THE ROYAL SOCIETY PUBLISHING

PROCEEDINGS B

# Electrostatic resistance to alpha-neurotoxins conferred by charge reversal mutations in nicotinic acetylcholine receptors

Richard J. Harris and Bryan G. Fry

**Article citation details**
*Proc. R. Soc. B* **288**: 20202703.
http://dx.doi.org/10.1098/rspb.2020.2703

**Review timeline**

| | |
|---|---|
| Original submission: | 29 October 2020 |
| Revised submission: | 10 December 2020 |
| Final acceptance: | 10 December 2020 |

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

# Review History

## RSPB-2020-2703.R0 (Original submission)

## Review form: Reviewer 1

**Recommendation**
Accept with minor revision (please list in comments)

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

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

**Quality of the paper: Is the overall quality of the paper suitable?**
Good

**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**
It is a very interesting paper providing new important insights about mechanisms of toxin resistance evolution. The conclusions are well supported by multiple experiments with mutated binding sites in different species.
I have rather minor comments.
1. I think it would be better to restructure the text and move the Fig 5 much earlier when the analysis of the published AChR sequences was first mentioned (line 106). Also, there are no details about how this analysis was done: how was the ancestral sequence identified? Was it reconstructed by analysing ACh receptor phylogeny among vertebrates? Xenopus laevis sequense on its own cannot be considered ancestral.
Also, it is not clear how the 10 convergent events of resistance evolution were identified. From the tree at Fig 5 it looks like an alternative scenario could be that the K residue evolved in the last common ancestor of the shown snake lineages and afterwards it was reversed in some of them to E/D. It would require only 9 reversal events at the position 195.
2. Line 58: phenylalanine is not polar
3. Line 90: I think it should be "189F mutant was not bound" according to the Fig 1B.
4. Line 95: repetitive “that”
5. Line 157: “diverse” – I guess “distant” would be more correct
6. Line 236: “addition” – probably should be “additional”
7. Line 237: “evolutions” – “evolutionary events” would be more correct
8. Line 254: “based from” - probably should be “based on”
9. Line 263: “synthesised” - I guess “connected” would be more correct
10. Line 274: “were” – should be “was”?
11. Fig 5: Not all the ancestral Ds are shown in blue, some are represented by dots (for example, Anilios)

## Review form: Reviewer 2

**Recommendation**
Major revision is needed (please make suggestions in comments)

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

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

**Quality of the paper: Is the overall quality of the paper suitable?**
Marginal

**Is the length of the paper justified?**
No

**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**
Review: RSPB-2020-2703

In this manuscript, the authors present comparative data on binding of neurotoxins from several snakes to the nAChR mimotopes of several potential snake prey and one snake predator. As such, it is an important comparative study that utilizes high-throughput technology to inform how toxins and resistance might evolve and coevolve repeatedly across lineages. Where it is heavy on novel results, it is currently lacking in its set-up…an apparent combination of original formatting for short-form journals and more importantly a lack of a broader hypothesis testing framework in the introduction. The hypotheses instead are named in a various points in the results section, where the reader (at least this reader) is already bogged down in the details of the results themselves and has to retroactively piece together what the hypothesis is and how it links to broader ideas in evolutionary biology. Additionally, claims regarding the number of times the K substitutions have evolved appear to require a more formal ancestral state reconstruction before they can be truly substantiated. In summary, these are wonderful and important results, and I endorse their rapid publication following efforts to 1) frame the results in a broader evolutionary context (such as repeatability vs. novelty in trait evolution) and 2) use a hypothesis-and-predictions lead in to each of the individual results so the reader knows why the test was done and what the specific results mean interms of a particular hypothesis. I give specific examples of these problems and make my best to propose solutions to them below. I will close here by emphasizing that while these critiques sound harsh, my general view of the prospectus for these results is not. I am excited by the results, and relative quick rewrite of the introduction, moving details already in the results to more of a lead-in position will make this a much more digestible

and impactful piece of work.

Major Comments:

Introduction –

The manuscript introduction (and paper overall) would greatly benefit from an effort to better frame a general biological question. The paper introduction essentially reads as "coevolution generates novelty -> venom coevolves with resistance -> nAChR's have evolved resistance -> here are some biological examples of resistance -> convergent resistance may involve different molecular/biophysical mechanisms -> we are going to explore resistance substitutions in the badger and also in several snakes". There is a broad literature on ion channel resistance evolution done in a comparative framework (I'm thinking of PNAS papers by Feldman on TTX resistance in snakes and subsequent broader taxonomic comparisons of TTX resistance by Joel McGlothin) that provide more clearly articulated concepts about the nature of constraint and convergence in repeated evolution of resistance traits. You seem to have a particularly novel result here, in showing that convergence phenotypes can come about through alternative biophysical mechanisms. More fleshing out of this idea as a question worth addressing would be welcome. The current draft is very short and appears written for a prior submission to a short form journal, so there is ample room to expand a bit on the broader biological questions at hand without seeming wordy.

Line 43-46: This paragraph, equal in length to the rest of the intro combined, reads a simply a list of examples. It seems that it could be reworked into two smaller paragraphs that subdivide the examples by type. For example, first list the confirmed examples of steric hindrance and the "minor forms". Then in the second paragraph, tantalize with the possible mechanism of charge repulsion and citations 4 and 11. This second paragraph will then end with the specific question in some form: Is there charge repulsion, really? In this way, the introduction moves the reader's understanding along in a more digestible manner.

Line 73-77 – The authors introduce their hypothesis in the same sentence in which they define biolayer interferometry. For readability, please separate and further flesh out both components.

Results and Discussion –

I think the readability of this section could be greatly improved by some subsectioning. The authors could then clearly delineated their presentation of honey badger mutations vs. snake mutations, and in so doing clearly divide the two types of resistance.

Line 104-117: Aside from the second sentence reporting on the authors' examination published sequences, this paragraph is all introductory material, and is specifically the kind of material I was wishing for when reading the introduction. It clearly lays out previous studies and posits a hypothesis at its final sentence. I strongly encourage that the bulk of this information is moved to the introduction.

Lines 161-190: In this section, it is clear that the authors are testing some form of a prediction stemming from a hypothesis of local adaptation due to coevolution, as they use terms such as sympatric and allopatric in describing their combination of venom and mimotope. The passage would read much more cleanly and comprehensibly if the authors introduced this in the form of predictions stemming from a hypothesis of local adaptation. E.g. "If resistance to a-neurotoxins involves local adaptation to specific toxin epitopes, then we expected to find stronger effects of the substitutions on binding affinity of sympatric venoms." This prediction then comes to bare in the comparison among the three Naja venoms, and the authors can then explicitly discuss the result as support for local adaptation playing a role in sequence substitutions…a truly novel result I believe! In this way, this paragraph is a microcosm of the broader issue with the paper: the punchline and impact an amazing dataset and results is buried by comparately poor set up.

These data are very complex to start with, and layered on top of that is that each species studied is idiosyncratic in the means by which resistance is achieved. A strong introduction with delineated hypotheses and predictions, followed by subsectioning and flagging phrases that link back to the prediction will all help turn this into a really impactful work. Just lead the reader by the nose through all the impacts.

Ancestral states – The term "ancestral" is used several times throughout the manuscript to polarize one state vs. another. Furthermore the claim of 10 independent evolution of K charge reversals in snakes is made and figure 5 is referenced. Despite all of this, there is no reference to any type of formal ancestral state reconstruction to support this claim. Since 10 of 19 snakes presented show a K substitutions, it may be equally or more parsimonious to assume the ancestral snake had a K that has subsequently been lost in lineages that don't have to deal with venomous predators, particularly since the tree is polarized against a frog, which is more distantly related than even a mammal (a lizard maybe is best?). This could be another case whether the scant introduction isn't making clear that a broader ancestral state reconstruction has already been published for snakes. Otherwise the claim of 10 independent evolutions – very cool if true – requires a formal ancestral state reconstruction with more sequences and is otherwise unsubstantiated by the present results. This clarification would add greatly to the paper.

Materials and Methods:

General comment - Despite previous method validation, it would still seem appropriate for the researchers to include intra-assay positive controls for each of the mimotope batches (i.e. a completely susceptible human or mouse mimotope) to provide context for how much, in a relative sense, these back-mutations are actually increasing binding affinities.

Line 268 – Given that the method is billed as a crucial innovation for the field in the abstract, and BLI is the key to the entire paper, it would be appropriate for at least a brief description of some of the basics of BLI in this section, or potentially in a short paragraph in the introduction that describes both what a mimotope is and how BLI works. The paper should be readable alone. While one would not have to describe something like Illumina sequencing process in a current paper, BLI is used much less frequently, and even more so in toxinology to date.

Line 278 – The aforementioned lack of detail is even more apparent in this section. I am attempting to evaluation your stats, but your raw data – apparently "association step" data – is mentioned bluntly and without explain. I can certainly piece together what the association step is without having done BLI, but honestly think a reader should be able to understand the basics of one's raw data without having to read a second paper. Furthermore, the author's need to include details about numbers of replicate runs done for input into their ANOVAs. Additional, which comparisons were made? Is there a need to correct for false discovery?

Figures –

Figure 1. Are only comparisons within each snake made? For example, it seems that comparisons of the wild-type binding to all four snakes (comparisons of all red bars) would be interesting to make, particularly if ecology suggests honey badgers should be more resistant to some of these species than others.

Figure 2. Same question as above. Is their utlity in comnparing not just within, put among the panels, in terms of binding levels? If not, potentially due to inter-assay variability or the like, this should be mentioned in the methods for readers to direct focus only within panels.

Figure 5. It would be helpful to the reader not familiar with snake systematics to code the tree with some color blocks and name the clades. For example, block of Pythonidae in a particular color and write "Pythonidae" or "Pythons" outside of the block, so that in text when readers are refered to substitutions occurring in pythons in Fig 5, then can look without having to further

cross-reference among binomials.

Minor -

Line 12: unclear why authors capitalize “Biolayer”

Line 17-18: The phrase “such a novel form of …has gone completely undiscovered” is circular – novel and undiscovered. But you have also already said it was novel on line 13, so this seems merely a two-line sales pitch for the paper. Instead, would be more useful to spend more time on biological relevance/implications for coevolution and convergence.

Line 23: Seems and undersell of the biological result to have the final sentence of the abstract to focus on the utility of the method. The biological result of charge reversal and its repeated convergence is fascinated and needs fleshing out even in the short space of the abstract.

Line 68: Change to “…confirmed to confer resistance”.

Line 68-71: Same sentence contains “in contrast”, “although”, and “since” transitions. It is therefore too complex and merits clarification and break-up.

Line 73: Change “theory” to “hypothesis”.

Lines 76: Extra comma after “snakes”.

Line 106: Remove a “that”

Lines 122 and 137: claim about charge reversal repeated in both spots. Choose one for brevity.

Lines 142-152: Some citations supporting the various claims made here are needed. In particular, regarding python terrestriality, juvenile malayopython arboreality, the idea that trees are devoid of elapids, and regarding Morelia and Liasis habits.

Line 161 and several places elsewhere: It occurs to me that significant wordiness in the many places that describe particular mutations could be cut by using the common shorthand (e.g. D191K). So here the sentence “Pseudaspis cana has evolved at position 191 a charge-reversal with the positively charged K in 162 place of negatively charged D at position 191, and with the introduction of an additional positively charged K at 196 which does not represent a charge reversal at this hypervariable position” could be shortened to “Pseudaspis can has evolved a D191K mutation, producing a charge reversal to a positive charge, while introduction of a 163K. does not reverse this hypervariable site’s charge”. This is both shorter and more understandable…hard to do! Something like this would be particularly refreshing in the bulkiest paragraph in the introduction, and the authors could maybe just spell things out the first time fully to show reader’s not familiar with AA replacement lingo how to read something like D191K.

Line 166: “was” to “were”

Line 210: A good place to start a general “Conclusions” subheading.

Line 213-214: Appears to be an unsubstantiated conclusion. It seems we cannot be sure that this was the original selection pressure, so this claim should be altered or better indicated to be speculative.

Line 236: Addition to additional

# Decision letter (RSPB-2020-2703.R0)

07-Dec-2020

Dear Dr Fry:

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.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" - in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:
If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article. Please see our Data Sharing Policies (https://royalsociety.org/journals/authors/author-guidelines/#data). 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).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy http://royalsocietypublishing.org/data-sharing.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Dr Maurine Neiman
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
This paper has now been seen by two reviewers, both of whom say that the work is important. I lean towards the more severe Referee, who also identifies a number of issues. This is a very thorough and intelligent review that identifies a number of cosmetic issues that should be attended to. In addition, there is the crucial issue of how the ancestral state was identified. I agree that additional work is needed in order both to explain how this was achieved and, I suspect, additional analyses to convince the reader that the conclusions are robust. It is certainly true that, at face value, an equally parsimonious explanation is that the K state was ancestral and has since been lost.

Reviewer(s)' Comments to Author:
Referee: 1

Comments to the Author(s)
It is a very interesting paper providing new important insights about mechanisms of toxin resistance evolution. The conclusions are well supported by multiple experiments with mutated binding sites in different species.
I have rather minor comments.
1. I think it would be better to restructure the text and move the Fig 5 much earlier when the analysis of the published AChR sequences was first mentioned (line 106). Also, there are no details about how this analysis was done: how was the ancestral sequence identified? Was it reconstructed by analysing ACh receptor phylogeny among vertebrates? Xenopus laevis sequense on its own cannot be considered ancestral.
Also, it is not clear how the 10 convergent events of resistance evolution were identified. From the tree at Fig 5 it looks like an alternative scenario could be that the K residue evolved in the last common ancestor of the shown snake lineages and afterwards it was reversed in some of them to E/D. It would require only 9 reversal events at the position 195.
2. Line 58: phenylalanine is not polar
3. Line 90: I think it should be "189F mutant was not bound" according to the Fig 1B.
4. Line 95: repetitive “that”
5. Line 157: “diverse” – I guess “distant” would be more correct
6. Line 236: “addition” – probably should be “additional”
7. Line 237: “evolutions” – “evolutionary events” would be more correct
8. Line 254: “based from” - probably should be “based on”
9. Line 263: “synthesised” - I guess “connected” would be more correct
10. Line 274: “were” – should be “was”?
11. Fig 5: Not all the ancestral Ds are shown in blue, some are represented by dots (for example, Anilios)

Referee: 2

Comments to the Author(s)
Review: RSPB-2020-2703

In this manuscript, the authors present comparative data on binding of neurotoxins from several snakes to the nAChR mimotopes of several potential snake prey and one snake predator. As such, it is an important comparative study that utilizes high-throughput technology to inform how toxins and resistance might evolve and coevolve repeatedly across lineages. Where it is heavy on novel results, it is currently lacking in its set-up…an apparent combination of original formatting for short-form journals and more importantly a lack of a broader hypothesis testing framework in the introduction. The hypotheses instead are named in a various points in the results section, where the reader (at least this reader) is already bogged down in the details of the results themselves and has to retroactively piece together what the hypothesis is and how it links to broader ideas in evolutionary biology. Additionally, claims regarding the number of times the K substitutions have evolved appear to require a more formal ancestral state reconstruction before they can be truly substantiated. In summary, these are wonderful and important results, and I endorse their rapid publication following efforts to 1) frame the results in a broader evolutionary context (such as repeatability vs. novelty in trait evolution) and 2) use a hypothesis-and-predictions lead in to each of the individual results so the reader knows why the test was done and what the specific results mean interms of a particular hypothesis. I give specific examples of these problems and make my best to propose solutions to them below. I will close here by emphasizing that while these critiques sound harsh, my general view of the prospectus for these results is not. I am excited by the results, and relative quick rewrite of the introduction, moving details already in the results to more of a lead-in position will make this a much more digestible and impactful piece of work.

Major Comments:

Introduction –

The manuscript introduction (and paper overall) would greatly benefit from an effort to better frame a general biological question. The paper introduction essentially reads as "coevolution generates novelty -> venom coevolves with resistance -> nAChR's have evolved resistance -> here are some biological examples of resistance -> convergent resistance may involve different molecular/biophysical mechanisms -> we are going to explore resistance substitutions in the badger and also in several snakes". There is a broad literature on ion channel resistance evolution done in a comparative framework (I'm thinking of PNAS papers by Feldman on TTX resistance in snakes and subsequent broader taxonomic comparisons of TTX resistance by Joel McGlothin) that provide more clearly articulated concepts about the nature of constraint and convergence in repeated evolution of resistance traits. You seem to have a particularly novel result here, in showing that convergence phenotypes can come about through alternative biophysical mechanisms. More fleshing out of this idea as a question worth addressing would be welcome. The current draft is very short and appears written for a prior submission to a short form journal, so there is ample room to expand a bit on the broader biological questions at hand without seeming wordy.

Line 43-46: This paragraph, equal in length to the rest of the intro combined, reads a simply a list of examples. It seems that it could be reworked into two smaller paragraphs that subdivide the examples by type. For example, first list the confirmed examples of steric hindrance and the "minor forms". Then in the second paragraph, tantalize with the possible mechanism of charge repulsion and citations 4 and 11. This second paragraph will then end with the specific question in some form: Is there charge repulsion, really? In this way, the introduction moves the reader's understanding along in a more digestible manner.

Line 73-77 – The authors introduce their hypothesis in the same sentence in which they define biolayer interferometry. For readability, please separate and further flesh out both components.

Results and Discussion –

I think the readability of this section could be greatly improved by some subsectioning. The authors could then clearly delineated their presentation of honey badger mutations vs. snake mutations, and in so doing clearly divide the two types of resistance.

Line 104-117: Aside from the second sentence reporting on the authors' examination published sequences, this paragraph is all introductory material, and is specifically the kind of material I was wishing for when reading the introduction. It clearly lays out previous studies and posits a hypothesis at its final sentence. I strongly encourage that the bulk of this information is moved to the introduction.

Lines 161-190: In this section, it is clear that the authors are testing some form of a prediction stemming from a hypothesis of local adaptation due to coevolution, as they use terms such as sympatric and allopatric in describing their combination of venom and mimotope. The passage would read much more cleanly and comprehensibly if the authors introduced this in the form of predictions stemming from a hypothesis of local adaptation. E.g. "If resistance to a-neurotoxins involves local adaptation to specific toxin epitopes, then we expected to find stronger effects of the substitutions on binding affinity of sympatric venoms." This prediction then comes to bare in the comparison among the three Naja venoms, and the authors can then explicitly discuss the result as support for local adaptation playing a role in sequence substitutions…a truly novel result I believe! In this way, this paragraph is a microcosm of the broader issue with the paper: the punchline and impact an amazing dataset and results is buried by comparately poor set up. These data are very complex to start with, and layered on top of that is that each species studied

is idiosyncratic in the means by which resistance is achieved. A strong introduction with delineated hypotheses and predictions, followed by subsectioning and flagging phrases that link back to the prediction will all help turn this into a really impactful work. Just lead the reader by the nose through all the impacts.

Ancestral states – The term "ancestral" is used several times throughout the manuscript to polarize one state vs. another. Furthermore the claim of 10 independent evolution of K charge reversals in snakes is made and figure 5 is referenced. Despite all of this, there is no reference to any type of formal ancestral state reconstruction to support this claim. Since 10 of 19 snakes presented show a K substitutions, it may be equally or more parsimonious to assume the ancestral snake had a K that has subsequently been lost in lineages that don't have to deal with venomous predators, particularly since the tree is polarized against a frog, which is more distantly related than even a mammal (a lizard maybe is best?). This could be another case whether the scant introduction isn't making clear that a broader ancestral state reconstruction has already been published for snakes. Otherwise the claim of 10 independent evolutions – very cool if true – requires a formal ancestral state reconstruction with more sequences and is otherwise unsubstantiated by the present results. This clarification would add greatly to the paper.

Materials and Methods:

General comment - Despite previous method validation, it would still seem appropriate for the researchers to include intra-assay positive controls for each of the mimotope batches (i.e. a completely susceptible human or mouse mimotope) to provide context for how much, in a relative sense, these back-mutations are actually increasing binding affinities.

Line 268 – Given that the method is billed as a crucial innovation for the field in the abstract, and BLI is the key to the entire paper, it would be appropriate for at least a brief description of some of the basics of BLI in this section, or potentially in a short paragraph in the introduction that describes both what a mimotope is and how BLI works. The paper should be readable alone. While one would not have to describe something like Illumina sequencing process in a current paper, BLI is used much less frequently, and even more so in toxinology to date.

Line 278 – The aforementioned lack of detail is even more apparent in this section. I am attempting to evaluation your stats, but your raw data – apparently "association step" data – is mentioned bluntly and without explain. I can certainly piece together what the association step is without having done BLI, but honestly think a reader should be able to understand the basics of one's raw data without having to read a second paper. Furthermore, the author's need to include details about numbers of replicate runs done for input into their ANOVAs. Additional, which comparisons were made? Is there a need to correct for false discovery?

Figures –

Figure 1. Are only comparisons within each snake made? For example, it seems that comparisons of the wild-type binding to all four snakes (comparisons of all red bars) would be interesting to make, particularly if ecology suggests honey badgers should be more resistant to some of these species than others.

Figure 2. Same question as above. Is their utlity in comnparing not just within, put among the panels, in terms of binding levels? If not, potentially due to inter-assay variability or the like, this should be mentioned in the methods for readers to direct focus only within panels.

Figure 5. It would be helpful to the reader not familiar with snake systematics to code the tree with some color blocks and name the clades. For example, block of Pythonidae in a particular color and write "Pythonidae" or "Pythons" outside of the block, so that in text when readers are refered to substitutions occurring in pythons in Fig 5, then can look without having to further cross-reference among binomials.

Minor -

Line 12: unclear why authors capitalize “Biolayer”

Line 17-18: The phrase “such a novel form of …has gone completely undiscovered” is circular – novel and undiscovered. But you have also already said it was novel on line 13, so this seems merely a two-line sales pitch for the paper. Instead, would be more useful to spend more time on biological relevance/implications for coevolution and convergence.

Line 23: Seems and undersell of the biological result to have the final sentence of the abstract to focus on the utility of the method. The biological result of charge reversal and its repeated convergence is fascinated and needs fleshing out even in the short space of the abstract.

Line 68: Change to “…confirmed to confer resistance”.

Line 68-71: Same sentence contains “in contrast”, “although”, and “since” transitions. It is therefore too complex and merits clarification and break-up.

Line 73: Change “theory” to “hypothesis”.

Lines 76: Extra comma after “snakes”.

Line 106: Remove a “that”

Lines 122 and 137: claim about charge reversal repeated in both spots. Choose one for brevity.

Lines 142-152: Some citations supporting the various claims made here are needed. In particular, regarding python terrestriality, juvenile malayopython arboreality, the idea that trees are devoid of elapids, and regarding Morelia and Liasis habits.

Line 161 and several places elsewhere: It occurs to me that significant wordiness in the many places that describe particular mutations could be cut by using the common shorthand (e.g. D191K). So here the sentence “Pseudaspis cana has evolved at position 191 a charge-reversal with the positively charged K in 162 place of negatively charged D at position 191, and with the introduction of an additional positively charged K at 196 which does not represent a charge reversal at this hypervariable position” could be shortened to “Pseudaspis can has evolved a D191K mutation, producing a charge reversal to a positive charge, while introduction of a 163K. does not reverse this hypervariable site’s charge”. This is both shorter and more understandable…hard to do! Something like this would be particularly refreshing in the bulkiest paragraph in the introduction, and the authors could maybe just spell things out the first time fully to show reader’s not familiar with AA replacement lingo how to read something like D191K.

Line 166: “was” to “were”

Line 210: A good place to start a general “Conclusions” subheading.

Line 213-214: Appears to be an unsubstantiated conclusion. It seems we cannot be sure that this was the original selection pressure, so this claim should be altered or better indicated to be speculative.

Line 236: Addition to additional

## Author's Response to Decision Letter for (RSPB-2020-2703.R0)

See Appendix A.

## Decision letter (RSPB-2020-2703.R1)

10-Dec-2020

Dear Dr Fry

I am pleased to inform you that your manuscript entitled "Electrostatic resistance to alpha-neurotoxins conferred by charge-reversal mutations in nicotinic acetylcholine receptors." 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.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

Open Access
You are invited to opt for Open Access, making your freely available to all as soon as it is ready for publication under a CCBY licence. Our article processing charge for Open Access is £1700. Corresponding authors from member institutions (http://royalsocietypublishing.org/site/librarians/allmembers.xhtml) receive a 25% discount to these charges. For more information please visit http://royalsocietypublishing.org/open-access.

Your article has been estimated as being 9 pages long. Our Production Office will be able to confirm the exact length at proof stage.

Paper charges
An e-mail request for payment of any related charges will be sent out after proof stage (within approximately 2-6 weeks). The preferred payment method is by credit card; however, other payment options are available

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

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 Maurine Neiman
Editor, Proceedings B

mailto: proceedingsb@royalsociety.org

Associate Editor:
Board Member
Comments to Author:
I find that the authors have satisfactorily addressed the points raised.

# Appendix A

Response to Reviewer 1's comments

Reviewer comments are in red, whilst our replies are in black.

It is a very interesting paper providing new important insights about mechanisms of toxin resistance evolution. The conclusions are well supported by multiple experiments with mutated binding sites in different species.

We appreciate that you find our work interesting and important. Thank you for reviewing the manuscript.

I have rather minor comments.

1. I think it would be better to restructure the text and move the Fig 5 much earlier when the analysis of the published AChR sequences was first mentioned (line 106). Also, there are no details about how this analysis was done: how was the ancestral sequence identified? Was it reconstructed by analysing ACh receptor phylogeny among vertebrates? Xenopus laevis sequense on its own cannot be considered ancestral.

Also, it is not clear how the 10 convergent events of resistance evolution were identified. From the tree at Fig 5 it looks like an alternative scenario could be that the K residue evolved in the last common ancestor of the shown snake lineages and afterwards it was reversed in some of them to E/D. It would require only 9 reversal events at the position 195.

Both these comments are fair. Firstly, we have moved the figure to where it is suggested as figure 2 and will be in the appropriate spot within the text.

Secondly, our figure highlighting these sequences was an abridged version of the full nAChR sequence analysis across the animal kingdom found in Kahn et al (2020). Widespread Evolution of Molecular Resistance to Snake Venom α-Neurotoxins in Vertebrates. - https://www.mdpi.com/2072-6651/12/10/638

In this paper they comprehensively assess nAChR sequences across the animal kingdom with that of a large proportion of reptiles including around 75 difference snake species. Given that these positively charged mutations have arisen approx. 10 separate times out of this number of species it is more parsimonious to suggest that these are independent coevolution events. The species possessing lysines are nested within broad clades of species without lysines. Thus lysine being the basal condition and species without lysines being derived state would require a greater number of secondary losses of lysine than the number of gains of lysine if lacking lysine was the basal state. Thus, the most parsimonious explanation is that lacking lysine is the basal state. Also given that other forms of alpha-neurotoxin resistance have arisen in snakes on multiple convergent occasions, such as the N-glycosylation motif, this implies resistance to these toxins is of a high selection within serpents and that evolution of resistance is a dynamic, readily evolved trait.

We fully understand how this might have been interpreted from our very small, abridged tree and thus we have expanded upon the figure legend to make this more explicit, adding this sentence – "This phylogeny is a smaller abridged version of the nAChR sequences tree found in Khan et al., 2020 [15]. – hopefully this will allow the readers to refer back to this paper and the much more comprehensive phylogeny within.

We have also expanded upon this within the introduction "However, a comprehensive examination of published nAChR sequences across the animal kingdom [15] revealed that within snakes (an

assessment of 75 different species), the positively charged amino acid lysine (K) has convergently evolved on at least 10 separate occasions…”.

2. Line 58: phenylalanine is not polar

Thanks for noticing this, it was supposed to say non-polar and has been changed accordingly

3. Line 90: I think it should be "189F mutant was not bound" according to the Fig 1B.

You are correct, this was a mistake. This sentence has been changed to “The 189F mutant did not show any binding increase, suggesting that…”

4. Line 95: repetitive “that”

This has been fixed.

5. Line 157: “diverse” – I guess “distant” would be more correct

This has been fixed.

6. Line 236: “addition” – probably should be “additional”

This has been fixed.

7. Line 237: “evolutions” – “evolutionary events” would be more correct

This has been fixed.

8. Line 254: “based from” - probably should be “based on”

This has been fixed.

9. Line 263: “synthesised” - I guess “connected” would be more correct

This has been fixed.

10. Line 274: “were” – should be “was”?

The preceding word was supposed to be plural, as in sensors not sensor which it originally was. This has been changed now and the ‘were’ was kept – “Streptavidin biosensors were hydrated…”

11. Fig 5: Not all the ancestral Ds are shown in blue, some are represented by dots (for example, Anilios).

This is an excellent spot. However, the Anilios sequence has an A at this position and not a D and thus we have corrected this and others similar in the figure. Thanks for noticing this.

Response to Reviewer 2's comments

Reviewer comments are in red, whilst our replies are in black.

In this manuscript, the authors present comparative data on binding of neurotoxins from several snakes to the nAChR mimotopes of several potential snake prey and one snake predator. As such, it is an important comparative study that utilizes high-throughput technology to inform how toxins and resistance might evolve and coevolve repeatedly across lineages. Where it is heavy on novel results, it is currently lacking in its set-up…an apparent combination of original formatting for short-form journals and more importantly a lack of a broader hypothesis testing framework in the introduction. The hypotheses instead are named in a various points in the results section, where the reader (at least this reader) is already bogged down in the details of the results themselves and has to retroactively piece together what the hypothesis is and how it links to broader ideas in evolutionary biology. Additionally, claims regarding the number of times the K substitutions have evolved appear to require a more formal ancestral state reconstruction before they can be truly substantiated. In summary, these are wonderful and important results, and I endorse their rapid publication following efforts to 1) frame the results in a broader evolutionary context (such as repeatability vs. novelty in trait evolution) and 2) use a hypothesis-and-predictions lead in to each of the individual results so the reader knows why the test was done and what the specific results mean interms of a particular hypothesis. I give specific examples of these problems and make my best to propose solutions to them below. I will close here by emphasizing that while these critiques sound harsh, my general view of the prospectus for these results is not. I am excited by the results, and relative quick rewrite of the introduction, moving details already in the results to more of a lead-in position will make this a much more digestible and impactful piece of work.

We thank the reviewer for seeing the importance and novelty in our research. We have endeavoured below to address the many very good points in which the reviewer has brought to our attention and hope that we have satisfied them adequately.

Major Comments:

Introduction –

The manuscript introduction (and paper overall) would greatly benefit from an effort to better frame a general biological question. The paper introduction essentially reads as "coevolution generates novelty -> venom coevolves with resistance -> nAChR's have evolved resistance -> here are some biological examples of resistance -> convergent resistance may involve different molecular/biophysical mechanisms -> we are going to explore resistance substitutions in the badger and also in several snakes". There is a broad literature on ion channel resistance evolution done in a comparative framework (I'm thinking of PNAS papers by Feldman on TTX resistance in snakes and subsequent broader taxonomic comparisons of TTX resistance by Joel McGlothin) that provide more clearly articulated concepts about the nature of constraint and convergence in repeated evolution of resistance traits. You seem to have a particularly novel result here, in showing that convergence phenotypes can come about through alternative biophysical mechanisms. More fleshing out of this idea as a question worth addressing would be welcome. The current draft is very short and appears written for a prior submission to a short form journal, so there is ample room to expand a bit on the broader biological questions at hand without seeming wordy.

We have now changed up the introduction as requested based upon this comment and further comments suggested below.

Line 43-46: This paragraph, equal in length to the rest of the intro combined, reads a simply a list of examples. It seems that it could be reworked into two smaller paragraphs that subdivide the examples by type. For example, first list the confirmed examples of steric hindrance and the "minor forms". Then in the second paragraph, tantalize with the possible mechanism of charge repulsion and citations 4 and 11. This second paragraph will then end with the specific question in some form: Is there charge repulsion, really? In this way, the introduction moves the reader's understanding along in a more digestible manner.

This is a fair assessment and we have done as the reviewer has suggested here. Please see the manuscript introduction [lines 41-71] as the section is too large to paste here in the response. We hope the reviewer is supportive of the new changes.

Line 73-77 – The authors introduce their hypothesis in the same sentence in which they define biolayer interferometry. For readability, please separate and further flesh out both components.

This has been fixed. Hopefully the new layout is more readable.

Results and Discussion –

I think the readability of this section could be greatly improved by some subsectioning. The authors could then clearly delineated their presentation of honey badger mutations vs. snake mutations, and in so doing clearly divide the two types of resistance.

We have taken this into consideration and have made two distinct subsections titled – "Assessing the arginine (R) resistance" which encompasses the honey badger testing. The second subsection is titled – "Assessing the charge-reversal lysine (L) resistance" which encompasses the snake resistance.

Line 104-117: Aside from the second sentence reporting on the authors' examination published sequences, this paragraph is all introductory material, and is specifically the kind of material I was wishing for when reading the introduction. It clearly lays out previous studies and posits a hypothesis at its final sentence. I strongly encourage that the bulk of this information is moved to the introduction.

This section has been moved to the introduction and incorporated to make the flow of the introduction and the hypothesis driven work clearer. We hope it now reads better.

Lines 161-190: In this section, it is clear that the authors are testing some form of a prediction stemming from a hypothesis of local adaptation due to coevolution, as they use terms such as sympatric and allopatric in describing their combination of venom and mimotope. The passage would read much more cleanly and comprehensibly if the authors introduced this in the form of predictions stemming from a hypothesis of local adaptation. E.g. "If resistance to a-neurotoxins involves local adaptation to specific toxin epitopes, then we expected to find stronger effects of the substitutions on binding affinity of sympatric venoms." This prediction then comes to bare in the comparison among the three Naja venoms, and the authors can then explicitly discuss the result as support for local adaptation playing a role in sequence substitutions…a truly novel result I believe! In this way, this paragraph is a microcosm of the broader issue with the paper: the punchline and impact an amazing dataset and results is buried by comparately poor set up. These data are very complex to start with, and layered on top of that is that each species studied is idiosyncratic in the means by which resistance is achieved. A strong introduction with delineated hypotheses and predictions, followed by subsectioning and flagging phrases that link back to the prediction will all help turn this into a really impactful work. Just lead the reader by the nose through all the impacts.

This is a fair assessment and we agree that we did miss out on mentioning this prior to setting up our hypothesis. We have added this sentence into the introduction [Lines 97-99] – "By further testing sympatric and allopatric snake venoms we can also assess if any resistance is likely caused by local adaptation due to coevolution". However, we didn't particularly want to delve too deep into this aspect since the literature (and some of our results) are pretty clear that alpha-neurotoxins in general tend to have a very similar binding to the orthosteric site and thus unless the site has specific resistance mechanisms then the alpha-neurotoxins (regardless of the species) is likely going to bind. The reasoning for the differences in binding affinity is likely due to the proportions of the toxins within the venoms, which is something we have spoken about in previous literature and also mentioned in this manuscript. It could be argued that these proportions and expression of these toxins is driven by local adaptation and the need for a greater or lesser proportion given specific prey types, however this is a whole other (and very deep) research area which is off topic here.

Regardless, we feel it is important to note that there doesn't seem to be any difference between sympatric and allopatric species, however this is likely due to the toxin binding convergence rather than local adaptation. We have noted in the conclusions section [Lines 236-240] – "The resistance to both sympatric and allopatric α-neurotoxic snake venoms suggests that although the coevolutionary selection pressures are likely due to predator-prey interactions between these species, the reduced susceptibility is also present across the allopatric α-neurotoxin types (Figs 1, 3-5) signifying similar binding mechanisms of these toxins, thus reinforcing the paradigm that sensitivities in the target provides the selection pressure for the evolution of the toxin."

Ancestral states – The term "ancestral" is used several times throughout the manuscript to polarize one state vs. another. Furthermore the claim of 10 independent evolution of K charge reversals in snakes is made and figure 5 is referenced. Despite all of this, there is no reference to any type of formal ancestral state reconstruction to support this claim. Since 10 of 19 snakes presented show a K substitutions, it may be equally or more parsimonious to assume the ancestral snake had a K that has subsequently been lost in lineages that don't have to deal with venomous predators, particularly since the tree is polarized against a frog, which is more distantly related than even a mammal (a lizard maybe is best?). This could be another case whether the scant introduction isn't making clear that a broader ancestral state reconstruction has already been published for snakes. Otherwise the claim of 10 independent evolutions – very cool if true – requires a formal ancestral state reconstruction with more sequences and is otherwise unsubstantiated by the present results. This clarification would add greatly to the paper.

Our figure highlighting these sequences was an abridged version of the full nAChR sequence analysis across the animal kingdom found in Kahn et al (2020). Widespread Evolution of Molecular Resistance to Snake Venom α-Neurotoxins in Vertebrates. - https://www.mdpi.com/2072-6651/12/10/638

In this paper they comprehensively assess nAChR sequences across the animal kingdom with that of a large proportion of reptiles including around 75 different snake species. The species with this mutation are nested deep within large clades of species without it. Thus, it would require a substantially larger number of secondary losses if lysine was the basal state and species without it are the ones with the mutation. Thus, the most parsimonious interpretation is multiple cases of convergent mutation for the lysine. In addition, the N-glycosylation form of resistance has been shown to have arisen on multiple occasions as well within snakes. Which provides supporting evidence for the evolution of resistance being a dynamic trait in snakes that arises on convergent occasions.

We fully understand how this might have been interpreted from our very small, abridged tree and thus we have expanded upon the figure legend to make this more explicit, adding this sentence – "This phylogeny is a smaller abridged version of the nAChR sequences tree found in Khan et al., 2020 [15]. – hopefully this will allow the readers to refer back to this paper and the much more comprehensive phylogeny within.

We have also expanded upon this within the introduction "However, a comprehensive examination of published nAChR sequences across the animal kingdom [15] revealed that within snakes (an assessment of 75 different species), the positively charged amino acid lysine (K) has convergently evolved on at least 10 separate occasions…".

We have also amended the figure to show the more closely related and basal reptile; Alligator sinesis instead of the amphibian Xenopus laevis, which (like the frog) also has negatively charged amino acids at these two key positions.

Materials and Methods:

General comment - Despite previous method validation, it would still seem appropriate for the researchers to include intra-assay positive controls for each of the mimotope batches (i.e. a completely susceptible human or mouse mimotope) to provide context for how much, in a relative sense, these back-mutations are actually increasing binding affinities.

We think that this notion from the reviewer is a fair assessment. However, each mimotope is separately synthesised, and sequence and purity are confirmed by mass spectrometry. We would like to point out that within the manuscript we have also cited some of our previous work in which we also test natural prey sequences to some of these exact same venoms and can indeed confirm that natural prey values (both AUCs and wavelength shifts) are comparable to that of the mutants that were bound high. In addition, binding to non-prey species (like mice or humans) is not a valid comparison as they may have reduced sensitivity.

Line 268 – Given that the method is billed as a crucial innovation for the field in the abstract, and BLI is the key to the entire paper, it would be appropriate for at least a brief description of some of the basics of BLI in this section, or potentially in a short paragraph in the introduction that describes both what a mimotope is and how BLI works. The paper should be readable alone. While one would not have to describe something like Illumina sequencing process in a current paper, BLI is used much less frequently, and even more so in toxinology to date.

We have added this paragraph to the methods section [lines 282-286] – "BLI is a label-free, microfluidics-free, optical technique that precisely measures the thickness of biomolecules accumulating on the interaction surface of an optical-fiber coated biosensor. The binding of molecules to the biosensor causes a measurable spectral shift in the wavelength of light being reflected through the fiber-optic biosensor, which yields quantitative, kinetic interaction information."

Line 278 – The aforementioned lack of detail is even more apparent in this section. I am attempting to evaluation your stats, but your raw data – apparently "association step" data – is mentioned bluntly and without explain. I can certainly piece together what the association step is without having done BLI, but honestly think a reader should be able to understand the basics of one's raw data without having to read a second paper. Furthermore, the author's need to include details about numbers of replicate runs done for input into their ANOVAs. Additional, which comparisons were made? Is there a need to correct for false discovery?

We understand the reviewers comments about the shortness of detail regarding this aspect. However, given that the full details of the assay design and set-up – including background knowledge and full methodology details - can be found in an assay validation paper in which we describe the use of this technology for the purpose of analyte-ligand interactions of venom and their targets, we have opted to make a very short, abridged version here. The original description of the methods and data processing is a full page worth of writing and we feel this is just superfluous and makes any preceding manuscript unnecessarily long.

To address the lack of information regarding the 'association step' we have added the parentheses afterward – "(a recording of the wavelength shift (nm) at 0.2 second intervals over a 120 second period)"- Hopefully this will allow the reader to understand this small aspect and if they want to further delve into the nitty gritty of the methods then the citation of the validation literature is available within text.

All additional information regarding the statistical outputs can be found in the supplementary material. These values and information are freely available for readers.

Figures –

Figure 1. Are only comparisons within each snake made? For example, it seems that comparisons of the wild-type binding to all four snakes (comparisons of all red bars) would be interesting to make, particularly if ecology suggests honey badgers should be more resistant to some of these species than others.

This is indeed a good notion by the reviewer, however as previously discussed in our comments, due to alpha-neurotoxins binding similarly to the orthosteric site it is only likely the proportion of alpha-neurotoxins that gives us these differences in binding observed. Thus, this is why we are only particularly interested in comparing the binding changes across the mimotopes within each venom rather than across each species tested.

Figure 2. Same question as above. Is their utlity in comnparing not just within, put among the panels, in terms of binding levels? If not, potentially due to inter-assay variability or the like, this should be mentioned in the methods for readers to direct focus only within panels.

See above comment.

Figure 5. It would be helpful to the reader not familiar with snake systematics to code the tree with some color blocks and name the clades. For example, block of Pythonidae in a particular color and write "Pythonidae" or "Pythons" outside of the block, so that in text when readers are refered to substitutions occurring in pythons in Fig 5, then can look without having to further cross-reference among binomials.

We agree that this would be helpful and have therefore amended the figure to show the family names to each clade. We hope this is much clearer for the readers and the reviewer.

Minor –

Line 12: unclear why authors capitalize "Biolayer"

This has been fixed.

Line 17-18: The phrase "such a novel form of …has gone completely undiscovered" is circular – novel and undiscovered. But you have also already said it was novel on line 13, so this seems merely a two-

line sales pitch for the paper. Instead, would be more useful to spend more time on biological relevance/implications for coevolution and convergence.

This has been fixed. We have removed the term novel in the first instance as not to oversell this word.

Line 23: Seems and undersell of the biological result to have the final sentence of the abstract to focus on the utility of the method. The biological result of charge reversal and its repeated convergence is fascinated and needs fleshing out even in the short space of the abstract.

This has been changed. The final two sentences now focus on the overall results outcome.

Line 68: Change to "…confirmed to confer resistance".

This has been fixed.

Line 68-71: Same sentence contains "in contrast", "although", and "since" transitions. It is therefore too complex and merits clarification and break-up.

We agree with this and have changed the sentence to read – "In contrast, the evolution of 187R in the honey badger has only been hypothesised to confer resistance to α-neurotoxins [4], due to them being predators of cobras (Naja) and based on natural history observations of them surviving cobra bites [4, 18]. However, this hypothesis has never been functionally tested."

Line 73: Change "theory" to "hypothesis".

Fixed.

Lines 76: Extra comma after "snakes".

This has been fixed.

Line 106: Remove a "that"

This has been fixed.

Lines 122 and 137: claim about charge reversal repeated in both spots. Choose one for brevity.

The second repeated instance at line 137 has been removed.

Lines 142-152: Some citations supporting the various claims made here are needed. In particular, regarding python terrestriality, juvenile malayopython arboreality, the idea that trees are devoid of elapids, and regarding Morelia and Liasis habits.

We have now added in the references requested and have further expanded upon this section [lines 154-165].

Line 161 and several places elsewhere: It occurs to me that significant wordiness in the many places that describe particular mutations could be cut by using the common shorthand (e.g. D191K). So here the sentence "Pseudaspis cana has evolved at position 191 a charge-reversal with the positively charged K in 162 place of negatively charged D at position 191, and with the introduction of an additional positively charged K at 196 which does not represent a charge reversal at this hypervariable position" could be shortened to "Pseudaspis can has evolved a D191K mutation, producing a charge reversal to a positive charge, while introduction of a 163K. does not reverse this hypervariable site's charge". This is both shorter and more understandable…hard to do! Something like this would be particularly refreshing in the bulkiest paragraph in the introduction, and the

authors could maybe just spell things out the first time fully to show reader's not familiar with AA replacement lingo how to read something like D191K.

We have amended this in some places throughout the manuscript where we feel it is easily understandable. The first instance of this has also been explained – "(the denotation of ancestral changes will be displayed as W187R throughout the manuscript)" [lines 109-110].

Line 166: "was" to "were"

This has been fixed.

Line 210: A good place to start a general "Conclusions" subheading.

This has been added.

Line 213-214: Appears to be an unsubstantiated conclusion. It seems we cannot be sure that this was the original selection pressure, so this claim should be altered or better indicated to be speculative.

We agree that this could be made more clearer as a speculative claim. We have added the sentence – "Future natural history field studies will be required to confirm this."

Line 236: Addition to additional

This has been fixed.