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Intraspecific sequence and gene expression variation contribute little to venom diversity in Sidewinder Rattlesnakes (*Crotalus cerastes*)

Rhett M. Rautsaw, Erich P. Hofmann, Mark J. Margres, Matthew L. Holding, Jason L. Strickland, Andrew J. Mason, Darin R. Rokyta and Christopher L. Parkinson

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Review timeline

Original submission:	8 April 2019
Revised submission:	28 May 2019
Final acceptance:	7 June 2019

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2019-0810.R0 (Original submission)

Review form: Reviewer 1 (Juan J Calvete)

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
Rhett M. Rautsaw and o-worker elaborate on. and expand, a previous study by the same research team on population venomics of the Sidewinder rattlesnakes (Crotalus cerastes). Tajima's D and FST across four populations comparing toxin and nontoxin loci showed little evidence of directional selection or differentiation between populations, suggesting that changes to protein sequences do not underlie the evolution of Sidewinder venom or that toxins are under extremely variant selection pressures. The authors conclude that given their generalist diet, moderate gene flow,
and environmental variation, the lack of differential expression and sequence divergence may suggest thart Sidewinders are under stabilizing selection which functions to maintain a generalist phenotype. Overall, there is very little genetic differentiation within Crotalus cerastes even between the most geographically distant lineages. In the analysis of Tajima's D, the authors identified six toxin transcripts that fell outside the 95th percentile generated from the nontoxin distribution. Only CTL-1 and SVMPIII-7 were found to be under significant positive selection, while CRISP-1, CTL-9, CTL-11, and SVSP-10 were found to be under significant balancing selection. On the other hand, using the nontoxins as a null distribution, three toxin transcripts that fell outside the 95th percentile were identified: CRISP-1, PLA2-1, and SVSP-10. Only CRISP-1 and SVSP-10 were proteomically verified by Hofmann EP, Rautsaw RM, Strickland JL, Holding ML, Hogan MP, Mason AJ, Rokyta DR, Parkinson CL. 2018 Comparative venom-gland transcriptomics and venom proteomics of four Sidewinder Rattlesnake (Crotalus cerastes) lineages reveal little differential expression despite individual variation. Sci. Rep. 8, 15534. This two proteins were the only two venom proteins shared between Tajima's D and FST analyses. A total of six unique
toxins were found to be significantly divergent between lineage comparisons. 3FTx-1 and CTL-9 were the only two toxins found to be significantly divergent in multiple lineage comparisons. However, these toxin transcripts have not been proteomically detected. This apparent contradiction deserves an (hypothetical) explanation.

- "These results conform to previous analyses in the Mojave Rattlesnake (C. scutulatus) and

Eastern Diamondback Rattlesnake (C. adamanteus) that both demonstrated fang length variation to be associated with population-level variation in venom composition". The authors may wish to elaborate on the link between fang length and venom variation.

- Please, discuss why "low-expression toxins are more likely to evolve via balancing selection than high-expression toxins".

- "Sidewinder venom, contrary to our primary hypothesis. Instead, nontoxins and toxins appear to evolve at similar rates, suggesting that Sidewinders have evolved a generalist venom arsenal as a result of stabilizing selection on both toxin sequences and expression levels". The authors use indistinctly the terms "stabilizing selection" and "balancing selection". However, usually, stabilizing selection is a concept that applies to a phenotypic trait while balancing selection is a concept that applies to a given locus. Balancing selection can either be due to negative-frequency dependence selection or due to overdominance (=heterozygous advantage at a single locus). Please, be consistent when using the terminology.

- In this manuscripts, authors clearly expose the results that point to a highly conserved venom phenotype across the distribution sampled in the United States. It is concluded that -given its generalist diet, moderate to high gene flow, and environmental variation– such venom conservation likely reflects the influence of stabilizing selection which functions to maintain a generalist phenotype, rather than involved in strong antagonistic coevolutionary interactions. Any idea of temporal and ecological frameworks underlying this type of natural selection?

Review form: Reviewer 2

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
N/A

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
Please refer to attached file: Comments to authors. (See Appendix A)

Decision letter (RSPB-2019-0810.R0)

10-May-2019

Dear Dr Parkinson:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

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It is a condition of publication that you make available the data and research materials supporting the results in the article. Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

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Best wishes,

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Associate Editor
Board Member: 1
Comments to Author:
Both reviewers are very positive about this study and agree that it makes an interesting and important contribution. Both reviewers make some useful suggestions for minor revisions. These are clearly described and should be addressed in a revised manuscript or rebutted convincingly.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
Rhett M. Rautsaw and o-worker elaborate on. and expand, a previous study by the same research team on population venomics of the Sidewinder rattlesnakes (Crotalus cerastes). Tajima’s D and FST across four populations comparing toxin and nontoxin loci showed little evidence of directional selection or differentiation between populations, suggesting that changes to protein sequences do not underlie the evolution of Sidewinder venom or that toxins are under extremely variant selection pressures. The authors conclude that given their generalist diet, moderate gene flow,
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However, these toxin transcripts have not been proteomically detected. This apparent contradiction deserves an (hypothetical) explanation.

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- Please, discuss why "low-expression toxins are more likely to evolve via balancing selection than high-expression toxins".

- "Sidewinder venom, contrary to our primary hypothesis. Instead, nontoxins and toxins appear to evolve at similar rates, suggesting that Sidewinders have evolved a generalist venom arsenal as a result of stabilizing selection on both toxin sequences and expression levels". The authors use indistinctly the terms "stabilizing selection" and "balancing selection". However, usually, stabilizing selection is a concept that applies to a phenotypic trait while balancing selection is a concept that applies to a given locus. Balancing selection can either be due to negative-frequency dependence selection or due to overdominance (=heterozygous advantage at a single locus). Please, be consistent when using the terminology.

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Referee: 2

Comments to the Author(s)
Please refer to attached file: Comments to authors.

Author's Response to Decision Letter for (RSPB-2019-0810.R0)

See Appendix B.

Decision letter (RSPB-2019-0810.R1)

07-Jun-2019

Dear Dr Parkinson

I am pleased to inform you that your manuscript entitled "Intraspecific sequence and gene expression variation contribute little to venom diversity in Sidewinder Rattlesnakes (*Crotalus cerastes*)" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact please let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

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Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,

Dr Sasha Dall
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor:
Board Member
Comments to Author:
The authors have responded comprehensively and positively towards the reviewers' suggestions for minor revisions. I see no remaining issues from initial review. This now looks like a strong and interesting contribution to the field, with considerable wider interest as well.

Appendix A

Comments to authors: RSPB-2019-0810

Authors Rautsaw et al. reported an interesting study that examined the relationship between gene expression and protein-coding changes in order to unravel the evolution of snake venom, using Sidewinder Rattlesnake (*Crotalus cerastes*) as the model. The venom gland transcriptomic data of the species (published) were analyzed with various tools to test for sequence variation/evolution, for the species from different geographical areas. In addition, fang morphology was included to study for association with sequence divergence of the toxins. The findings were somewhat contrary to the primary hypothesis (and what commonly perceived to be true by many), as the coding sequence evolution and gene expression seem to contribute very little to the evolution of Sidewinder venom. Author thus concluded that the venom is remarkably similar across the distribution in US and is under the influence of stabilizing selection. The work provides a new insight into the evolution of snake venom and the scientifically commendable. There are only a few queries which the authors should try to address for readers from different backgrounds, and a few suggestions for the authors to consider.

Abstract: Concise.

Line 41-46: “However, recent research at the intraspecific level suggests that snake venom evolution might be driven by changes in toxin gene expression (i.e. toxin quantity) rather than coding sequence evolution (i.e. toxin function) [7,17–19].” – Authors referred this phenomenon to a few examples of intraspecific studies of the new world species (esp. viperids). Suggestion: Authors to include similar case observed at intraspecific venom-gland transcriptomics of Asian/Old World species, for example the medically important cobra e.g. *Naja kaouthia*, where differential gene expression (transcriptomically and proteomically verified) rather than coding sequence evolution has been shown to drive the discrepancies in neurotoxicity and antivenom response (Ref: https://peerj.com/articles/3142/)

Line 100-109: “few genes were differentially expressed between lineages of Sidewinders, and overall venom expression was similar across the range of the species. The lack of differential expression among toxin genes could be a product of a relatively flat adaptive landscape and stabilizing selection functioning to maintain a generalist venom arsenal. ….. Sidewinders prey on a variety of small mammals and lizards, and there is no evidence of geographic variation in their diet.”

Comment: The considerable lack of differential expression of toxin genes in Sidewinder Rattlesnakes has led the authors to hypothesize that their venoms have diverged in protein sequences (Line 117-120). Can the authors explain why divergence in protein sequences is anticipated - is it prompted by any differences in functional properties, or Sidewinders from different geographical areas cause variable clinical symptom/sign of toxicity? A note on the clinical manifestation of toxicity of this species, if available, would be helpful and provide insights into population health issue.

Line 483-484: “These results suggest weak differences between toxins and nontoxins.” – Can the authors elaborate more specifically what “differences” were referred to? (in what?)

Line 498-501: “Therefore, using average values might not have provided informative differences between toxins and nontoxins in some tests.” Can authors propose what suitable alternative can be possibly used? (instead of average values)

Line 501-504: “Nonetheless, few toxin outliers were shared across tests which further supports that toxins are under diverse selection pressures and evolving at similar rates as nontoxins.” – Was the categorization of toxins and nontoxins followed the name of toxin annotated, as in most venom-gland transcriptomic studies? Could it be possible that some transcripts grouped under ‘toxins’ actually do not serve toxic function, and possibly represent the physiological genes before selection or neo-functionalization?

Line 523-525: “…reveal variation in fang size that was concordant with the highest amounts of divergence in toxin sequences.” – Is there any postulation to relate the fang size and the particular toxins with high sequence divergence? What toxins were those and if they were functionally related to certain fang morphology?

Appendix B

CLEMSON
College of SCIENCE

27 May 2018

Dear Editors:

We would like to thank you and the reviewers for the helpful comments and suggestions on RSPB-2019-0810, "**Intraspecific sequence and gene expression variation contribute little to venom diversity in Sidewinder Rattlesnakes (*Crotalus cerastes*)**". We are writing to resubmit our revised manuscript for consideration for publication in *Proceedings of the Royal Society B: Biological Sciences*. As a result of the reviewers' comments, we have made revisions to the text of the manuscript. Please find point-by-point responses to the reviewers' comments on the next page (reviewer comments in italics, our responses in bold). We have also made minor changes throughout the manuscript for clarity and brevity.

As with our original submission, all authors have approved the revised manuscript and we declare no conflicts of interest. This research was funded by the National Science Foundation and numerous small grants including Sigma Xi Grants-in-aid-of-research and the American Museum of Natural History Theodore Roosevelt Memorial Fund. The funding sources are all acknowledged in the manuscript and there will be no direct financial benefits resulting from publication of this manuscript. Lastly, all research was conducted following ethical and legal guidelines and regulations.

Thank you for your time and consideration of our revised manuscript for publication. We look forward to your response.

Sincerely,

Christopher L. Parkinson, Ph.D.
Professor

Dept. of Biological Sciences & Dept. of Forestry and Environmental Conservation, Clemson University,
190 Collings St., 157B Life Sciences Facility, Clemson, SC 29634
Phone: 864-656-3058
viper@clemson.edu

Response to Reviewers:

Reviewer #1 (Remarks to the Author):

*Rhett M. Rautsaw and co-worker elaborate on and expand, a previous study by the same research team on population venomics of the Sidewinder Rattlesnakes (*Crotalus cerastes*). Tajima's D and* F_{ST} *across four populations comparing toxin and nontoxin loci showed little evidence of directional selection or differentiation between populations, suggesting that changes to protein sequences do not underlie the evolution of Sidewinder venom or that toxins are under extremely variant selection pressures. The authors conclude that given their generalist diet, moderate gene flow, and environmental variation, the lack of differential expression and sequence divergence may suggest that Sidewinders are under stabilizing selection which functions to maintain a generalist phenotype. Overall, there is very little genetic differentiation within* Crotalus cerastes *even between the most geographically distant lineages. In the analysis of Tajima's D, the authors identified six toxin transcripts that fell outside the 95th percentile generated from the nontoxin distribution. Only CTL-1 and SVMPIII-7 were found to be under significant positive selection, while CRISP-1, CTL-9, CTL-11, and SVSP-10 were found to be under significant balancing selection. On the other hand, using the nontoxins as a null distribution, three toxin transcripts that fell outside the 95th percentile were identified: CRISP-1, PLA2-1, and SVSP-10. Only CRISP-1 and SVSP-10 were proteomically verified by Hofmann EP, Rautsaw RM, Strickland JL, Holding ML, Hogan MP, Mason AJ, Rokyta DR, Parkinson CL. 2018 Comparative venom-gland transcriptomics and venom proteomics of four Sidewinder Rattlesnake (*Crotalus cerastes*) lineages reveal little differential expression despite individual variation. Sci. Rep. 8, 15534. These two proteins were the only two venom proteins shared between Tajima's D and* F_{ST} *analyses. A total of six unique toxins were found to be significantly divergent between lineage comparisons. 3FTx-1 and CTL-9 were the only two toxins found to be significantly divergent in multiple lineage comparisons. However, these toxin transcripts have not been proteomically detected. This apparent contradiction deserves an (hypothetical) explanation.*

*"These results conform to previous analyses in the Mojave Rattlesnake (*C. scutulatus*) and Eastern Diamondback Rattlesnake (*C. adamanteus*) that both demonstrated fang length variation to be associated with population-level variation in venom composition". The authors may wish to elaborate on the link between fang length and venom variation.*
Author Response: Although much is still unknown about the link between morphology and venom variation, we have discussed the possible link that myotoxin expression and fang length might be correlated (L550 – 557).

Please, discuss why "low-expression toxins are more likely to evolve via balancing selection than high-expression toxins".
Author Response: We have clarified this throughout the manuscript. In the specific section the reviewer is referring (Results: Effect of Toxin Expression on Sequence Evolution) we have added "High-expression toxins are constrained by selection to ensure translational efficiency and produce the focal phenotype [43]. Our results support that low-expression toxins have higher sequence variation and evolve via balancing selection." (L326 – 330)

"Sidewinder venom, contrary to our primary hypothesis. Instead, nontoxins and toxins appear to evolve at similar rates, suggesting that Sidewinders have evolved a generalist venom arsenal as a result of stabilizing selection on both toxin sequences and expression levels". The authors use indistinctly the terms "stabilizing selection" and "balancing selection". However, usually, stabilizing selection is a concept that applies to a phenotypic trait while balancing selection is a

concept that applies to a given locus. Balancing selection can either be due to negative-frequency dependence selection or due to overdominance (=heterozygous advantage at a single locus). Please, be consistent when using the terminology.
Author Response: We agree with the reviewer that balancing selection refers to alleles while stabilizing selection refers to phenotypes. We have clarified this throughout the manuscript to ensure stabilizing selection is only referred to in reference to the venom phenotype. In this specific section we have removed "… on both toxin sequences and expression levels".

In this manuscripts, authors clearly expose the results that point to a highly conserved venom phenotype across the distribution sampled in the United States. It is concluded that -given its generalist diet, moderate to high gene flow, and environmental variation- such venom conservation likely reflects the influence of stabilizing selection which functions to maintain a generalist phenotype, rather than involved in strong antagonistic coevolutionary interactions. Any idea of temporal and ecological frameworks underlying this type of natural selection?
Author Response: We have added two sentences (L604 – 610) in the last paragraph of our discussion regarding the temporal and ecological frameworks underlying these selection pressures. We believe the generalist diet of Sidewinders is a strong ecological influence on the maintenance of their current venom composition. With regards to the temporal framework, the populations of Sidewinders diverged approximately 1.5 million years ago and have little variation in venom. We compare this to the Mojave and Sonoran populations of the Mojave Rattlesnake which diverged approximately 0.5 to 1 million years ago and display striking differences in venom due to diversifying selection pressures.

Reviewer #2 (Remarks to the Author):
*Authors Rautsaw et al. reported an interesting study that examined the relationship between gene expression and protein-coding changes in order to unravel the evolution of snake venom, using Sidewinder Rattlesnake (*Crotalus cerastes*) as the model. The venom gland transcriptomic data of the species (published) were analyzed with various tools to test for sequence variation/evolution, for the species from different geographical areas. In addition, fang morphology was included to study for association with sequence divergence of the toxins. The findings were somewhat contrary to the primary hypothesis (and what commonly perceived to be true by many), as the coding sequence evolution and gene expression seem to contribute very little to the evolution of Sidewinder venom. Author thus concluded that the venom is remarkably similar across the distribution in US and is under the influence of stabilizing selection. The work provides a new insight into the evolution of snake venom and the scientifically commendable. There are only a few queries which the authors should try to address for readers from different backgrounds, and a few suggestions for the authors to consider.*

Abstract: Concise.

Line 41-46: "However, recent research at the intraspecific level suggests that snake venom evolution might be driven by changes in toxin gene expression (i.e. toxin quantity) rather than coding sequence evolution (i.e. toxin function) [7,17–19]." – Authors referred this phenomenon to a few examples of intraspecific studies of the new world species (esp. viperids). Suggestion: Authors to include similar case observed at intraspecific venom-gland transcriptomics of Asian/Old World species, for example the medically important cobra e.g. Naja kaouthia, *where differential gene expression (transcriptomically and proteomically verified) rather than coding*

sequence evolution has been shown to drive the discrepancies in neurotoxicity and antivenom response (Ref: https://peerj.com/articles/3142/)
Author Response: We have added the recommended citation.

Line 100-109: "few genes were differentially expressed between lineages of Sidewinders, and overall venom expression was similar across the range of the species. The lack of differential expression among toxin genes could be a product of a relatively flat adaptive landscape and stabilizing selection functioning to maintain a generalist venom arsenal. ….. Sidewinders prey on a variety of small mammals and lizards, and there is no evidence of geographic variation in their diet."
Comment: The considerable lack of differential expression of toxin genes in Sidewinder Rattlesnakes has led the authors to hypothesize that their venoms have diverged in protein sequences (Line 117-120). Can the authors explain why divergence in protein sequences is anticipated - is it prompted by any differences in functional properties, or Sidewinders from different geographical areas cause variable clinical symptom/sign of toxicity? A note on the clinical manifestation of toxicity of this species, if available, would be helpful and provide insights into population health issue.
Author Response: We have added a sentence at the end of this paragraph on why this hypothesis is anticipated. L118 – 121: "A single case of neurotoxicity of Sidewinder venom [44], together with data showing that expression and sequence evolution are highly correlated [4], favor this hypothesis."

Line 483-484: "These results suggest weak differences between toxins and nontoxins." – Can the authors elaborate more specifically what "differences" were referred to? (in what?)
Author Response: We have elaborated that the differences are in reference to estimates of Tajima's D and Fst.

Line 498-501: "Therefore, using average values might not have provided informative differences between toxins and nontoxins in some tests." Can authors propose what suitable alternative can be possibly used? (instead of average values)
Author Response: We have added a sentence (L512 – 519) stating that looking for outliers likely provided more informative results for detecting selection and that having a larger sample size to look across Sidewinder's full distribution would allow for better statistical analyses.

Line 501-504: "Nonetheless, few toxin outliers were shared across tests which further supports that toxins are under diverse selection pressures and evolving at similar rates as nontoxins." – Was the categorization of toxins and nontoxins followed the name of toxin annotated, as in most venom-gland transcriptomic studies? Could it be possible that some transcripts grouped under 'toxins' actually do not serve toxic function, and possibly represent the physiological genes before selection or neo-functionalization?
Author Response: It is possible, but unlikely. Additionally, any falsely-categorized toxins would make up a very small proportion of the overall dataset (e.g. Ficolin [n = 2]). Transcripts were annotated via blastx searches against the UniProt animal venom proteins and toxins database (http://www.uniprot.org/program/Toxins) and clustering with previously annotated snake toxins. Therefore, the categorization of toxins and nontoxins was based on previous annotations, but from a database of known toxins. Additionally, we previously used proteomics to confirm the secretion of ~80% of transcriptome-annotated

toxins in *C. cerastes* (Hofmann and Rautsaw *et al.* 2018). Therefore, we anticipate almost all of the toxins to have some toxic functions.

Line 523-525: "…reveal variation in fang size that was concordant with the highest amounts of divergence in toxin sequences." – Is there any postulation to relate the fang size and the particular toxins with high sequence divergence? What toxins were those and if they were functionally related to certain fang morphology?
Author Response: The toxins with highest differentiation between populations were identified in Figure 2D. We do not know how or if these three toxins (CRISP-1, PLA2-1, SVSP-10) relate to fang size, especially given that the function of CRISP is unclear (see Results: Selection on Individual Toxins). Much is still unknown about the link between morphology and venom variation; however, we have discussed the possibility that fang size may be related to myotoxin expression (L550 – 557).