher than LD50.

Strengthening this point, for seven ophidian species, both LD_{min} and LD_{max} were reported as LDLo. For 24 ophidian species, the LDmin was an LDLo, and for 20 ophidian species, the LDmax was an LDLo, as previously mentioned. The median number of DB entries for an ophidian species with one or more LDLo values was 10.

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

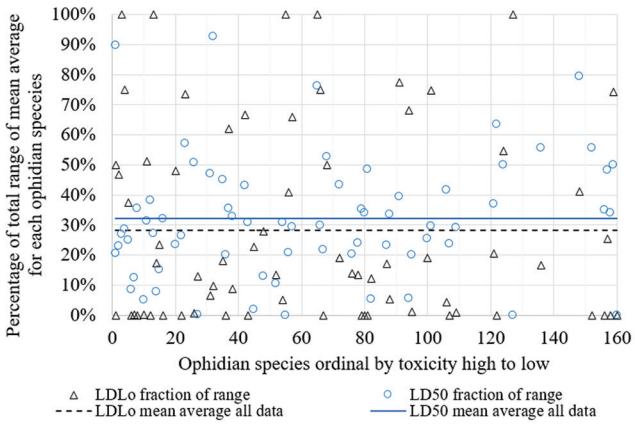

Figure 2. LDLo fraction of range compared to LD50 fractions of range. Fifty-one out of 160 ophidian species had at least one LDLo value and at least one LD50 value. The average of the LDLo fractions was 28.3%, and the average of the LD50 fractions was 32.2%. They differed by roughly the standard error. One would expect LDLo to average considerably below LD50.

4.2. Route of inoculation minimum and maximum lethal dose

Figure 4 strongly supports the concept that IC < IV < IP < IM < SC. This is the only hypothesis that was not falsified in this analysis. However, there were contradictory instances.

Out of 29 IC injections, 16 were LD_{min} values, which was as expected. Hence, for approximately half the instances, an IC injection was the minimum, and the *N* should be meaningful at 22. Using a synthetic X-axis, the 0.85 R^2 coefficient suggested that approximately 85% of the distribution fit the assumption that the route of inoculation varies as IC < IV < IP < IM < SC. The linear fit for LD_{max} showed the opposite trend, with a good R^2 suggesting that 89% of results could be attributed to the route of inoculation distributed in this manner.

However, 13 out of 249 SC injections were LD_{min} values, which made this unexpectedly the route of highest toxicity for 7.8% of the 160 ophidian species. Out of these 13, four venoms had strong hemotoxic or nephrotoxic effects, and the other nine were neurotoxic.

Of the IC inoculations, two were LD_{max}, which was opposite to expectations (*Notechis scutatus* and *Naja atra*) and was 1.2% of the 160 ophidian species. *N. scutatus* and *N. atra* contain both pre- and post-synaptic neurotoxins. *N. scutatus* had 26 DB entries and *N. atra* had 17. This should probably not be an artifact of a low number of studies performed for each. The percentages of these paradoxical SC and IC inverted cases were about the same, at 7% and 5% of their respective routes of inoculation.

4.3. Venom toxicity range fold change

The range of venom toxicity per ophidian species within the mouse test species had a peak value of 2.15 logs (141.33-fold change) and the mean average of 0.94 logs (8.89-fold change) within a single test-animal species, as shown in Figure 5. This peak was 3.53 times the 1.6 logs (40-fold change) documented in the literature for toxicity differences between test-animal species, as discussed above. For all test-animal species together, the peak value was 4.76 logs (57,471.26-fold change) and the mean range was 3.1 logs (1139.6-fold change). This peak was ~1,437 times, and the mean was ~28 times what current literature indicates.

The largest range fold change seen for an ophidian species tested in mouse for one route of inoculation was in *Crotalus horridus*, being 2.96 logs (916.7-fold change), *N (studies)* = 20. The largest range fold change for an

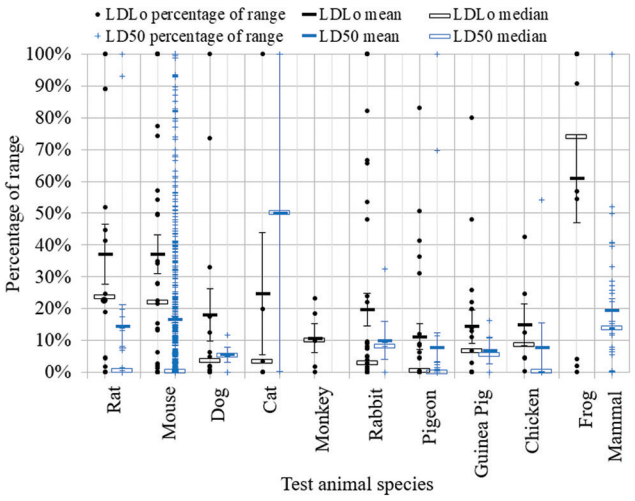

Figure 3. Fractions of range by test animal. Error bars show the standard error of the mean. Higher N animal datasets such as mouse, continued to show LDLo above LD50 for the median as well, and this was significant.

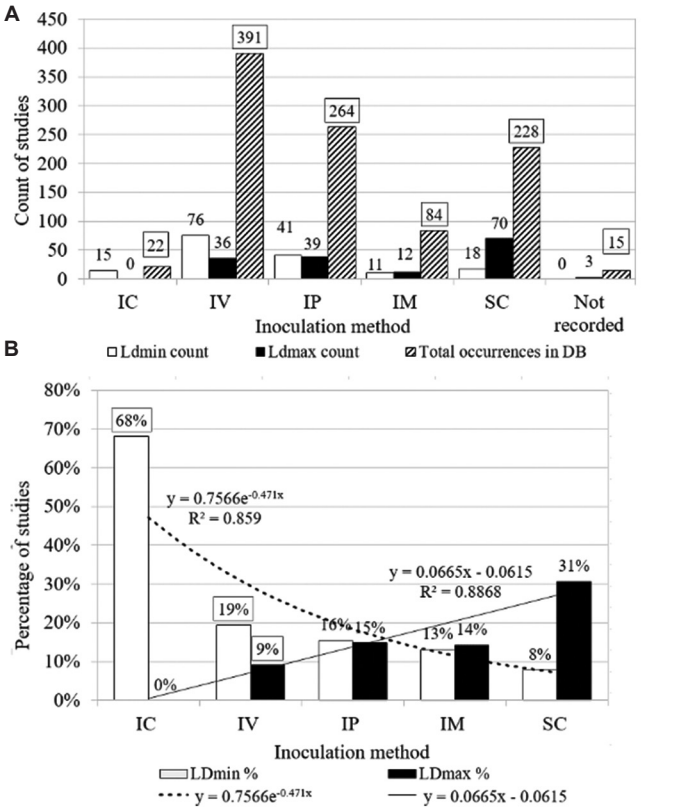

Figure 4. Route of inoculation distribution: Minimum and maximum lethal doses in the order of the hypothesis. Graph A shows the total number of inoculation route records in our curated DB, and how many occurrences of LD_{min} and LD_{max} for each inoculation route. Graph B shows what fraction of ophidian venoms had an LD_{min} (the minimum lethal dose) that is by the route of administration of the column. Conversely, LD_{max} shows the reverse. For instance, over half of ophidian species that had an intracerebral (IC) administration recorded show that the IC was the LD_{min}. Since not all ophidian species had an IC, this was not perfect, but it was supportive. The curves, fitted to a synthetic x-axis scale, confirmed what was visible by inspection, and that the degree of conformance of the LD_{min} values to the hypothesis was similar to LD_{max}. The confounding data, for instance, 7% of IC being the LD_{min} and 5% of SC being LD_{min}, are difficult to explain. IC: Intracerebral; IM: Intramuscular; IV: Intravenous; IP: Intraperitoneal; SC: Subcutaneous.

ophidian species tested only in mouse for all routes of inoculation is also *Crotalus horridus*, being 3.62 logs (4,150-fold change). For routes of inoculation, IM had the lowest, and SC had the highest LD. Examining single ophidian species, the data for all test-animal-species, including all routes of inoculation, showed that the largest range fold change is in *Naja naja*, being 4.46 logs (57,471-fold change), *N (studies)* = 35, *N (test-animal-species)* = 10 and this was for an unknown route of inoculation in rabbit and an IC inoculation in rat. These are among the highest *N* (number of studies) counts for ophidian species. Note that a specific ophidian species being mentioned here does not mean this species has been conclusively determined to be the single most venomous or to have the widest range of all.

One might ask whether the range fold change increase holds up when a single species and single route of inoculation is examined. In Figure 6, 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.

Figure 7, which looks at single test species for multiple routes of inoculation, shows an exponential regression trend that reaches significance for the range fold change increase as the *N* (the number of studies) gets larger. The fold change best-fit equation being exponential means it is not tending to flatten out yet, and flattening out is expected as more studies are done. This graph appears to signal the same thing that a set of ecological diversity transects continuing to increase exponentially would – data collection was insufficient. It indicates that to fully characterize ophidian venom lethal doses probably requires more than 50 different studies.

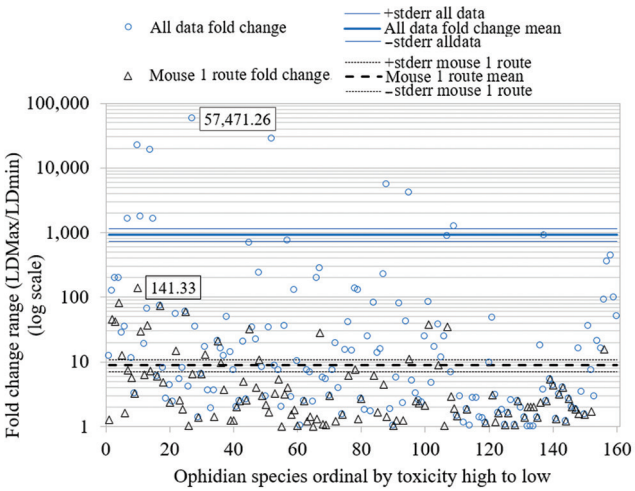

Figure 5. Mouse versus all test species LD_{min} and averages. The standard error of the means is shown as light-solid or light-dotted bars above and below mean average strong-solid and strong-dashed lines.

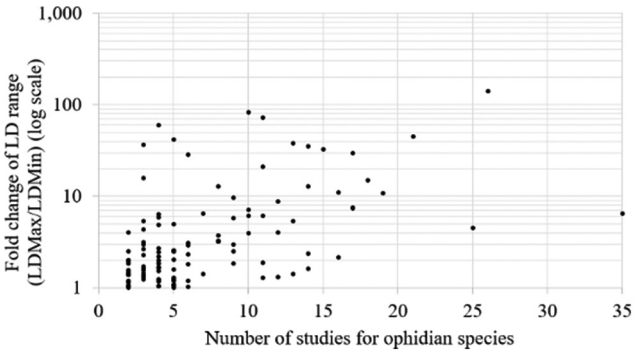

Figure 6. Range fold change (log scale) versus the number of studies for one test-animal species by one route of inoculation. N = 113 ophidian species that had two or more studies for the same route of inoculation.

4.4. Regressions of LD_{min}

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

Alternate hypothesis: Minimum lethal dose varies by more than a factor of two, and minimum lethal dose correlates with the number of times the dose has been tested. Table 1 summarizes the results of Figures 8-10.

4.4.1. Correlation of the number of routes of inoculation with LD_{min}

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

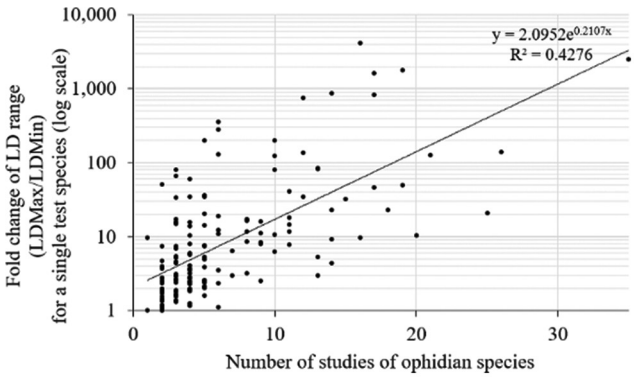

Figure 7. Venom range fold change for single test-animal species and multiple routes of inoculation. N = 160 ophidian species with 2 or more reports for the same test species. There was an exponential trend of increase in the range as the number of studies rose. In this graph, within each ophidian species, for test species with two or more entries, the test species with the largest fold change is shown.

lethal dose value, which rises as the number of inoculation routes goes to five, because, as is seen above, IC < IV < IP < IM < SC does tend to hold true.

4.4.2. Correlation of number of test-animal species per ophidian species to LD_{min}

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

In addition, 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 were multiple instances of the same test-animal species occupying LD_{min} and LD_{max}, and quite a few were very close to this state, which suggests that, indeed, the number of times an ophidian species is tested is a major factor.

Table 1. Regressions curve fit summary for LD_{min} rounded

Correlation examined	R^2
Number of routes of inoculation	0.26
Number of test species tested	0.31
Number of LD DB entries (number of studies)	0.37

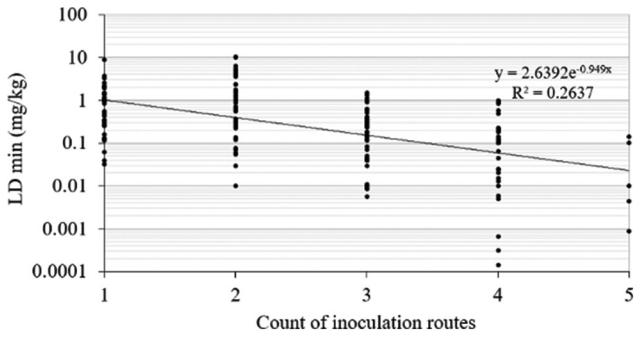

Figure 8. Minimum lethal dose versus number of routes of inoculation (Table 1, first entry).
The N for the number of routes of inoculation 1–5 are, respectively, 30, 36, 61, 33, and 7.

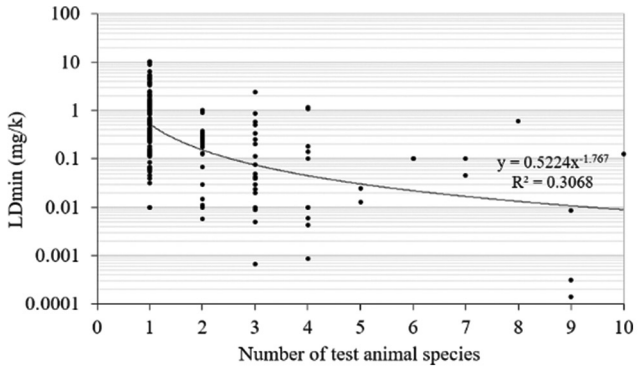

Figure 9. Minimum lethal dose versus number of test-animal species (Table 1, second entry).

4.4.3. Correlation of the number of DB entries (studies reported) per ophidian species to LD_{min}

In Figure 10, the "All data LD_{min}" fitted curve does not control for different test species. To address this criticism, "Mouse LD_{min}" shows the same graph filtered for inoculation of mice only. The R^2 value of 0.25 was not as good as the 0.37 R^2 value for all data. However, the N was lower, and by inspection, there was an excellent match for the curves for the region where they both had data. If there were no correlation by number of studies per species, then the fitted curves should be flat, whereas, both fitted curves spanned over a log and had quite close exponents and constants. Consequently, these data suggest that the primary correlate for lethality was the number of studies that had been performed.

4.5. Regressions on range fold change

The range fold change is $R_{max} \div R_{min}$. The R^2 values for these regressions were larger than what we see above. These data indicate that there is a correlation for all measures with the number of DB entries for lethal doses (number of studies reported). The number of routes of inoculation bore a meaningful correlation for all data and for single test species. We do not show that these graphs since they are redundant.

Table 2 confirms that the number of studies done is probably the primary correlation of toxicity for whole venom,

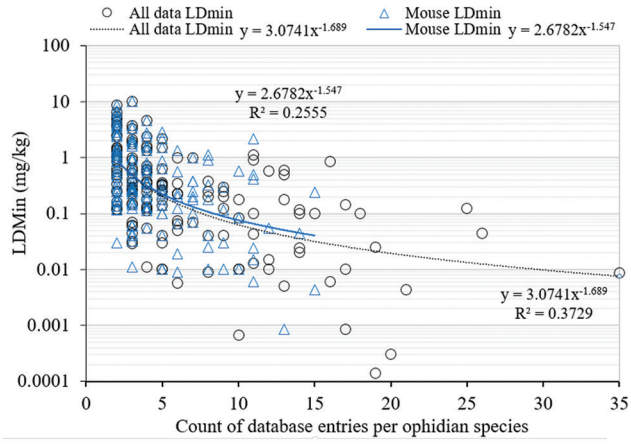

Figure 10. LD_{min} for all data versus mouse only data (Table 1, third entry). Minimum lethal dose versus count of database entries per ophidian species. For all data, the fitted curve spans ~2.15 logs. For mouse data only, the fitted curve spans ~1.4 logs (ophidian species N = 160). What is visible by inspection is that when data are 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 a higher number of DB entries.

Table 2. Regressions on all data for range fold changes of the highest over the lowest dose. All data (LD-ADrf), single species (LD-SSrf), single species, and single route (LD-SRrf)

Data, by species	LD-ADrf R^2	LD-SSrf R^2	LD-SRrf R^2
Number of routes of inoculation	0.429	0.34	
Number of species tested	0.5464		
Number of LD DB entries	0.5622	0.4042	0.2271

R^2 below 0.20 is not shown. N = 160 ophidian species.

and just above the correlation of the number of test-animal species used. The number of routes of inoculation is a mildly distant third correlate, and the relationship of routes of inoculation to the number of studies persists when restricted to a single species (center column).

5. DISCUSSION OF POSSIBLE CONFOUNDERS

There might be errors in database record entries, or some papers or books got the numbers wrong. However, Sascha Steinhoff has made strenuous efforts to validate the data entries as evidenced by the sourcing of each one. We checked and corrected every data entry in our curation process, and we have discussed this with him in personal communications as well. Hence, if there is an error in reporting, the error should be in the original source publication. We do not believe that this is a significant source of error.

Biomedical science in general has a reproducibility problem [31]. 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 IC 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, SC 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 SC injection hits a vein more often than thought. Similarly, veins may be missed and either become SC injections or IM injections. In animals such as mice, muscles can be missed.

However, for this study, there was no way to know what reproducibility issues there might be, and rejecting data *post hoc* because it does not fit preconceptions is unreasonable. We 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 or the rate of such error.

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 $LD_{max} \div LD_{min}$. To test this hypothesis, several methods were used. The top 14 venomous snakes listed as a threat to humans were plotted relative to their ordinal in lethality and the number of studies in the order of threat. Of these 14 ophidian species, 11 were in the bottom (more toxic) quartile of the 160 ophidian species included in this study, and 10 of the 14 were above the median of five studies, with a median of 10 studies among this set. Hence, there might be some effects from studies being directed at snakes dangerous to humans.

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.

We found that 22 out of the 160 species had subspecies listed. Only seven out of those 22 could have had an impact because the LD_{min} and LD_{max} were possibly different subspecies. Just two of these seven ophidian species had a range fold change that changed by a factor of two or more, and just one changed by a factor of 20. The mean range fold change for the seven that changed out of the 22 with listed subspecies was 0.88.

The mean LD_{min} fold change for the seven species out of 22 was 5.29. For all 22 species, the mean LD_{min} 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 to this dataset.

6. 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 [32,33].

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, we 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 suggests 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.

7. CONCLUSION

The import of this meta-analysis is several. First, the correlation between the number of times a venomous ophidian's lethal dose is studied and the range of lethal dose indicates that 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 discovery of variants in the transect area.

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

7.1. LD50 estimations

We developed a method of estimating lethal dose range and probability to support a research application for human study. Inspecting the density plots of Figure 3, it is apparent that the mouse data are the most populated and should be best for estimating a general-purpose probability distribution curve. This distribution is a histogram binned by tenths from 0.1 to 0.9 using all of the mouse LD50 data. Fitted to that histogram, equation 2 provides a simple logarithmic distribution (graph not shown) that declines in density from low LD to high LD. This logarithmic distribution may not hold for a specific species of venom. We caution that extending equation 2 below an *x* of 0.1 will be misleading. We expect that in the region between 0 and 0.1, there is likely to be a logarithmic rise, and that the true form of this distribution is probably an F distribution [34].

For general use, we can say that there is probably a rough logarithmic decline in the toxicity level over the range, with approximately 40% of the distribution in the lowest 10% of doses. Solving for *x* yields equation 3. The lethal dose at any *x* is provided by equation 4.

$$y = -0.26 \cdot \ln(x) \quad (2)$$

$$x = e^{-3.84615\,y} \quad (3)$$

$$L_D = x \cdot K + m \quad (4)$$

Where: **K** is the range of lethal doses in mg/kg;
x is a fraction of K, $0.1 < x < 0.9$;
y is the remainder fraction of the distribution from x to 1, and $y < 1$;
$\mathbf{L_D}$ is the lethal dose in mg/kg for a specific point on the x axis;
m is minimum lethal dose for a species in mg/kg

The correct way to define LD50 at this time is to use a meta-dataset, and to treat LDLo the same as LD50, to provide LD mean, median, minimum, range, and standard deviation, along with the *N* for the number of studies per test species used. We provide this in columns A and B in all-test-species form in the supplement. If there are sufficient data to make a reasonable estimation of the total LD range (*K*), then using equation 2, a nominal toxicity distribution can be estimated. Here is a working example.

Example: *Daboia russelii*

n	17 (minimum 0.01 mg/kg)
range	16.24 mg/kg (maximum is the last LD in column D.)
mean	1.923 mg/kg
median	0.260 mg/kg
σ	4.187 mg/kg

7.1.1. Use of the probability distribution function to find LD at midpoint

Let us determine what the approximate L_D will be at the midpoint of the distribution. If *y* is set to 50%, then $x = e^{-3.8461 \cdot 0.5} = 0.146$ or 14.6%. Using the definition of *x*, equation 4 will provide the lethal dose at this point. Thus, assuming that equation 2 holds for *Daboia russelii* venom, the 50% L_D should be $14.6\% \cdot 16.24\ \text{mg/kg} + 0.01\ \text{mg/kg} = 2.38\ \text{mg/kg}$. This is a rough determination, so let us call it ≈2.4 mg/kg.

7.1.2. Use of the distribution to find probability of a snakebite at a specific LD or less

Let us ask what the approximate probability of a snakebite victim experiencing an LD of 0.5 mg/kg or less should be. To do this, we first need to find what *x* will be for a 0.5 mg/kg lethal dose. Solving equation 4 for *x* yields $x = \frac{L_D - m}{K}$, compute

$$x = \frac{0.5 - 0.01}{16.24} = 0.0302.$$

Since equation 2 provides the fraction of the distribution after the *x* point, finding the region below the *x* point is $1 - y$.

Compute $y = -0.26 \cdot \ln(0.0302) = 91\%$ then compute $1 - 0.91 = 9\%$. Again, this is a rough estimate, so we can call it an approximately 1 in 10 chance.

Safety could be estimated by specifying *N* standard deviations, depending on the desired safety margin. If an LDLo value is desired, this is simply the minimum lethal dose in the meta-dataset and should be referred to that way (e.g., LDmin) to avoid confusion. This can be done separately for each route of inoculation and test species if there are sufficient data.

7.2. 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 from that of other animals, including monkeys. It would be desirable to develop a basis for relating animal lethal dose studies to our knowledge of human lethal doses. The work we did falls short of that, and what is desirable remains out of reach. Consequently, we must make do with what is available now.

It may be reasonable to consider the 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, we see that a sufficient *N* should be more than 50 different studies, and this is unlikely to happen soon. Furthermore, there are multiple instances in larger ophidian species datasets where non-mammal data were bracketed with mammalian data. Consequently, the most reasonable course requires a judgment call by those who are creating the estimate as to whether it is best to use aggregate data for all species, and compute as discussed above for the general case, or if non-mammalian data should be excluded.

It has been argued that humans cannot receive IP or IC inoculations, except in the case of infants. However, there were cases of bites to the thorax/abdomen in adults that appeared to progress more quickly, which may be similar enough to include IP for that instance.

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.

7.3. LD50 estimations with little data and computational research to support it

We recommend, for estimating LD50 for an ophidian with little data, the use of a customized meta-dataset for related species and factor proportionally from the mean of the minimal known data. To do this, a related ophidian meta-dataset can be curated based on what is known of venom makeup and/or genetic distance, plus the prey species. For that meta-dataset, the LD mean, minimum, range, and standard deviation are determined.

Estimation of the 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.

7.4. Human bite treatment: confirmation and caution

This meta-dataset tells us that, controlling for dose, the envenomation effect can vary by over 57,000 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, 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 confirmed that snakebites should always be treated symptomatically and that this should be done aggressively with antivenom when feasible because sooner or later an outlier should appear.

These results also confirmed that antivenom manufacturers should use mixtures from a variety of snakes of the same species for the immunization of animals. It is our understanding that this is, in fact, normal procedure.

7.5. 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 with recreational cobra bites, that are suggestive of inbred strains being less toxic [35,36]. Research of this issue will require many samples from wild 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 variations. This is a question that could take many years to answer.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Conceptualization: Brian P. Hanley
Formal analysis: All authors
Investigation: All authors
Methodology: All authors
Writing-original draft: Brian P. Hanley
Writing-review & editing: All authors

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA

Data analyzed in this study are primarily sourced from the Steinhoff database [28]. 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 materials. References for each data entry are in column AK of the supplement spreadsheet. This is the 37th column.

FURTHER DISCLOSURE

Second part of this study can be accessed at https://doi.org/10.14440/jbm.2024.0038.

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