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PROCEEDINGS B

The cost of chemical defence: the impact of toxin depletion on growth and behaviour of cane toads (*Rhinella marina*)

Ryann A. Blennerhassett, Kim Bell-Anderson, Richard Shine and Gregory P. Brown

Article citation details
Proc. R. Soc. B **286**: 20190867.
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Review timeline

Original submission:	21 March 2019
1st revised submission:	12 April 2019
2nd revised submission:	19 April 2019
Final acceptance:	24 April 2019

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

Review History

RSPB-2019-0676.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Major revision is needed (please make suggestions 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?
Acceptable

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.
Yes

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
General comments:
This manuscript uses a combination of laboratory and field-based experiments to examine the impact of toxin replenishment in Cane toads. The authors use accurate statistics although I have some minor concerns about a few unmentioned assumptions. The data suggest that toads are impacted, in terms of morphology and performance, after toxins have been forcibly removed and the toads are, presumably, replenishing them. The data are hard to interpret as they are transformed though. The manuscript is extremely well written, and the amount of data presented is impressive. It is not clear throughout the manuscript what the authors mean when they mention "aspects of physiology" (e.g., L80). Organ masses and SUL are considered morphological traits [L215] and behavioral traits are obvious but it's very unclear what aspects of physiology were measured in this study. I expect readers will also have trouble with their use of the term "fitness-related" throughout. There were no true metrics of biological/Darwinian fitness in this study. The fittest toads might not necessarily be the largest, fastest, or have the most toxin, they just need to survive, mate, and produce offspring. The authors will either need to add more justification in the intro/discussion of consider alternate phrasing such as 'performance' throughout. The manuscript could also be greatly improved by adding much more justification for why the metrics they selected are useful compared to measuring metabolism. There is a brief paragraph (L71-74) but then the data suggests metabolic measures may have explained a lot of the variance detected. While I appreciate the authors admittance in the discussion (L350-351) that metabolic data would have helped, the context of the study could be re-phrased to explain that metrics *in addition to* metabolism might help explain toad energy budget dynamics during toxin replenishment. It is also very difficult to make sense of the findings based on the figures and I strongly recommend plotting raw data so that readers can grasp the biological relevance.

Specific Comments:
L52-54: Please be careful here and consider using 'typically' as I imagine some venom/poison/toxin experts will get flustered. The most concrete definition of 'venomous' I've seen so far – "a complex substance produced in a specialized gland and delivered by an associated specialized apparatus that is deleterious to other organisms in a given dosage and is actively used in the subjugation and/or digestion of prey and/or in defense." (Minton 1974)
L61: insert oxford comma

L67: insert oxford comma
L68: should that read "toxins" or is it mostly homogeneous?
L74: "a far wider array of traits" is strangely worded. My understanding is that you're trying to say 'there are more ways to measure energy expenditure than metabolic rates' although the use of trait doesn't help get this point across, probably because I don't think readers would equate metabolic rate as a 'trait' per se. Consider re-wording
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L84: delete second use of "in" and consider changing to "laboratory" or "laboratory setting"
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L99-101: please insert citation for the meteorological data
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L117: Do you have any citation to verify that you can, in fact, squeeze out all of their toxins? Was the squeezing done by hand or did you use a tool? I would appreciate reading about how you tried to limit subjectivity when extracting toxins, especially because you were weighing them to such a fine degree. It's hard to know
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L217: with sex and body size both treated as indepdent variables I'm a little concerned that the assumptions of multicollinearity and homoscedasticity were violated. Can you please provide, in the ms or as supplementary materials, VIF for your tests? Sex, SUL, mass, organ mass, and toxin hopefully don't have VIF > 10. In terms of homoscedasticity, I don't necessary buy into needing a GQ test but a supplemental scatterplot might be useful.
L222: oxford comma

L234: perhaps I missed it, but where in your methods do you describe the factors for your ANCOVA?
L237-241: this, with some re-wording, seems better suited for the methods
L244: oxford comma
L251: please describe if this is SD or SEM
L258-263: please do a better job of justifying why energy and body size were not related when you actually quantified them (L256) but it did when you extended the data [L263]. It's unclear if you were interpolating or extrapolating to find this p value. Without a relationship in your measured data, did you extrapolate as a linear relationship? This section needs to either be removed or backed up with much more justification.
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L281 & L295: I assume the mass of the toxin removed was accounted for and not included in the mass loss measurements but that is unclear, or I missed it. Ie If you squeezed out 4 g of toxin I want to make sure 'start weight' was after removing the toxin and not before
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L386: change to "threat is on"
L393: remove name from citation
Table 1: please explain in caption that wt = "weight" for clarity
L578: oxford comma
Figure 2/3/4: it is unclear why your figures visualize the transformed data which make it difficult for the reader to comprehend. This transformed data has no biological meaning and it's fine to mention that your stats were on transformed data but I want to see the actual data to make sense of it.
Figure 2/Figure 3: Insert "positive" to figure 2. please add units to axes. Please clarify in legend that individual points represent individual toads and if this is data from field, lab, or both. Also describe what line of best fit is with an R2 value.
Figure 3/4: it is unclear why the open circles are outliers from the figure and fig legend alone, those points look very close to the line of best fit.
Figure 5: describe line of best fit in caption and include relevant stats.
Figure 6: I'm very glad that you plotted the raw data, it would also be useful to have boxplots with error bars overlaid so readers can quickly

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?
Excellent

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

Quality of the paper: Is the overall quality of the paper suitable?
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Is the length of the paper justified?
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Yes

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Yes

Is it adequate?
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Do you have any ethical concerns with this paper?
No

Comments to the Author
Chemical defences in vertebrate animals is an under-studied topic, and the toxin biology of cane toads is of particular importance due to the conservation impact of this species. Thus, this study contributes valuable results to the field of chemical ecology. Overall, the manuscript is neatly written and I see no major flaw in the design, execution and interpretation of the study. I have several relatively minor comments which need attention though, especially regarding the clarification of some methodological aspects. Also, I recommend that the theoretical framework of the study should be better focused on chemical defences, and mixing up the latter with "offensive" toxins (venoms) should be avoided (or, at the very least, the two should be clearly distinguished). There is a bunch of recent literature directly relevant to this study (see Hettyey et al. 2014. Oikos 123:1025-1028 and further citations below) that would be more appropriate to cite and build the introduction on instead of the venom literature.

Detailed comments:

L21: This is not a good start for the abstract. Do we have evidence that "many animals that produce chemical defences are reluctant to deploy them"? In the manuscript you only mention cane toads' reluctance. Many of the chemical defences in nature are not even deployable, although their levels can be adjusted facultatively. Many of the deployable toxins are actually venoms used for hunting (not defence). Mixing these two kinds of toxins throughout the paper can be misleading.

L46-50: It is unfortunate to mix toxins with venoms, as the latter are not (primarily) for defence. You need not rely (only) on the 'venom optimization hypothesis': the same general concept of trade-offs has been formulated for chemical defences under the name 'optimal defence theory'. It has been developed for plants but also can, and has been, applied to animals like amphibians (e.g. Bokony et al. 2016. 42:329-338).

L51-52: It is strange that after saying "most studies", you cite a single case study, when there are several citations available (e.g. Benard & Fordyce 2003. Ecology 84:68-78; Fordyce et al. 2006. Oecologia 149:101-106; Kurali et al. 2016. Biol. J. Linnean Soc. 119:1000-1010; Toth et al. 2019. J. Chem. Ecol. doi: 10.1007/s10886-019-01045-9). Notably, these latter studies all looked at defensive chemicals (not venoms) and found little evidence for the costs of chemical defences.

L52-54: While this definition seems accurate, I think there is another important distinction: venoms are used mainly for immobilizing or killing prey while defensive toxins are not used in a foraging context. This is not necessarily trivial for all readers, so it would be helpful to clarify this here.

L62-70: A third line of evidence is that the compressed parotoid gets injured; the bleeding and immune reaction have physiological costs, as does the healing of the tissues (see Jared et al. 2014. Toxicon 87:92-103).

L71-77: Again, I find it awkward that you talk about venoms when your paper is about defensive toxins. For example, lost foraging opportunities seem irrelevant for defensive toxins. Why talk about these when you can talk directly about the potential costs of defensive toxins? I agree that "consequences for fitness-related traits may be of greater ecological and evolutionary significance", but this sentence might be vague for readers; you could be a bit more specific, e.g. give an example (see previous studies cited above).

L116: Looking at Fig. 1 it is not trivial what the "length and width of each parotoid" are, as the gland looks almost spherical. Can you indicate these measurements of the photo? Also, please explain what these measurements were used for. Was there a correlation between parotoid size and toxin amount?

L117-118 (also L94): The logic here is not 100% clear. Are you saying that you removed ALL toxins from these animals' parotoids? In this case, please provide evidence that the entire toxin store can be depleted by manual compression. If you cannot show that, then can you validate that the sample you took is representative for the entirety of each individual's total toxin amount? In other words, does the amount of squeezed-out toxin correlate well with the total amount of toxins? Theoretically it is possible to squeeze out a small amount from a large gland and a larger amount from a smaller gland. This should not affect the comparisons between de-toxined and control toads, but it is a concern when you are correlating the amount and caloric content of toxins with other morphological traits.

L122: For comparisons of de-toxined and control toads to be valid, especially because the sample sizes were relatively small, it is important to show that any difference between the two experimental groups was not due to accidental differences in other factors. For both free-ranging and captive toads, please report the distribution of body size/mass and sex in the two experimental groups. I understand that you used these two traits as independent variables in your statistical analyses, which is fine, but it does not demonstrate that these traits were indeed independent of the toxin variables (if they are not, you may have to deal with multicollinearity). For behavioural assays, please report if the exact time of day differed between the two groups. Order effects might also confound these assays: did you clean the arenas of olfactory cues between trials of different individuals?

L209: Please clarify if toxin content of the left and right glands were analysed as repeated measures of the same individual or were summed (or averaged, or what).

L251 and throughout: Please define what measure of variability is given after the ± and please provide such a measure for all estimates of mean values in the Results text (e.g. L272-273, L281-282, L295-296)

L250-254: Please explain these analyses in more detail in the Methods. First, please explain what "energy density" is; this phrase is not trivial to all readers. Second, please explain why and how you tested whether any of the 13 individuals differed in toxin energy density. How can this analysis yield exactly the same statistics as the comparison between left and right glands? Third, the comparison between left and right glands should be a paired test (e.g. Wilcoxon test); to the best of my knowledge the Kruskal-Wallis test is inappropriate here. Finally, please report the df for each chi-squared statistic.
Altogether, these non-parametric analyses seem rather inefficient, because you cannot investigate more than one predictor at a time. If females are systematically larger than males, you cannot really test the effects of size and sex with two separate tests. You had 26 toxin samples from 13 toads, which is not that bad: I would try using the left and right data as repeated measures in a mixed model with SUL and sex as predictors (and you can compare left/right by adding that as another predictor). The statistical assumptions should be assessable for such a model.

L267-273: Please clarify in the Methods if telemetry bout (Day # in Table 2) was used as a numeric variable or a categorical factor in this analysis.
L269-271: What exactly was the effect of toxin depletion on movement? It is difficult to grasp from this description. It would be helpful if you moved here the sentence from the caption of Fig. 5 (i.e. de-toxined toads remained at a constant distance from their point of capture but control toads moved increasingly further away over the five days).
L271-273: Was the difference seen on the final day significant?

L319: "storage" is unclear; please clarify.

L321: I might have missed something but where are the results showing that "larger parotoid glands carry more toxin than smaller ones"?

L324-325: It may be worth adding that the liver is also important for immune defence.

L330-332: It is not evident how/why decreased dispersal would mean reduced foraging; please elaborate a bit.

L334-335: To be fair, this speculation is not supported by the results of your behavioural assays.

In contrast, the lower activity observed in the field is marginally supported by the captive study. Ideally, the discussion should make these connections between the different parts of the study.

L347-348: I recommend rephrasing this sentence to something like "facultative adjustments of chemical defence in response to predation risk have been reported in amphibians". I would not say they are well documented in bufonids. Only two studies have found some changes in toad toxin levels after manipulation of predation risk, but those results are not very clear because predation risk was manipulated in the larval stage and the changes were only seen after metamorphosis (Benard & Fordyce 2003; Hagman et al. 2009. Functional Ecology 23:126-132). Another experiment did not find any effect of predator cues on toad toxins (Üveges et al. 2017). You cite a study that was done on salamanders (Bucciarelli et al. 2017) and a field study that found no relationship between predation pressure and toad toxins (Bokony et al. 2016).

L349: The last part of the sentence is unclear.

L366-367: I wonder why you cite only this Chen & Chen study which was done on a single animal. Jared et al. (2014) did a more detailed study, showing that much of the expressed glands are empty even after 90-105 days and some collapsed tissues still do not show any indication of recovery.

L388-396: There is a further interesting aspect of your results: you found a negative correlation between gonad size and toxin content. This may be due to a general trade-off between toxins and growth, as you speculate, but it might also be related to the fact that bufadienolide toxins are synthesized from the same precursor as sex hormones, starting with the same biochemical steps (see Bokony et al. 2019. Scientific Reports 9:3163).

There are minor typos throughout, e.g. L84: as in captivity, L93: Fig. 1, L101: °C, L140: they were, L220: subtlely (?), L294: there is no Fig. 7, L320: expectations, L352: beetle, L354: weighing... Reference 47: Some of the authors are missing.

Fig. 2 and 4: You could make these graphs more informative by showing males and females with different symbols.

Table 1: Could you please report the parameter estimates instead of (or in addition to) the F statistics? The parameter estimates are more informative because they show the direction of the effect. If you choose to do this, please do not forget to report the intercept as well. Also, you could remove the df columns because they are redundant; these two pieces of information can be given in the caption or footnotes.

Fig. 6: Wouldn't this graph look better as a box plot? Or, if you added a 95% confidence interval on these dot plots, we could see if there was significant growth in any of the groups.

Decision letter (RSPB-2019-0676.R0)

03-Apr-2019

Dear Dr Brown:

I am writing to inform you that your manuscript RSPB-2019-0676 entitled "The cost of chemical defence: the impact of toxin depletion on fitness-relevant traits of cane toads (Rhinella marina)." has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

1) A 'response to referees' document including details of how you have responded to the comments, and the adjustments you have made.
2) A clean copy of the manuscript and one with 'tracked changes' indicating your 'response to referees' comments document.
3) Line numbers in your main document.

To upload a resubmitted manuscript, 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 Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,

Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
The manuscript 'The cost of chemical defence: the impact of toxin depletion on fitness-relevant traits of cane toads (Rhinella marina)' was reviewed by myself and two experts in the field of chemical defense and physiology. As you can see from their reviews, the reviewers both liked the manuscript and agree that the manuscript was well written and the ideas and general framework are of broad interest. However, there are some important points and clarifications that were raised and that should be addressed prior to publication including the following: 1) There are some clarifications in the methodology that should be made, including specifically how measurements were acquired and how toxin content was quantified; 2) The interchangeable discussion of toxins versus venoms is mispleading to reviewers and should be carefully distinguished; 3) there are various issues with word-choice throughout the manuscript that may be misleading to readers, in particular the use of 'fitness-measures' throughout the manuscript may not be appropriate when no direct fitness assessments where made; and 4) better justification of the measurements chosen and their importance in this system would greatly benefit readers. In addition to these issues, there is a long list of specific, more minor suggested changes the authors should address and important references/citations include.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
General comments:
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L386: change to “threat is on”
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Table 1: please explain in caption that wt = “weight” for clarity
L578: oxford comma
Figure 2/3/4: it is unclear why your figures visualize the transformed data which make it difficult for the reader to comprehend. This transformed data has no biological meaning and it’s fine to mention that your stats were on transformed data but I want to see the actual data to make sense of it.
Figure 2/Figure 3: Insert “positive” to figure 2. please add units to axes. Please clarify in legend that individual points represent individual toads and if this is data from field, lab, or both. Also describe what line of best fit is with an R2 value.
Figure 3/4: it is unclear why the open circles are outliers from the figure and fig legend alone, those points look very close to the line of best fit.
Figure 5: describe line of best fit in caption and include relevant stats.
Figure 6: I’m very glad that you plotted the raw data, it would also be useful to have boxplots with error bars overlaid so readers can quickly

Referee: 2

Comments to the Author(s)
Chemical defences in vertebrate animals is an under-studied topic, and the toxin biology of cane toads is of particular importance due to the conservation impact of this species. Thus, this study contributes valuable results to the field of chemical ecology. Overall, the manuscript is neatly written and I see no major flaw in the design, execution and interpretation of the study. I have several relatively minor comments which need attention though, especially regarding the clarification of some methodological aspects. Also, I recommend that the theoretical framework of the study should be better focused on chemical defences, and mixing up the latter with "offensive" toxins (venoms) should be avoided (or, at the very least, the two should be clearly distinguished). There is a bunch of recent literature directly relevant to this study (see Hettyey et

al. 2014. Oikos 123:1025-1028 and further citations below) that would be more appropriate to cite and build the introduction on instead of the venom literature.

Detailed comments:

L21: This is not a good start for the abstract. Do we have evidence that "many animals that produce chemical defences are reluctant to deploy them"? In the manuscript you only mention cane toads' reluctance. Many of the chemical defences in nature are not even deployable, although their levels can be adjusted facultatively. Many of the deployable toxins are actually venoms used for hunting (not defence). Mixing these two kinds of toxins throughout the paper can be misleading.

L46-50: It is unfortunate to mix toxins with venoms, as the latter are not (primarily) for defence. You need not rely (only) on the 'venom optimization hypothesis': the same general concept of trade-offs has been formulated for chemical defences under the name 'optimal defence theory'. It has been developed for plants but also can, and has been, applied to animals like amphibians (e.g. Bokony et al. 2016. 42:329-338).

L51-52: It is strange that after saying "most studies", you cite a single case study, when there are several citations available (e.g. Benard & Fordyce 2003. Ecology 84:68-78; Fordyce et al. 2006. Oecologia 149:101-106; Kurali et al. 2016. Biol. J. Linnean Soc. 119:1000-1010; Toth et al. 2019. J. Chem. Ecol. doi: 10.1007/s10886-019-01045-9). Notably, these latter studies all looked at defensive chemicals (not venoms) and found little evidence for the costs of chemical defences.

L52-54: While this definition seems accurate, I think there is another important distinction: venoms are used mainly for immobilizing or killing prey while defensive toxins are not used in a foraging context. This is not necessarily trivial for all readers, so it would be helpful to clarify this here.

L62-70: A third line of evidence is that the compressed parotoid gets injured; the bleeding and immune reaction have physiological costs, as does the healing of the tissues (see Jared et al. 2014. Toxicon 87:92-103).

L71-77: Again, I find it awkward that you talk about venoms when your paper is about defensive toxins. For example, lost foraging opportunities seem irrelevant for defensive toxins. Why talk about these when you can talk directly about the potential costs of defensive toxins? I agree that "consequences for fitness-related traits may be of greater ecological and evolutionary significance", but this sentence might be vague for readers; you could be a bit more specific, e.g. give an example (see previous studies cited above).

L116: Looking at Fig. 1 it is not trivial what the "length and width of each parotoid" are, as the gland looks almost spherical. Can you indicate these measurements of the photo? Also, please explain what these measurements were used for. Was there a correlation between parotoid size and toxin amount?

L117-118 (also L94): The logic here is not 100% clear. Are you saying that you removed ALL toxins from these animals' parotoids? In this case, please provide evidence that the entire toxin store can be depleted by manual compression. If you cannot show that, then can you validate that the sample you took is representative for the entirety of each individual's total toxin amount? In other words, does the amount of squeezed-out toxin correlate well with the total amount of toxins? Theoretically it is possible to squeeze out a small amount from a large gland and a larger amount from a smaller gland. This should not affect the comparisons between de-toxined and

control toads, but it is a concern when you are correlating the amount and caloric content of toxins with other morphological traits.

L122: For comparisons of de-toxined and control toads to be valid, especially because the sample sizes were relatively small, it is important to show that any difference between the two experimental groups was not due to accidental differences in other factors. For both free-ranging and captive toads, please report the distribution of body size/mass and sex in the two experimental groups. I understand that you used these two traits as independent variables in your statistical analyses, which is fine, but it does not demonstrate that these traits were indeed independent of the toxin variables (if they are not, you may have to deal with multicollinearity). For behavioural assays, please report if the exact time of day differed between the two groups. Order effects might also confound these assays: did you clean the arenas of olfactory cues between trials of different individuals?

L209: Please clarify if toxin content of the left and right glands were analysed as repeated measures of the same individual or were summed (or averaged, or what).

L251 and throughout: Please define what measure of variability is given after the ± and please provide such a measure for all estimates of mean values in the Results text (e.g. L272-273, L281-282, L295-296)

L250-254: Please explain these analyses in more detail in the Methods. First, please explain what "energy density" is; this phrase is not trivial to all readers. Second, please explain why and how you tested whether any of the 13 individuals differed in toxin energy density. How can this analysis yield exactly the same statistics as the comparison between left and right glands? Third, the comparison between left and right glands should be a paired test (e.g. Wilcoxon test); to the best of my knowledge the Kruskal-Wallis test is inappropriate here. Finally, please report the df for each chi-squared statistic.
Altogether, these non-parametric analyses seem rather inefficient, because you cannot investigate more than one predictor at a time. If females are systematically larger than males, you cannot really test the effects of size and sex with two separate tests. You had 26 toxin samples from 13 toads, which is not that bad: I would try using the left and right data as repeated measures in a mixed model with SUL and sex as predictors (and you can compare left/right by adding that as another predictor). The statistical assumptions should be assessable for such a model.

L267-273: Please clarify in the Methods if telemetry bout (Day # in Table 2) was used as a numeric variable or a categorical factor in this analysis.
L269-271: What exactly was the effect of toxin depletion on movement? It is difficult to grasp from this description. It would be helpful if you moved here the sentence from the caption of Fig. 5 (i.e. de-toxined toads remained at a constant distance from their point of capture but control toads moved increasingly further away over the five days).
L271-273: Was the difference seen on the final day significant?

L319: "storage" is unclear; please clarify.

L321: I might have missed something but where are the results showing that "larger parotoid glands carry more toxin than smaller ones"?

L324-325: It may be worth adding that the liver is also important for immune defence.

L330-332: It is not evident how/why decreased dispersal would mean reduced foraging; please elaborate a bit.

L334-335: To be fair, this speculation is not supported by the results of your behavioural assays. In contrast, the lower activity observed in the field is marginally supported by the captive study. Ideally, the discussion should make these connections between the different parts of the study.

L347-348: I recommend rephrasing this sentence to something like "facultative adjustments of chemical defence in response to predation risk have been reported in amphibians". I would not say they are well documented in bufonids. Only two studies have found some changes in toad toxin levels after manipulation of predation risk, but those results are not very clear because predation risk was manipulated in the larval stage and the changes were only seen after metamorphosis (Benard & Fordyce 2003; Hagman et al. 2009. Functional Ecology 23:126-132). Another experiment did not find any effect of predator cues on toad toxins (Üveges et al. 2017). You cite a study that was done on salamanders (Bucciarelli et al. 2017) and a field study that found no relationship between predation pressure and toad toxins (Bokony et al. 2016).

L349: The last part of the sentence is unclear.

L366-367: I wonder why you cite only this Chen & Chen study which was done on a single animal. Jared et al. (2014) did a more detailed study, showing that much of the expressed glands are empty even after 90-105 days and some collapsed tissues still do not show any indication of recovery.

L388-396: There is a further interesting aspect of your results: you found a negative correlation between gonad size and toxin content. This may be due to a general trade-off between toxins and growth, as you speculate, but it might also be related to the fact that bufadienolide toxins are synthesized from the same precursor as sex hormones, starting with the same biochemical steps (see Bokony et al. 2019. Scientific Reports 9:3163).

There are minor typos throughout, e.g. L84: as in captivity, L93: Fig. 1, L101: °C, L140: they were, L220: subtlely (?), L294: there is no Fig. 7, L320: expectations, L352: beetle, L354: weighing... Reference 47: Some of the authors are missing.

Fig. 2 and 4: You could make these graphs more informative by showing males and females with different symbols.

Table 1: Could you please report the parameter estimates instead of (or in addition to) the F statistics? The parameter estimates are more informative because they show the direction of the effect. If you choose to do this, please do not forget to report the intercept as well. Also, you could remove the df columns because they are redundant; these two pieces of information can be given in the caption or footnotes.

Fig. 6: Wouldn't this graph look better as a box plot? Or, if you added a 95% confidence interval on these dot plots, we could see if there was significant growth in any of the groups.

Author's Response to Decision Letter for (RSPB-2019-0676.R0)

See Appendix A.

RSPB-2019-0867.R0

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?
Good

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

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

The authors did an exceptional job addressing most of the comments and concerns raised by the handling editor and reviewers. I reiterate my concern with using 'fitness-related', especially in the title. The authors did an excellent job of replacing 'fitness-relevant' with 'growth and behavior'. However, leaving it in the title is misleading to the reader. The usage of fitness-relevant traits' in L25 is acceptable because it is followed up with an explanation that the authors are referring to 'growth rate and behavior' (L28). Including this term in the title, which is the first thing potential readers view, suggests that direct fitness assessments were made – which is not

the case. I have carefully read through the revised manuscript and I only have a few additional, minor concerns:

L21: change to "Many animals capable…"

L60: oxford comma after 'parasites'

L85-89: I suggest removing these lines or re-wording for multiple reasons. It is not clear what you mean by 'activation' of the immune system. The immune system is a remarkably complex network and many of its facets are constitutively active. Normal immunocompetence, in the absence of pathogens, is an active process with dynamic turnover and unknown energetic costs. Activation of innate and adaptive immune does not require the presence of pathogens (e.g., atopic and autoimmune diseases). Pyrogenic and non-febrile responses are typically associated with ~10% increase in metabolic rate. However, that is an average value, and primary, secondary, and tertiary immune responses involve different components which can have drastically different impacts on metabolic rates, especially given different lag rates, type of responding cells (naïve B or T vs memory), type and number of antibodies, or whether or not the response is thymus-dependent.

118: it appears that your Lat/Long are not in the same format

L145: please include citation and remove colon and extra space after "after-effects"

L168: the range of SUL is 50-80mm so I don't understand how the ranges changed to 61.2-84.9mm (L179-180) and 62.2-85.4mm (L181). Is this due to growth in the outdoor enclosures over two weeks (L175)? The minimum SUL increased from 50 to 61.2mm over this time?

L188: comma after e.g.

L251: insert space with "week1"

L402/446: remove parenthesis

L445-446: Please clarify the directionality of these advantages based on your citations. I assume larger toads have higher fecundity, mating success and more foraging opportunities. It is also not clear what you mean by "thermal and hydric relations". Does this mean that larger toads have greater access to Tset/Tpref? What about their size changes hydric relations? Does this mean larger toads have greater access to hydrically preferred refugia because of behavior or is it physiological (I would assume SA:V would favor smaller toads absorbing physiological relevant levels of water through pelvic patches)?

L623: oxford comma

L623: please clarify if these were radio-tracked or lab toads

Table 2: I might have missed something but it is unclear why 'distance between diurnal refugia' df for sex is 1,32 and for SUL is 1,31. Below in 'distance for capture site' the df for sex is 1,31 and SUL is 1,32. Is this switch a typo?

It is also unclear why the ESM (Table 1) has 1,32 df for all of the radio-tracked toads but there is 1,31 df for Table 2.

Fig 2-4: I understand that you don't want to include statistical information about the line of best fit. However, the figure legends should at least describe something along the lines of 'Circles represent individual animals, and the line of best fit is in black'. It is also not clear if the line of best fit includes or excludes the outliers. For example, Fig3, top panel might have a very different slope depending on if the outliers were included.

Figure 2: Please explain the difference between open and closed circles

Figure 3: Please specify that the outliers are open circles.

Figure 4: Please explain the difference between open and closed circles. Y axis says 'residuals' and X axis says 'residual' – please remain consistent

Figure 5: Please re-word to clarify that your figure represents average distance moved, what the error bars represent (SD or SE) and what the lines represent (best fit)

Figure 6: please re-check the control boxplot in the top panel (radio-tracked) as it looks like the top 'whisker' doesn't reach the maximum value. I may have missed something in the manuscript but I only recall two outliers (removed from the analysis), is the whisker off or were there other suspected outliers too?

Decision letter (RSPB-2019-0867.R0)

17-Apr-2019

Dear Dr Brown:

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. 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,
Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
The authors have done a great job addressing most reviewer concerns and these changes have greatly improved the quality and clarity of the manuscript. However, there are a still a few important changes that should be made prior to publication. In particular, 1) the use of the term fitness in the title is misleading based on the metrics use in the study. 2) several of the figures and tables present redundant information with one another and so scan be moved to supplemental material. It is also difficult to interpret the transformed data in Fig 3, 4. In addition to these issues, there are more specific changes suggested by the review that should also be addressed.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).
The authors did an exceptional job addressing most of the comments and concerns raised by the handling editor and reviewers. I reiterate my concern with using 'fitness-related', especially in the title. The authors did an excellent job of replacing 'fitness-relevant' with 'growth and behavior'. However, leaving it in the title is misleading to the reader. The usage of fitness-relevant traits' in L25 is acceptable because it is followed up with an explanation that the authors are referring to 'growth rate and behavior' (L28). Including this term in the title, which is the first thing potential readers view, suggests that direct fitness assessments were made – which is not the case. I have carefully read through the revised manuscript and I only have a few additional, minor concerns:

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Figure 6: please re-check the control boxplot in the top panel (radio-tracked) as it looks like the

top 'whisker' doesn't reach the maximum value. I may have missed something in the manuscript but I only recall two outliers (removed from the analysis), is the whisker off or were there other suspected outliers too?

Author's Response to Decision Letter for (RSPB-2019-0867.R0)

See Appendix B.

Decision letter (RSPB-2019-0867.R1)

24-Apr-2019

Dear Dr Brown

I am pleased to inform you that your manuscript entitled "The cost of chemical defence: the impact of toxin depletion on growth and behaviour of cane toads (Rhinella marina)." 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

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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,

Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Author response to reviewer and editorial comments

Thank you for the opportunity to revise our manuscript. We found the comments and copy-editing of both reviewers to be very helpful and we have incorporated their suggestions into our revision. Our responses to each comment appear below, preceded by asterisks.

Associate Editor
Board Member: 1
Comments to Author:
The manuscript 'The cost of chemical defence: the impact of toxin depletion on fitness-relevant traits of cane toads (Rhinella marina)' was reviewed by myself and two experts in the field of chemical defense and physiology. As you can see from their reviews, the reviewers both liked the manuscript and agree that the manuscript was well written and the ideas and general framework are of broad interest. However, there are some important points and clarifications that were raised and that should be addressed prior to publication including the following:
1) There are some clarifications in the methodology that should be made, including specifically how measurements were acquired and how toxin content was quantified;

* We have clarified our methodology- see details below.

2) The interchangeable discussion of toxins versus venoms is mispleading to reviewers and should be carefully distinguished;

* We have distinguished between venoms and toxins and shifted the emphasis of the Introduction away from venoms - see details below.

3) there are various issues with word-choice throughout the manuscript that may be misleading to readers, in particular the use of 'fitness-measures' throughout the manuscript may not be appropriate when no direct fitness assessments where made;

* We have replaced most instances of 'fitness-related' with 'growth and behaviour'- see below.

4) better **justification of the measurements chosen** and their importance in this system would greatly benefit readers.

* We clarify that the measurements chosen have previously proven effective at detecting differences among groups of toads- see below.

In addition to these issues, there is a long list of specific, more minor suggested changes the authors should address and important references/citations include.

* A long list of changes have been incorporated and references added- see below.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
General comments:
This manuscript uses a combination of laboratory and field-based experiments to examine the impact of toxin replenishment in Cane toads. The authors use accurate statistics although I have some minor concerns about a few unmentioned assumptions. The data suggest that toads are impacted, in terms of morphology and performance, after toxins have been forcibly removed and the toads are, presumably, replenishing them. The data are hard to interpret as they are transformed though. The manuscript is extremely well written, and the amount of data presented is impressive.

It is not clear throughout the manuscript what the authors mean when they mention “aspects of physiology” (e.g., L80). Organ masses and SUL are considered morphological traits [L215] and behavioral traits are obvious but it’s very unclear what aspects of physiology were measured in this study.

* Physiological performance is implied by some of our measures (e.g. locomotor performance), but it is correct that we didn't actually make any physiological measurements. We have removed mention of 'physiology’ in the revision.

I expect readers will also have trouble with their use of the term “fitness-related” throughout. There were no true metrics of biological/Darwinian fitness in this study. The fittest toads might not necessarily be the largest, fastest, or have the most toxin, they just need to survive, mate, and produce offspring. The authors will either need to add more justification in the intro/discussion of consider alternate phrasing such as ‘performance’ throughout.

* We have removed most instances of 'fitness-related' and replaced the term with the more specific 'growth and behaviour'.

The manuscript could also be greatly improved by adding much more justification for why the metrics they selected are useful compared to measuring metabolism. There is a brief paragraph (L71-74) but then the data suggests metabolic measures may have explained a lot of the variance detected. While I appreciate the authors admittance in the discussion (L350-351) that metabolic data would have helped, the context of the study could be re-phrased to explain that metrics in addition to metabolism might help explain toad energy budget dynamics during toxin replenishment.

* We have added an explanation that we chose our metrics based on their ability to detect differences in other attributes (e.g. disease status, invasion history) among toads. We have also paraphrased the comment about energy budget dynamics and inserted this point into the Introduction.

It is also very difficult to make sense of the findings based on the figures and I strongly recommend plotting raw data so that readers can grasp the biological relevance.

* We have revised our figures, in some cases to show raw data

Specific Comments:

L52-54: Please be careful here and consider using ‘typically’ as I imagine some venom/poison/toxin experts will get flustered. The most concrete definition of ‘venomous’ I’ve seen so far – “a complex substance produced in a specialized gland and delivered by an associated specialized apparatus that is deleterious to other organisms in a given dosage and is actively used in the subjugation and/or digestion of prey and/or in defense.” (Minton 1974)

* 'typically' inserted.

L61: insert oxford comma

* Comma inserted

L67: insert oxford comma

* Comma inserted

L68: should that read “toxins” or is it mostly homogeneous?

* Changed to 'toxins'

L74: “a far wider array of traits” is strangely worded. My understanding is that you’re trying to say ‘there are more ways to measure energy expenditure than metabolic rates’ although the use of trait doesn’t help get this point across, probably because I don’t think readers would equate metabolic rate as a ’trait’ per se. Consider re-wording

* Reworded to-
" However, increased oxygen consumption may only represent part of the cost of venom regeneration. Other potential costs could include..."

L78: start new paragraph

* New paragraph started

L79: remove “a sample of”

* Removed

L81: change to “mimicking an encounter”

* Changed

L83: oxford comma

* Comma added

L84: delete second use of “in” and consider changing to “laboratory” or “laboratory setting”

* Deleted and changed

L78-L86: you switch tense throughout. E.g. " we investigate" (L78) vs "we dissected" (L79). Please stay consistent throughout

* Changed to past tense throughout

L86: oxford comma

* Comma added

L99-101: please insert citation for the meteorological data

* Citation inserted

L97 & L104: As written, it isn't clear why you mention both study sites. Please clarify the roles of Fogg Dam (laboratory study) and Leaning Tree Lagoon (field collected toads and field study) in this sub-section

* Clarified as suggested

L117: Do you have any citation to verify that you can, in fact, squeeze out all of their toxins? Was the squeezing done by hand or did you use a tool? I would appreciate reading about how you tried to limit subjectivity when extracting toxins, especially because you were weighing them to such a fine degree. It's hard to know

* We have added detail on how we squeezed glands and references on its efficacy. We attempted to limit subjectivity by squeezing until no more toxin emerged.

L120: oxford comma after "patted dry"

* Comma added

L128: delete "Hudson, Brown" as you already have "[40]"

* Author names removed

L131: it's not clear how you can squeeze the gland without expressing any toxin. Please clarify here, or earlier in your methods so it's possible to understand your methods (this also relates to my comment on L117)

* We've added further description and a small diagram to illustrate how a gland can be squeezed without expressing toxin. Essentially squeezing in a manner that compresses (rather than expands) the outer surface of the gland will not cause the contents to be released.

L140: change to "they were euthanized, weighed, and…"

* Comma added

L141: oxford comma

* Comma added

L151: remove “Finnerty, Shine”

* Author names removed

L157: consider rewording – “the toads had toxin expressed from their…”

* Changed wording to "... toads were de-toxined (as above)..."

L158: insert space – “12 l”

* Space inserted

L160: oxford comma

* Comma added

L166: change to “…described by Finnerty et al. [41]…”

* Changed as suggested

L172: change to “its”

* Apostrophe removed

L166-176: I assume you used more than one enclosure because 20 toads at 30 min per toad would be 10 hrs of trials and you say all trials took place in ~5 hours (L175). Did you clean the enclosures between trials and did you account for enclosure as a variable in your behavioral stats? It’s fine if you did not, but it would be prudent to point that out.

* We have clarified these points- " Four arenas were filmed simultaneously, and arenas were sprayed and wiped with 10% ethanol between trials to remove scent cues. But we did not point out that arena was not included in analysis. It is more efficient to indicate that factors that are included in analyses, rather than list all the factors that were not included.

L180,181,183,184: space between number an ‘cm’

* Spaces added in front of 'cm'

L186: change to “during the daytime”

* Changed

L187 & L193: please include approximate time of day here as you did in L175 if you have it.

* Times of day added

L211: oxford comma

* Comma added

L212: oxford comma

* Comma added

L212-213: clean up the citations, no name needed for Proc B just [##]

* Citations now corrected

Stats: did you account for number of crickets eaten in any of your statistics? That is unclear in the ms. I assume this might have been nested within individual but it isn't clear

* We did not quantify the number of crickets eaten. All toads were provided with the same amount of food each day, and all crickets were usually gone by the next feeding.

L217: with sex and body size both treated as indepdent variables I'm a little concerned that the assumptions of multicollinearity and homoscedasticity were violated. Can you please provide, in the ms or as supplementary materials, VIF for your tests? Sex, SUL, mass, organ mass, and toxin hopefully don't have VIF > 10. In terms of homoscedasticity, I don't necessary buy into needing a GQ test but a supplemental scatterplot might be useful.

* The highest VIF in any of our multiple regressions was 1.81. We have added a statement to the 'Statistical analyses' section to indicate this.

L222: oxford comma

* Comma added.

L234: perhaps I missed it, but where in your methods do you describe the factors for your ANCOVA?

* We now refer to this test as a multiple regression.

L237-241: this, with some re-wording, seems better suited for the methods

* Although information on the outliers might appropriately belong under 'statistical analyses', we believe that presenting it together with the overall toxin vs size relationship is easier for the reader to understand.

L244: oxford comma

* Comma added.

L251: please describe if this is SD or SEM

*SE added.

L258-263: please do a better job of justifying why energy and body size were not related when you actually quantified them (L256) but it did when you extended the data [L263]. It's unclear if you were interpolating or extrapolating to find this p value. Without a relationship in your measured data, did you extrapolate as a linear relationship? This section needs to either be removed or backed up with much more justification.

* Energy density of toxin is constant (10.06kJ/g); it doesn't change with body size (as indicated at L256). But the total mass of toxin produced by a toad does depend on body size (L235 in original ms). The relationship described at L258 simply multiplies the mass of toxin produced by each of the 30 toads by the energy density constant. Larger toads had a higher caloric investment in toxin simply because they produced more toxin. We have attempted to clarify this point in the revised ms.

Results: please be consistent with your significant digits through the results section

* We have changed p values to two decimal places, in all but one instance.

L281 & L295: I assume the mass of the toxin removed was accounted for and not included in the mass loss measurements but that is unclear, or I missed it. Ie If you squeezed out 4 g of toxin I want to make sure 'start weight' was after removing the toxin and not before

* We now clarify that 'initial mass' excluded toxin mass. The average amount of toxin removed (0.1g) was very small compared to toad body mass.

L279: In your methods you describe using these variables as independent factors (L213), why did you change them here?

*We use the terms 'independent variable' and 'covariate' interchangeably. But to avoid confusion we now use 'independent variables' here.

L302: oxford comma

* Comma added.

L306: oxford comma

* Comma added.

L306: specify if you mean partially or completely emerged from shelters as you do in L307.

* We have clarified that males both partially and fully emerged more quickly than did females.

L319: oxford comma

* Comma added.

L320: chane to “expectations”

* Changed.

L326: can you please clarify what other, non-energetic substances you mean here? Besides glycogen what other substance might contribute to the massive mass loss in your data? Vitamin, metals, or blood protein (albumin)?

* Glycogen is likely to be the major component of liver mass loss. We have added that soluble proteins are also stored in the liver. This is unlikely to be an important contribution because protein levels in cane toad toxin are low.

L335: you released the toads back to their original site of capture though, so I don’t understand how you consider foraging here a ‘novel environment’.

* Novel was an incorrect term to use. We have reworded this to read - '... potentially risky forays away from their home site'.

L349: this needs to be re-worded, if I understand correctly – you collected all toads form the same site so there shouldn’t be any impact of predators on their toxin amounts. Correct? Please re-phrase, as written it’s a little confusing.

* Reworded to read - " Because all toads used in the present study were collected from the same site, differences in predator encounters are unlikely to have impacted toxin levels.'

L352-354: remove names in citations

* Author names removed.

L372: be careful stating what selection ‘should’ do

* We have reworded this to read- '... we would expect selection to favour...'

L377-381: Are there other species that have been studied?

* Not to our knowledge.

L380-381 should be moved up and it would be helpful to cite research on other toxic animals that secret toxins.

* We have moved this sentence up to the end of the preceding paragraph.

L377-379 make it unclear if toads exude their toxins it all comes out or are they able to control the amount? Please provide a brief justification for this line of reasoning. If they can

control the volume exuded then I don't follow your line of reasoning, if they can't control the amount then it makes sense.

* We have clarified that this statement. We assume that toads cannot control the amount of toxin released.

L386: change to "threat is on"

* Changed to 'threat is on'.

L393: remove name from citation

* Author name removed.

Table 1: please explain in caption that wt = "weight" for clarity

* We have replaced 'wt' with 'weight' in table 1.

L578: oxford comma

* Comma added.

Figure 2/3/4: it is unclear why your figures visualize the transformed data which make it difficult for the reader to comprehend. This transformed data has no biological meaning and it's fine to mention that your stats were on transformed data but I want to see the actual data to make sense of it.

*We have now plotted untransformed data for Fig. 2. Unfortunately plotting raw data for Figs 3 and 4 would not reveal the trade-offs between variables depicted, because the residual values plotted in Figs 3 and 4 remove the effect of body size from each variable. This mimics the effects of including body size as an independent variable in the multiple regression analyses. If we plotted out raw data for ovary mass vs toxin mass for instance, it would show a strong positive relationship because both ovary mass and toxin mass are higher in larger toads. Only when the effects of body size are removed (by using residuals) are the negative trade-offs able to be depicted graphically.

Figure 2/Figure 3: Insert "positive" to figure 2. please add units to axes. Please clarify in legend that individual points represent individual toads and if this is data from field, lab, or both. Also describe what line of best fit is with an R2 value.

*We have revised the heading to include the suggested information. We have not added statistical information related to the lines of fit because none of these results are what we discuss in the text. For example, the multiple regression results in Table 1 indicate that the significance of the negative relationship between toxin mass and liver mass is $p = 0.282$. But the significance of the simple regression line depicted in Fig 4 is $p= 0.0542$. We think that presenting two p-values for the same relationship, one from a statistically appropriate analysis and the other just for a Figure, would be confusing for most readers.

Figure 3/4: it is unclear why the open circles are outliers from the figure and fig legend alone, those points look very close to the line of best fit.

* We have clarified that these are the outliers from the relationship depicted in Figure 2; that is, toads that had an unusually low amount of toxin for their body size.

Figure 5: describe line of best fit in caption and include relevant stats.
* Because the Figures now depict raw data, instead of the transformed values used in our analyses, statistics associated with lines of fit are not relevant.

Figure 6: I'm very glad that you plotted the raw data, it would also be useful to have boxplots with error bars overlaid so readers can quickly

* We have now changed this figure to include box plots.

Referee: 2

Comments to the Author(s)
Chemical defences in vertebrate animals is an under-studied topic, and the toxin biology of cane toads is of particular importance due to the conservation impact of this species. Thus, this study contributes valuable results to the field of chemical ecology. Overall, the manuscript is neatly written and I see no major flaw in the design, execution and interpretation of the study. I have several relatively minor comments which need attention though, especially regarding the clarification of some methodological aspects. Also, I recommend that the theoretical framework of the study should be better focused on chemical defences, and mixing up the latter with "offensive" toxins (venoms) should be avoided (or, at the very least, the two should be clearly distinguished). There is a bunch of recent literature directly relevant to this study (see Hettyey et al. 2014. Oikos 123:1025-1028 and further citations below) that would be more appropriate to cite and build the introduction on instead of the venom literature.

Detailed comments:

L21: This is not a good start for the abstract. Do we have evidence that "many animals that produce chemical defences are reluctant to deploy them"? In the manuscript you only mention cane toads' reluctance. Many of the chemical defences in nature are not even deployable, although their levels can be adjusted facultatively. Many of the deployable toxins are actually venoms used for hunting (not defence). Mixing these two kinds of toxins throughout the paper can be misleading.

* We have changed this opening sentence to specify animals that are capable of deploying chemical defences, rather than just producing them.

L46-50: It is unfortunate to mix toxins with venoms, as the latter are not (primarily) for defence. You need not rely (only) on the 'venom optimization hypothesis': the same general concept of trade-offs has been formulated for chemical defences under the name 'optimal defence theory'. It has been developed for plants but also can, and has been, applied to animals like amphibians (e.g. Bokony et al. 2016. 42:329-338).

* We have revised the Introduction along the suggested lines. We have clarified that venom has a role in immobilizing prey as well as in defence. We also refer to 'optimization hypotheses' more generally, to incorporate both venom optimization and defence optimization hypotheses.

L51-52: It is strange that after saying "most studies", you cite a single case study, when there are several citations available (e.g. Benard & Fordyce 2003. Ecology 84:68-78; Fordyce et al. 2006. Oecologia 149:101-106; Kurali et al. 2016. Biol. J. Linnean Soc. 119:1000-1010; Toth et al. 2019. J. Chem. Ecol. doi: 10.1007/s10886-019-01045-9). Notably, these latter studies all looked at defensive chemicals (not venoms) and found little evidence for the costs of chemical defences.

* We have now added additional references to this section.

L52-54: While this definition seems accurate, I think there is another important distinction: venoms are used mainly for immobilizing or killing prey while defensive toxins are not used in a foraging context. This is not necessarily trivial for all readers, so it would be helpful to clarify this here.
*This is indeed an important distinction to make here, and we now outline its importance here.

L62-70: A third line of evidence is that the compressed parotoid gets injured; the bleeding and immune reaction have physiological costs, as does the healing of the tissues (see Jared et al. 2014. Toxicon 87:92-103).

*We have added this point as a further line of evidence.

L71-77: Again, I find it awkward that you talk about venoms when your paper is about defensive toxins. For example, lost foraging opportunities seem irrelevant for defensive toxins. Why talk about these when you can talk directly about the potential costs of defensive toxins? I agree that "consequences for fitness-related traits may be of greater ecological and evolutionary significance", but this sentence might be vague for readers; you could be a bit more specific, e.g. give an example (see previous studies cited above).

* We have revised this paragraph to de-emphasise venom as suggested.

L116: Looking at Fig. 1 it is not trivial what the "length and width of each parotoid" are, as the gland looks almost spherical. Can you indicate these measurements of the photo? Also, please explain what these measurements were used for. Was there a correlation between parotoid size and toxin amount?

*We have indicated the approximate location of length and width measurements in Fig 1 and now include results on the correlations between toxin mass and gland dimensions.

L117-118 (also L94): The logic here is not 100% clear. Are you saying that you removed ALL toxins from these animals' parotoids? In this case, please provide evidence that the entire toxin store can be depleted by manual compression. If you cannot show that, then can you validate that the sample you took is representative for the entirety of each individual's total toxin amount? In other words, does the amount of squeezed-out toxin correlate well with

the total amount of toxins? Theoretically it is possible to squeeze out a small amount from a large gland and a larger amount from a smaller gland. This should not affect the comparisons between de-toxined and control toads, but it is a concern when you are correlating the amount and caloric content of toxins with other morphological traits.

* We clarify that manipulating parotoid toxin is more feasible than manipulating widely distributed skin glands. We also clarify that we squeezed glands until no more toxin came out, but that we did not subsequently examine glands histologically to determine if any residual toxin remained.

L122: For comparisons of de-toxined and control toads to be valid, especially because the sample sizes were relatively small, it is important to show that any difference between the two experimental groups was not due to accidental differences in other factors. For both free-ranging and captive toads, please report the distribution of body size/mass and sex in the two experimental groups. I understand that you used these two traits as independent variables in your statistical analyses, which is fine, but it does not demonstrate that these traits were indeed independent of the toxin variables (if they are not, you may have to deal with multicollinearity). For behavioural assays, please report if the exact time of day differed between the two groups. Order effects might also confound these assays: did you clean the arenas of olfactory cues between trials of different individuals?

* We have added details on the range of body size and sex ratios for de-toxined and control toads in both the free-ranging and wild toads. We have also specified the time of day of the behavioural trials and clarified that arenas were cleaned with 10% ethanol between successive trials.

L209: Please clarify if toxin content of the left and right glands were analysed as repeated measures of the same individual or were summed (or averaged, or what).

* The mass of toxin obtained from left and right glands were summed to give a total toxin weight for each toad. We have clarified this procedure in the revision.

L251 and throughout: Please define what measure of variability is given after the ± and please provide such a measure for all estimates of mean values in the Results text (e.g. L272-273, L281-282, L295-296)

* We have clarified that means are followed by standard errors and have added these measures of variation at the indicated points.

L250-254: Please explain these analyses in more detail in the Methods. First, please explain what "energy density" is; this phrase is not trivial to all readers.

* We now explain that caloric density is a measure of energy content corrected for mass of the sample.

Second, please explain why and how you tested whether any of the 13 individuals differed in toxin energy density.

* We clarify that we used the Kruskall-Wallis test to determine if there were differences in toxin caloric density among the 13 toads.

How can this analysis yield exactly the same statistics as the comparison between left and right glands?

* We now use the mixed model suggested below to compare left vs right glands.

Third, the comparison between left and right glands should be a paired test (e.g. Wilcoxon test); to the best of my knowledge the Kruskal-Wallis test is inappropriate here. Finally, please report the df for each chi-squared statistic.

* We now use the Kruskal-Wallis test only to determine if caloric content of toxin varied among 13 individual toads. We have replaced the other nonparametric tests with the model suggested below, to assess effects of SUL, sex and left vs right glands.

Altogether, these non-parametric analyses seem rather inefficient, because you cannot investigate more than one predictor at a time. If females are systematically larger than males, you cannot really test the effects of size and sex with two separate tests. You had 26 toxin samples from 13 toads, which is not that bad: I would try using the left and right data as repeated measures in a mixed model with SUL and sex as predictors (and you can compare left/right by adding that as another predictor). The statistical assumptions should be assessable for such a model.

* We now use this suggested model in place of the nonparametric tests.

L267-273: Please clarify in the Methods if telemetry bout (Day # in Table 2) was used as a numeric variable or a categorical factor in this analysis.

* We have revised the Methods to clarify that Day# was included as a continuous fixed effect in the model and Bout (first week vs second week) was included as a nominal random effect.

L269-271: What exactly was the effect of toxin depletion on movement? It is difficult to grasp from this description. It would be helpful if you moved here the sentence from the caption of Fig. 5 (i.e. de-toxined toads remained at a constant distance from their point of capture but control toads moved increasingly further away over the five days).

* We have added the sentence from the figure heading to the text as suggested.

L271-273: Was the difference seen on the final day significant?

*No. Although Control toads were further way from their original capture sites than were de-toxined toads on the last day, the difference was NS (p=0.24)

L319: "storage" is unclear; please clarify.

* We have clarified that we refer here to liver size.

L321: I might have missed something but where are the results showing that "larger parotoid glands carry more toxin than smaller ones"?

* This result was accidentally removed. We have now reinserted the positive correlations between gland size and toxin mass.

L324-325: It may be worth adding that the liver is also important for immune defence.

* We now mention the liver's role in biosynthesis.

L330-332: It is not evident how/why decreased dispersal would mean reduced foraging; please elaborate a bit.

* We did not intend to causally link reduced dispersal to reduced foraging. We now clarify that some aspects of behaviour (dispersal) are affected by toxin removal and conceivably other behaviours (foraging) could be affected as well.

L334-335: To be fair, this speculation is not supported by the results of your behavioural assays. In contrast, the lower activity observed in the field is marginally supported by the captive study. Ideally, the discussion should make these connections between the different parts of the study.

* We now discuss the contrasting behavioural results seen in the lab vs field.

L347-348: I recommend rephrasing this sentence to something like "facultative adjustments of chemical defence in response to predation risk have been reported in amphibians". I would not say they are well documented in bufonids. Only two studies have found some changes in toad toxin levels after manipulation of predation risk, but those results are not very clear because predation risk was manipulated in the larval stage and the changes were only seen after metamorphosis (Benard & Fordyce 2003; Hagman et al. 2009. Functional Ecology 23:126-132). Another experiment did not find any effect of predator cues on toad toxins (Üveges et al. 2017). You cite a study that was done on salamanders (Bucciarelli et al. 2017) and a field study that found no relationship between predation pressure and toad toxins (Bokony et al. 2016).

* We have changed this sentence as suggested.

L349: The last part of the sentence is unclear.

* We have re-worded it to read " Because all toads used in the present study were collected from the same site, differences in predator encounters are unlikely to have impacted toxin levels."

L366-367: I wonder why you cite only this Chen & Chen study which was done on a single animal. Jared et al. (2014) did a more detailed study, showing that much of the expressed glands are empty even after 90-105 days and some collapsed tissues still do not show any indication of recovery.

* We have now cite Jared et al here. We had cited only the Chen & Chen MS because it specifically involved *R. marina*, but we agree that the Jared et al paper is much more detailed and better documented and *R. icterus* is a close relative.

L388-396: There is a further interesting aspect of your results: you found a negative correlation between gonad size and toxin content. This may be due to a general trade-off between toxins and growth, as you speculate, but it might also be related to the fact that bufadienolide toxins are synthesized from the same precursor as sex hormones, starting with the same biochemical steps (see Bokony et al. 2019. Scientific Reports 9:3163).
*Thank you very much for this information. We have added this point and citation to the first paragraph of the Discussion.

There are minor typos throughout, e.g.
L84: as in captivity,
L93: Fig. 1,
L101: °C,
L140: they were,
L220: subtlely (?),
L294: there is no Fig. 7,
L320: expectations,
L352: beetle,
L354: weighing...

* These typos have all been fixed, except 'subtlely' which appears to be the correct spelling.

Reference 47: Some of the authors are missing.

* We have added the five missing authors.

Fig. 2 and 4: You could make these graphs more informative by showing males and females with different symbols.

* We have revised these Figures to show separate symbols for males and females.

Table 1: Could you please report the parameter estimates instead of (or in addition to) the F statistics? The parameter estimates are more informative because they show the direction of the effect. If you choose to do this, please do not forget to report the intercept as well. Also, you could remove the df columns because they are redundant; these two pieces of information can be given in the caption or footnotes.

* We have added parameter estimates and intercepts to Table 1.

Fig. 6: Wouldn't this graph look better as a box plot? Or, if you added a 95% confidence interval on these dot plots, we could see if there was significant growth in any of the groups.

* We have now configured Fig 6 to a boxplot.

**We very much appreciate these constructive and insightful comments from the editors and reviewers.

Appendix B

Thank you for the opportunity to revise our manuscript. As requested, we have changed the title and moved two Figures to ESM. We have also acted on all the other reviewer suggestions; our specific responses to each comment appear below, preceded by asterisks.

Dear Dr Brown:

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.

Associate Editor
Comments to Author:
The authors have done a great job addressing most reviewer concerns and these changes have greatly improved the quality and clarity of the manuscript. However, there are a still a few important changes that should be made prior to publication. In particular,
1) the use of the term fitness in the title is misleading based on the metrics use in the study.

* We have removed 'fitness-related' from the title and replaced it with 'growth and behaviour'.

2) several of the figures and tables present redundant information with one another and so scan be moved to supplemental material. It is also difficult to interpret the transformed data in Fig 3, 4.

* We have moved Figs 3 and 4 to ESM and have expanded their headings to try to aid interpreting the residual values.

In addition to these issues, there are more specific changes suggested by the review that should also be addressed.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).
The authors did an exceptional job addressing most of the comments and concerns raised by the handling editor and reviewers. I reiterate my concern with using 'fitness-related', especially in the title. The authors did an excellent job of replacing 'fitness-relevant' with 'growth and behavior'. However, leaving it in the title is misleading to the reader. The usage of fitness-relevant traits' in L25 is acceptable because it is followed up with an explanation that the authors are referring to 'growth rate and behavior' (L28). Including this term in the title, which is the first thing potential readers view, suggests that direct fitness assessments were made – which is not the case. I have carefully read through the revised manuscript and I only have a few additional, minor concerns:

* We have removed 'fitness-related' from the title and replaced it with 'growth and behaviour'.

L21: change to “Many animals capable…”

* Changed

L60: oxford comma after ‘parasites’

* Comma added

L85-89: I suggest removing these lines or re-wording for multiple reasons. It is not clear what you mean by ‘activation’ of the immune system. The immune system is a remarkably complex network and many of its facets are constitutively active. Normal immunocompetence, in the absence of pathogens, is an active process with dynamic turnover and unknown energetic costs. Activation of innate and adaptive immune does not require the presence of pathogens (e.g., atopic and autoimmune diseases). Pyrogenic and non-febrile responses are typically associated with ~10% increase in metabolic rate. However, that is an average value, and primary, secondary, and tertiary immune responses involve different components which can have drastically different impacts on metabolic rates, especially given different lag rates, type of responding cells (naïve B or T vs memory), type and number of antibodies, or whether or not the response is thymus-dependent.

* Our intent here was to use 'activation' in the sense as in the titles of the cited papers- experimentally triggered immune responses using LPS. However, to avoid the analogy causing confusion, we have deleted this passage.

118: it appears that your Lat/Long are not in the same format

* We have replaced the extra degree symbol with a decimal.

L145: please include citation and remove colon and extra space after “after-effects”

* Changed as suggested

L168: the range of SUL is 50-80mm so I don’t understand how the ranges changed to 61.2-84.9mm (L179-180) and 62.2-85.4mm (L181). Is this due to growth in the outdoor enclosures over two weeks (L175)? The minimum SUL increased from 50 to 61.2mm over this time?

* '50' was a typo here, the correct minimum size at initial capture of these toads was 60 mm. We have corrected this.

L188: comma after e.g.
* Comma added

L251: insert space with “week1”
* Space inserted.

L402/446: remove parenthesis
* We added an extra parenthesis at L402 and removed a parenthesis at L 446.

L445-446: Please clarify the directionality of these advantages based on your citations. I assume larger toads have higher fecundity, mating success and more foraging opportunities. It is also not clear what you mean by "thermal and hydric relations". Does this mean that larger toads have greater access to Tset/Tpref? What about their size changes hydric relations? Does this mean larger toads have greater access to hydrically preferred refugia because of behavior or is it physiological (I would assume SA:V would favor smaller toads absorbing physiological relevant levels of water through pelvic patches)?

* We have added directionality details and further references here. Larger toads have lower rates of desiccation (due to SA:V), and reduced rates of heating and cooling (due to greater thermal mass).

L623: oxford comma

* Comma added

L623: please clarify if these were radio-tracked or lab toads

* Clarified

Table 2: I might have missed something but it is unclear why 'distance between diurnal refugia' df for sex is 1,32 and for SUL is 1,31. Below in 'distance for capture site' the df for sex is 1,31 and SUL is 1,32. Is this switch a typo?

*We have clarified that we used the Kenward-Rogers df approximation method for all our Mixed model analyses. Thus, degrees of freedom approximations for different terms in these models can vary subtly, from 31.06 to 31.82, and which were rounded to 31 and 32 respectively. We have clarified this in the Statistical Analyses section and in the heading of Table 2

It is also unclear why the ESM (Table 1) has 1,32 df for all of the radio-tracked toads but there is 1,31 df for Table 2.

* See above. ESM Table 1 was not a mixed model, so used conventional df values

Fig 2-4: I understand that you don't want to include statistical information about the line of best fit. However, the figure legends should at least describe something along the lines of 'Circles represent individual animals, and the line of best fit is in black'. It is also not clear if the line of best fit includes or excludes the outliers. For example, Fig3, top panel might have a very different slope depending on if the outliers were included.

* We have re-done Figs 2-4 and their headings to better indicate that the outliers were excluded from lines of fit. We have also added the phrase 'Circles represent individual animals, and the line of best fit is in black' to the headings

Figure 2: Please explain the difference between open and closed circles

* We have now clarified that open symbols represent females and closed symbols represent males.

Figure 3: Please specify that the outliers are open circles.

* We have clarified that the open symbols represent outliers

Figure 4: Please explain the difference between open and closed circles. Y axis says ‘residuals’ and X axis says ‘residual’ – please remain consistent

* All 'residuals' replaced with 'residual'

Figure 5: Please re-word to clarify that your figure represents average distance moved, what the error bars represent (SD or SE) and what the lines represent (best fit)
* We have revised this figure heading to include the suggested information.

Figure 6: please re-check the control boxplot in the top panel (radio-tracked) as it looks like the top ’whisker’ doesn’t reach the maximum value. I may have missed something in the manuscript but I only recall two outliers (removed from the analysis), is the whisker off or were there other suspected outliers too?

* We have fixed the indicated whisker in this Figure. Our attempt to exclude the low values from the plot inadvertently exclude the high values as well.