**Response to reviewer comments for JOVE-S-18-01574**

**Note: all responses to reviewer comments are in bold and immediately follow the comment that was addressed.**

**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.   
2. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. **We added this.**  
3. Please remove commercial language: Alconox. **We removed this.**  
4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). **We checked the protocol text and removed pronouns.**  
5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. **We removed many of the notes and removed all phrases in the protocol that were not in imperative tense.**  
6. 1.1.1: Please specify the animal used in the protocol. **We changed this to “reptile.”**  
7. 2.2: Please specify the volume of hexane used to solubilize the lipids. **We changed this to provide a minimal target concentration. The volume of hexane for this step is dependent on the yield of lipids from the extraction.**  
8. 3.1.5: How is the sand washed and dried? **This is sold as washed dried sand.**   
9. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step. **We combined many of the protocol steps to yield fewer total steps in each section.**  
10. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.  
11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. **We corrected this.**  
12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted. **We did this.**  
13. Figure 1: Please combine all panels of one figure into a single image file. **We did this.**  
14. Discussion: Please discuss any limitations of the technique. **We added a section where we discuss limitations.**  
15. References: Please do not abbreviate journal titles. **We spelled out all of the journal titles.**

**Reviewer comments:**  
  
Reviewer #1:  
  
It is with great interest and enthusiasm that I have read the manuscript, entitled "Chemical isolation, quantification, and separation of skin lipids from reptiles' by Baedke and colleagues. While chemical communication is a well-established field in biology, it is still biased in its focus on chemical signals produced and used by invertebrates. Nevertheless, reptiles, especially squamates, are known to strongly rely on chemical signals to communicate with conspecifics. Since the study of chemical communication in reptiles is an upcoming field, I believe this methodological paper might be of utmost importance. Although I've ample experience in the field of lizard chemical communication, I must admit that I don't have the expertise to comment on the purely methodological aspect of the paper. I believe others will be more suited to do this. Nevertheless, aside from the methods, it is still very important to frame the current study well, and appropriately.  
  
Although I only have a couple of minor comments, my main comment is about the introduction (of both the abstract and main document). The authors clearly have a tendency to integrate applicable science or a certain economical value into this (L54: understanding the waterproofing role of lipids in the evolution of terrestrial life and wildlife management of invasive species via pheromone-based lures"),. While there is nothing wrong with that, I don't think there is enough literature at this point to talk about the true possibilities of the use of reptile lipids for economical/medical value. Also, the manuscript will not be less interesting (some might say the contrary!) without the applicable science. Unraveling the origin and evolution of the massive animal diversity (including chemical signal diversity) on earth is an important thing on its own. Because also the references used regarding this (ref 4, 5, 6) are not really proper ones, I believe this part of the manuscript should be removed. It is already plenty if the authors solely focus on the importance of understand animal diversity and the evolution of chemical signals. When this is tackled (together with some minor issues), I believe I can recommend this paper for publication.

**We reframed the utility of our methods for wildlife managers and believe it should be stated given its applicability, and another reviewer of this manuscript asked us to elaborate on that point specifically. Therefore we kept this section but refined it. We removed Ref 6 because we agree the relevance to dermatological research is less germane. We also removed Ref 4 in this section because Ref 3 is sufficient. We appreciate the comments about better references for lizard chemical ecology and have included these.**   
  
Some minor comments:  
  
Introduction  
L51: Change "Reptiles produce lipid compounds in their skin that serve …." By "Reptiles are equipped with specialized epidermal glands that produce lipids used for social communication, such as intra- and interspecific recognition, territoriality, and mate assessment. " **We revised this sentence.**

Also. I believe the papers cited here are odd ones. Reference 2, 3 and 4 are appropriate, but 1 and 5 are not. Rather, cite reviews like:  
-> Mayerl, C., Baeckens, S. & Van Damme, R. 2015. Evolution and role of the follicular epidermal gland system in non-ophidian squamates. Amphibia-Reptilia 36: 185-206. **We added this reference.**  
-> Martín, J., López, P. (2011): Pheromones and reproduction in reptiles. In: Hormones and Reproduction in Verte- brates, p. 141-167. Norris, D.O., Lopez, K.H., Eds, Aca- demic Press, USA. **We did not include this reference because the 2014 Martin and Lopez paper is a better fit.**  
-> Martín, J., López, P. (2014): Pheromones and chemical communication in lizards. In: The Reproductive Biol-ogy and Phylogeny of Lizards and Tuatara, p. 43-77. Rheubert, J.L., Siegen, D.S., Trauth, S.E., Eds, CRC Press, USA. **We added this reference.**

**It should be noted that glands are responsible for pheromone production in most if not all lizards, but this is not true for snakes. Most species of snakes have skin devoid of glands. Instead, skin lipids are produced en masse by the epithelium.**   
  
L62: I agree. Especially in squamates, thus, lizards and snakes. See the recent papers on lacertid lizards (from the authors mentioned above).  
**We have added referrals to those citations.**  
  
  
Reviewer #2:  
  
Manuscript Summary:  
The authors present a protocol for extracting lipids from reptile skins and then separating fractions of lipids based on differences in polarity. The resulting fractions can then be used in bioassays to determine which fractions are critical for mediating social interactions followed by chemical analyses to identify compounds of interest. In general, the protocol is very clear and easy to follow. Materials are adequately described and alternatives are provided in some cases. While a copy-edit is recommended, there are no major concerns for this manuscript and only a few minor comments are provided.  
  
Major Concerns:  
No major concerns for this manuscript.  
  
Minor Concerns:  
  
Line 110 - Authors state that shed skin mass should be weighed to 0.01 g, however, the authors point out in line 499 that this may not be adequate for subsequent GC-MS analysis. What is the precision of the balance the authors use for this step? Is a different balance with different precision used in subsequent steps? **We added this in the protocol (step 3.2.9).**  
  
Line 174 - Extracts can be stored at -20 C. It would be informative for some readers to mention how long extracts can be stored and still be useful. Some readers may assume that -80 C is necessary if samples will be stored for extended periods. Perhaps the authors can clarify whether this is necessary or not. **In our experience, the lipids are quite stable for years at -20C, and we have never stored them at lower temperatures. Therefore, we would not recommend -80C since we have no experience doing so. We added a note after step 1.2.5 to address this.**   
  
Line 262 - Authors refer to steps that should be repeated, but the numbers are for steps not presented in the protocol. Should read (steps 2.1.2.4 - 2.1.2.8)? **This was a typo and was corrected.**  
  
Line 408 - Meaning of the first sentence beginning on this line not clear. **This was rewritten for clarity.**  
  
  
Reviewer #3:  
  
Manuscript Summary:  
The manuscript by Baedke et al. describes a detailed protocol to extract and fraction lipids from the skin in reptiles. In the manuscript the authors also present some data showing that is possible to obtain lipid fractions separated by their polarity. The extracted lipids can then be used for further analysis or experiments. I find that the protocol is well written and I think that it could be useful for other researchers working on chemical ecology. However, in my opinion, there are some things that could be better explained or clarified. I also have a couple of major issues or questions.  
  
Major Concerns:  
My main major concern is that the authors did not perform any quality control to corroborate what is in the different fractions of lipids. In fact the authors stated in Table 2 that "... GC-MS quality control checks should be run on 6 and 8 to ensure proper elution of the ketones". So if the authors have ran such controls they should state that and also show the results, even in a brief manner just to show how straight is that protocol. **We included a new figure, Figure 3, to show two GC traces of representative positive and negative results.**   
A second thing that needs more detail is how the standardization process was done. In lines 204-207 the authors already mention different ways to do that but I think they do not give enough details. Then they use the proportion of lipids of shed skin mass in Figure 1B (is that the weight of lipids in sample divided by the total weight of the sample?). Please give more details on that and also explain why this method to standardize might be better than others. **We provided more details about the standardization options.**   
  
Minor Concerns:  
  
There are a few questions, comments or suggestions that came to my mind while reading the manuscript:  
  
Lines 32, 55-56: how can wildlife managers benefit of isolating these lipids? It would be good to hear a bit more about that. And I also wonder how sensitive is this method, like for example to recover lipids from the environment? **We elaborated, though briefly, to explain the use of chemical cues in wildlife management. As for sensitive in terms of recovery of cues from the environment, that has never been tested to our knowledge but would be useful to know.**   
  
Line 65: Sometimes the addition of polar solvents like methanol is also used to recover polar lipids **We revised this.**  
  
Line 71: There are also other nonpolar solvents used **We rephrased this to indicate these are only examples.**  
  
Lines 78-80: Maybe it would be good to explain the similarities or advantages respect other techniques of separation like TLC. **We added a sentence comparing these techniques.**   
  
Lines 145-146: I think it is correct and fine to store at -20 C. However for a long term storage I think it could be good to keep the lipids in ultrafreezer (around -70 or -80 C). **We addressed this as note after step 1.2.5.**  
  
Lines 204-207: See major concerns **We clarified this.**  
  
Line 293: Maybe is good to say that other solvents could be used to clean the glassware, especially if the solvent to extract the lipid is also the same. **We modified this.**   
  
Line 370/Table 2: Would be good to know what is in each of the fractions, but for that one needs to do some more chemical analyses (like GC/MS). Even I know the manuscript focuses on the extraction and separation you should say something about how to confirm which kind of lipids are in each fraction and how we can be sure of that (see major concerns). **The goal of the elution protocol is to provide separated fractions that can be used either for compound identification or directly in bioassays to guide discovery of bioactive compounds. Each species will yield slightly or completely different compounds depending on the elution protocol used, but we addressed the methyl ketone isolation specifically with Figure 3.**  
  
Lines 447-445: How did you standarize? (see major concerns) **We addressed this.**  
  
Line 460: But if you consider all the fractions together (except 1-3) they have a higher proportion that the fractions 1-3, and therefore polar lipids (4-12) are then the dominant, or maybe I am missing something here? **We clarified this. Each set of fractions represents a different group of polar compounds, therefore we group the fractions based on common polarity and treat them as separate elutions (not simply polar vs. nonpolar).**   
  
line 484: That might be very interesting to get chemosignals from dead specimens, I wonder how feasible is to look for potential pheromones or chemosignals from dead skin specimens? **The methyl ketones in garter snakes and brown treesnakes can be extracted from shed(=dead) skin and have bioactive properties.**  
  
Lines 506-512: I think the authors would make the protocol much more stronger if they perform the quality controls, as I stated in the major concerns part.  
  
Lines 520-522: This might be an interesting point for researchers working with very small reptiles and that need to pool samples because of the small amount of lipids present. By pooling samples one can get enough amount of lipids for chemical analysis. When the authors say that this might be not optimal can they be more specific on which might be the related problems? **We added to this.**  
  
  
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