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RESPONSE TO LETTER

Response to Heethoff, Norton, and Raspotnig: Ant and Mite Diversity Drives Toxin Variation in the Little Devil Poison Frog and Erratum

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Abstract Our recent publication titled "Ant and Mite Diversity Drives Toxin Variation in the Little Devil Poison Frog" aimed to describe how variation in diet contributes to population differences in toxin profiles of poison frogs. Some poison frogs (Family Dendrobatidae) sequester alkaloid toxins from their arthropod diet, which is composed mainly of ants and mites. Our publication demonstrated that arthropods from the stomach contents of three different frog populations were diverse in both chemistry and species composition. To make progress towards understanding this trophic relationship, our main goal was to identify alkaloids that are found in either ants or mites. With the remaining samples that were not used for chemical analysis, we attempted to identify the arthropods using DNA barcoding of cytochrome oxidase 1 (CO1). The critique of Heethoff, Norton, and Raspotnig refers to the genetic analysis of a small number of mites. Here, we respond to the general argument of the critique as well as other minor issues detailed by Heethoff, Norton, and Raspotnig.

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Our recent publication titled "Ant and Mite Diversity Drives Toxin Variation in the Little Devil Poison Frog" aimed to describe how variation in diet contributes to population differences in toxin profiles of poison frogs. Some poison frogs (Family Dendrobatidae) sequester alkaloid toxins from their arthropod diet, which is composed mainly of ants and mites (Darst et al. 2005). Our publication demonstrated that arthropods from the stomach contents of three different frog populations were diverse in both chemistry and species composition. The specific species of ants and mites that contribute alkaloid toxins to poison frogs is not well understood, although outstanding scientists in the field of poison frog chemical ecology are pursuing this question (Saporito et al. 2004, 2007, 2012). To make progress towards understanding this trophic relationship, our main goal was to identify alkaloids that are found in either ants or mites. With the remaining samples that were not used for chemical analysis, we attempted to identify the arthropods using DNA barcoding of cytochrome oxidase 1 (CO1). The critique of Heethoff, Norton, and Raspotnig refers to the genetic analysis of a small number of mites (Fig. 3 in McGugan et al. 2016). Here, we respond to the general argument of the critique as well as other minor issues detailed by Heethoff, Norton, and Raspotnig.

Using Caution when Interpreting Stomach Content Data

In our analysis of frog stomach contents, we were able to isolate 104 mites from 32 frogs. Most of these mites were used for detection of alkaloids using liquid chromatography/mass spectrometry. The remaining 20 mites were used for genetic analysis and we were only able to obtain CO1 sequence for 9 mites. The main critique by Heethoff, Norton, and Raspotnig is that none of these 9 mite samples are members of mite families known to produce alkaloids (Saporito et al. 2015).

Contrary to the claims by Heethoff et al., we never make the assumption that these 9 mites contain alkaloids, especially *Archegozetes longisetosus*, which is a non-alkaloid mite. The argument by Heethoff et al. is based on the incorrect assumption that every arthropod isolated from poison frog stomach contents contributes alkaloids, which is unlikely. As we stated in our discussion, readers should take "caution against assuming … that every ingested arthropod found in a poison frog stomach contributes to the alkaloid repertoire of the frog." Stomach contents represent a snapshot in time and we attempted to profile every prey item in the hopes of providing this important information for more thorough diet studies in the future. Moreover, our goal was not to link specific toxins to specific arthropod species, but to determine which alkaloids were derived from mites *generally*. Since analyzing the arthropod samples using liquid chromatography/mass spectrometry prevents subsequent use of those samples for CO1 sequencing, we are not making any claims that those mites identified by CO1 sequencing are those from which the alkaloids were sequestered as that direct relationship cannot be demonstrated. Clearly some mites recovered from the frog stomachs do have alkaloid toxins, as we were able to identify two alkaloids common to frogs and pooled mite samples. As we further outline below, the critique and analysis presented by Heethoff et al. is constructed on a shaky foundation, which does not invalidate our results and interpretations presented in McGugan et al. (2016).

Mite Identification by Morphological vs. Molecular Methods Mites are an incredibly diverse taxon/group. As Heethoff et al. point out, over 10,000 species have been described (Schatz 2002; Subías 2004); however, Schatz (2002, 2008) estimates that there are 50,000 to 100,000 mite species globally (Schatz and Behan-Pelletier 2008), suggesting only 10–20 % of mite species have been described. We obtained mites from the stomachs of *Oophaga sylvatica* in northwestern Ecuador. Ecuador has one of the highest number of endemic species per hectare (Brehm et al. 2008), making it one of the most biodiverse areas in the world. Even in the last few years several new mite species have been characterized in Ecuador (Ermilov and Kalúz 2012a, b, c; Ermilov et al. 2013a, b). As mites are relatively unexplored compared to other arthropod taxa, we approached this project with the expectation that many mite samples would be genetically undescribed species.

Our research goal was to determine which poison frog alkaloids were derived from ants or mites generally, and we used DNA barcoding to identify remaining mites that were not included in our chemical analysis. Although mite taxonomy was not the main driving force of our research project, we included tentative identification of a small number of mites to the Oribatid Subfamily. Heethoff, Norton, and Raspotnig make several comments regarding the specimen photographs and descriptions that need to be addressed. Heethoff et al assign family or genus identification based on the photographs of a few mites, which is greatly appreciated, although none was confidently identified to the species level. They also suggest that the lack of traditional morphological identification of mites is "unacceptable". As only a handful of people in the world are able to confidently perform that task, we instead pursued the idea that we could assign a tentative identification to the mites using DNA barcode (CO1) sequencing. As we recovered these arthropods from frog stomachs, many specimens were partially digested, making morphological identification difficult. Furthermore, DNA barcoding can be useful for identifying cryptic species where morphological identification can be misleading (Hebert et al. 2004). As the authors point out, thousands of mite species have been identified, but few have been barcoded and this is a major bottleneck in the field of mite ecology. Most of the mites for whom we successfully obtained CO1 sequence are at least genetically undescribed. We were hesitant to assign a sample to a particular genus given the low percent matches of our mite CO1 sequences to those in the GenBank database as well as our overall low sample size. We merely reported the closest GenBank match, which does not imply the mites belong to that matched genus given the low percent identity. However, our CO1 sequence information was sufficient to identify the specimens as Oribatid mites, which is consistent with the identifications proposed by Heethoff et al. DNA barcoding studies are not a replacement for morphological studies and these methods should be regarded as complementary (Hajibabaei et al. 2007). We agree with Heethoff et al. that CO1 sequences are severely lacking for mites, and we hope that our study, along with necessary future morphological work, will contribute to this need.

Heethoff et al. also commented that some specimen photographs in Figure 3 are mistakes. We double-checked our records and confirmed that we accurately reported the work of undergraduate and high school students involved in this research project. While we cannot rule out cross-contamination or mislabeling, the specimen photographs reflect our records. Furthermore, these are still images of partially digested arthropods, which may make identification by morphometric means more difficult than collections of pristine mites from the leaf litter.

Phylogenic Analysis Our goal with this study was to determine which alkaloids (if any) were derived from mites recovered from poison frog stomachs. Of the remaining mite samples that were not used for chemical analysis, we obtained CO1 sequences for 9 mites and presented a phylogenetic tree containing mite sequences from our study

with representative genera from the closest GenBank matches. Our hope with this data was to add mite CO1 sequences to the sparse mite DNA barcode database. Heethoff et al. present a phylogeny that includes our data with a number of other mite species that do and do not contain alkaloids. The only nodes of high confidence mirror our results in Figure 3, which we highlighted with bold lines. We coded branches below a bootstrap of 70 as dotted lines because no confidence should be put in those nodes. This is a rather non-traditional way of displaying a phylogeny, but we used this visualization so the reader could immediately understand where low and high confidence nodes are located. Given our overall research goal was not mite taxonomy, we consider the phylogeny presented in McGugan et al. to be sufficient. Of course the phylogeny could be improved with increased DNA barcoding information for neotropical mites, and we hope our contribution is one step forward towards progress.

Minor Comments by Heethoff et al. Heethoff et al. made a few other minor comments that do not alter our results or interpretations of data, but still need to be clarified and are corrected in an erratum:

1. The scale bars in Figure 3 and Online Resource Figure 1 are 0.5 mm, not 5 mm. This mistake was only made as a typo in the figure legend; there was no such error in our calculations of mite volume. For example the average mite length and width used in our calculations of volume was (average ± standard deviation) 0.947 ± 0.276 mm and 0.613 ± 0.221 mm, respectively.
2. Heethoff et al. remark that the mite in Online Resource Figure 1a is not an Oribatid mite and that this was somehow in conflict with our report. We did not report on the taxonomic group of this mite because we did not use DNA barcoding methods to identify it; this specimen was instead used for chemical analysis. We used this photograph as an example of mite shape used for volume calculations and it is not connected to the genetic data.
3. The mite phylogeny was generated by Neighbor Joining methods with default options in MEGA 6.
4. We double-checked our mite CO1 data and confirmed all sequences are correct and a BLAST for each one retrieves many mite sequences from the GenBank database. Heethoff et al. found a single typo in Online Resource Table 2 where the closest BLAST hits to each mite sequence are listed. There is an error for one sample where the accession number listed is a bacterium, not a mite, due to a mistake in the accession number (the correct accession number is KF293453, not KM293453). However, this mite sequence BLASTs exclusively to mites, and a simple BLAST of this data retrieves the relevant closest mite matches.

Summary Our primary research goal was to identify which poison frog alkaloids are derived from ants or mites *generally*. We used pooled ants or mites for chemical analysis and identified two poison frog alkaloids that are also found in mites. In this sense, mites do contribute to the alkaloid profile of a poison frog. As we also obtained individual mites from stomach contents that were not included in the mite chemical analysis, we hoped to tentatively identify some of the mites in the frogs' diet to at least the subfamily level and help fill the gap in mite DNA barcoding. We remain confident that DNA barcoding is a reliable way to provide new insights into the trophic ecology of poison frogs and their ant and mite prey. It is our opinion that trophic ecology research communities utilizing morphometric or molecular identification should work together within a collegial, professional, and supportive framework that encourages future scientists to move this field forward.

We would like to thank Heethoff, Norton, and Raspotnig for providing us with an opportunity to clarify misinterpretations of our data on the trophic ecology of poison frogs and correct minor typos in our publication. We would also like to thank John Romeo, the Editor of the Journal of Chemical Ecology, for allowing a response letter.

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