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Roughing It: A Mantellid Poison Frog Shows Greater Alkaloid Diversity in Some Disturbed Habitats[#]

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Four five-skin alkaloid extracts of the Madagascan poison frog *Mantella baroni* from three disturbed collection sites were compared with four five-skin extracts from three undisturbed sites. The number of alkaloids (diversity) was significantly different in *M. baroni* between undisturbed and disturbed collection sites, with more alkaloids generally being found in frogs from disturbed sites. Two undisturbed sites did not differ from two disturbed sites, but the third disturbed site (coded **6**) had more than twice the alkaloid diversity found in frogs from the third undisturbed site (coded **5a/5b**). There was no difference in the quantity of alkaloids in *M. baroni* between undisturbed and disturbed collection sites. The hypothesis that an undisturbed habitat confers a benefit to poison frogs dwelling therein, in allowing for the sequestration of greater alkaloid diversity and amounts, is challenged by our results. In the course of our study, we found that collections of frogs separated by an interval of three months at an undisturbed site differed by only 4% in alkaloid composition over this period, whereas frogs collected at a disturbed site and collected approximately three months later already had a 26% difference in alkaloid composition between the two collections. This constancy of skin alkaloid composition likely reflects a constancy of dietary prey items consumed by frogs at undisturbed sites.

Over 350 alkaloids, mainly of branched-chain structures, have been detected in the skins of small, brightly colored frogs of the family Mantellidae (subfamily Mantellinae) found only in Madagascar.[1−8] Mantellinae is a large subfamily of ca. 100 species, of which only the 17 currently known species of the genus *Mantella* are known to contain skin alkaloids. The alkaloids in mantellids are typically of a molecular weight less than 400 amu and are mainly of the "izidine" classes, e.g., pyrrolizidines, indolizidines, and quinolizidines, the latter two sometimes elaborated with complex side-chains as in the pumiliotoxin and homopumiliotoxin classes, respectively (Figure 1).[1] Many alkaloids show potent activity against various subtypes of nicotinic acetylcholine receptors or interact with Ca^{2+}, K^+, or Na^+ ion channels, functions that are likely responsible for their deterrence of predators, parasites, or microorganisms.[9−11] Since nearly all of these skin alkaloids are sequestered from a diet of small arthropods, mainly mites, ants, beetles, and millipedes,[12] the availability of particular arthropods in a given habitat and its consequent effect on the alkaloid content in frogs has been investigated, directly by analysis of frog stomach contents (cf. ref 12) and indirectly by inferences drawn from the structures of alkaloids present in arthropods and frog skins.[1] One mantellid species, *Mantella baroni*, in particular, has been the subject of several studies of habitat quality and its effect on the number, amount, and type of alkaloids in frog skin. *M. baroni* (Boulenger, 1888)[13] is found in a wide diversity of habitats, ranging from upland riparian forests at higher elevations to dry shrubby hillsides, where it is in temporary residence.[14] Interestingly, this species possesses remarkable genetic uniformity across this large distribution range.[15] The sites of our present study of *M. baroni* in different habitats vary in elevation from 500 to 1300 m and range in distance over 400 km in eastern Madagascar (see Figure 2a and Table 1).

One early study of the skin alkaloids in mantellids among habitats[3] employed quantitation of alkaloids using packed-column gas chromatography with flame ionization detection (FID). This study included *M. baroni* extracts combined from an average of 9 ± 5 skins from six habitats of varying states of disturbance, ranging from relatively undisturbed to more or less disturbed. While notable differences in alkaloid profiles were observed in *M. baroni* by Daly et al.,[3] there appeared to be no discernible relationship between the type, number, or amount of alkaloids detected in these frogs and the disturbance level of the collection sites (habitats). For example, average numbers of 17 ± 4 versus 20 ± 3 alkaloids were reported for the two undisturbed and four disturbed sites, respectively. Since alkaloid amounts were measured in the Daly study[3] using FID rather than mass spectrometric ion currents, the work has provided the most accurate quantitation yet reported, although no internal standards were employed (see gas chromatograms of Figure 8 in ref 3).

In 2006, Clark et al. formulated[7] a hypothesis that stated simply that mantellid frogs from a "pristine" site (i.e., an undisturbed location) will have a greater alkaloid content and variety in their skins relative to frogs from disturbed sites. While habitats of *M. baroni* only were studied by Clark et al.,[7] the authors extrapolated their findings to poison frogs in general. While, *ab initio,* this hypothesis seemed possible and was certainly environmentally attractive, even utilitarian, we could not confirm it in our own work,[8,16] and we challenged[8] the methodology and data handling of the Clark et al. study. We found and reported[3,8,12] in fact that skin extracts of *M. baroni* from a disturbed habitat usually had a similar or often a greater and *not* a smaller number of alkaloids than those from an undisturbed habitat. For example, we observed[8] that extracts of three collections of *M. baroni* (each $n = 2$) from widely separated disturbed habitats (Figure 2b, sites **A**, **B**, **D**) had an average of 25 ± 4 alkaloids, whereas *M. baroni* extracts ($n = 6$; $n = 1$) from two undisturbed habitats (Figure 2b, site **G** and nearby Ranomafana National Park) had an average of 18 ± 1

[#] Dedicated to the late Dr. John W. Daly of NIDDK, NIH, Bethesda, Maryland, for his pioneering work on bioactive natural products.

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[⊥] Deceased March 5, 2008. This paper is dedicated to the memory of John William Daly, who played a major role in unraveling the structures of hundreds of alkaloids in the skins of the poison frogs of Madagascar and had the satisfaction of seeing this project, begun in 1984, mature into one of the most fascinating stories in chemical ecology, but did not live to glimpse the final chapters.
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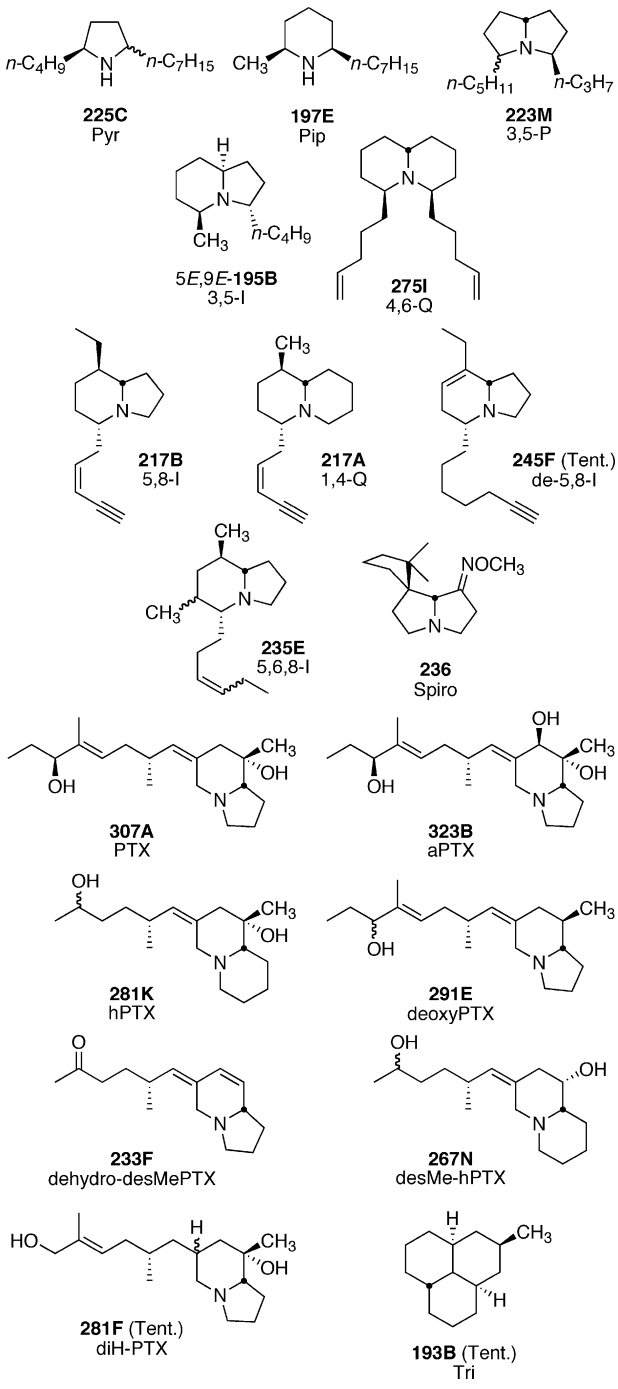

Figure 1. Representative alkaloid structures of each class observed in the current study. Abbreviations (see caption for Table 2) used for each class are given below the structure, which is identified by the code system used in ref 1.

alkaloids. Nevertheless, Clark continued to promote the "habitat hypothesis" [our emphasis] in the popular press[17] with no additional supporting evidence having been published of which we are aware.

In our present study, aimed at more critically addressing the issue of frog-skin alkaloids and habitat quality, we selected six sites where the most widely studied mantellid Madagascan frog, *M. baroni*, is found. Three of the sites were clearly disturbed, either clear-cut, burned over, or in agricultural use, and three of the sites were undisturbed forest (see photos of one disturbed and one undisturbed site in Figure 3). To further investigate the relationship between habitat disturbance and alkaloid content in this frog species, we have specifically examined the difference in alkaloid number (i.e., diversity) and quantity in *M. baroni* between these disturbed and undisturbed habitats. Using GC-MS techniques, including chemical ionization mass spectrometry, we found again that *contrary* to the habitat hypothesis of Clark et al.[7] a disturbed site has at least as many but often a greater number and amount of alkaloids than any of the undisturbed sites we investigated (see Table 2 and Figures 2a, 4). Nearly all of the alkaloid classes detected were of branched-chain, presumably mite-derived, structures (see Figure 1 for specific examples of structural types reported here).

Results and Discussion

At the present time, many of the 350 alkaloids identified in mantellids are similar to, if not identical with, alkaloids from the 20 or so alkaloid classes found in New World dendrobatid poison frogs,[1] although some key differences exist.[16] The structures of these alkaloids can be visualized as being of two principal types: alkaloids with a straight-chain skeletal backbone and those with one or more branch points. The former likely arise from a polyacetylene; the latter from isoprenoid precursors. Among the former are α,α′-dialkyl "izidines" such as 3-butyl-5-methylindolizine (**195B**, Table 2), of which one diastereoisomer (5*E*,9*E*) was one of the first alkaloids detected in an insect,[18] in this case a trail marker component of the ant *Monomorium pharaonis*. All four diastereomers have been detected in frogs, and two have been detected in ants. Among the latter is a large class of 5,8-disubstituted indolizidines, typified by **217B**, a 5-(pent-2-en-4-ynyl)-8-methylindolizidine. The straight-chain alkaloids are considered to derive from ant prey, where nearly every ant alkaloid known is of this type.[19,20] The branched-chain alkaloids, prominent in mantellids, are likely of mite origin.[16,21]

Our 2008 study[8] of mantellid alkaloids, which included single skins of *M. baroni* from two populations, detected 56 alkaloids (including diastereomers) of 12 structural types and three alkaloids of unknown class(es); the majority of the alkaloids were of branched-chain structures. Furthermore, in this 2008 study, there was a similar number of alkaloids (35 vs 33, respectively) in frogs ($n = 4$) from a disturbed habitat (Besariaka) when compared to frogs ($n = 15$) from an undisturbed habitat (Ranomafana National Park; see Figure 2b for both sites). Only 10 alkaloids were shared between frogs from both habitats.

In the present study, a total of 121 alkaloids (including diastereomers) of 20 structural types were detected in *M. baroni*, nearly all of which are known, and approximately 78% (94/121) are of branched-chain structures, which interestingly appear uniformly throughout the eight collections (Table 2). The 108 alkaloids were accompanied by a total of 13 diastereomers (a few of which may be positional isomers) that occur apparently randomly among the sites (Table 2). Among the diastereomers, branched-chain structures represented 77% of the structures.

Over 65% of the alkaloids were found in limited molecular weight ranges that included 217−219 (6 alkaloids), 221−223 (8), 237 (5), 243−249 (18 alkaloids, of which 74% were of branched-chain structures), 251−267 (16), 281 (5), 307 (9), and 323 (4). In the MW range 241−247, seven 5,8-disubstituted indolizidines were found in frogs from disturbed site **6**. Of the 121 alkaloids, we have identified eight dendrobatid alkaloids, including one diastereomer, not previously seen in poison frogs of Madagascar. The new alkaloids are marked with an asterisk in Table 2. Four are of straight-chain structures, whereas the others are of branched-chain or unknown structure.

The "Habitat Hypothesis". In this study, the numbers of alkaloids in *M. baroni* extracts were significantly different between undisturbed and disturbed habitats, albeit marginally ($t = 2.47$; p value $= 0.048$). The amount of alkaloids in *M. baroni* extracts was not significantly different between undisturbed and disturbed habitats ($t = 1.86$; p value $= 0.113$). The largest number of alkaloids was detected in the frog extract ($n = 5$) from the disturbed site Andohan'i Sity An'ala (site **6** in Figures 2a, 4), which contained

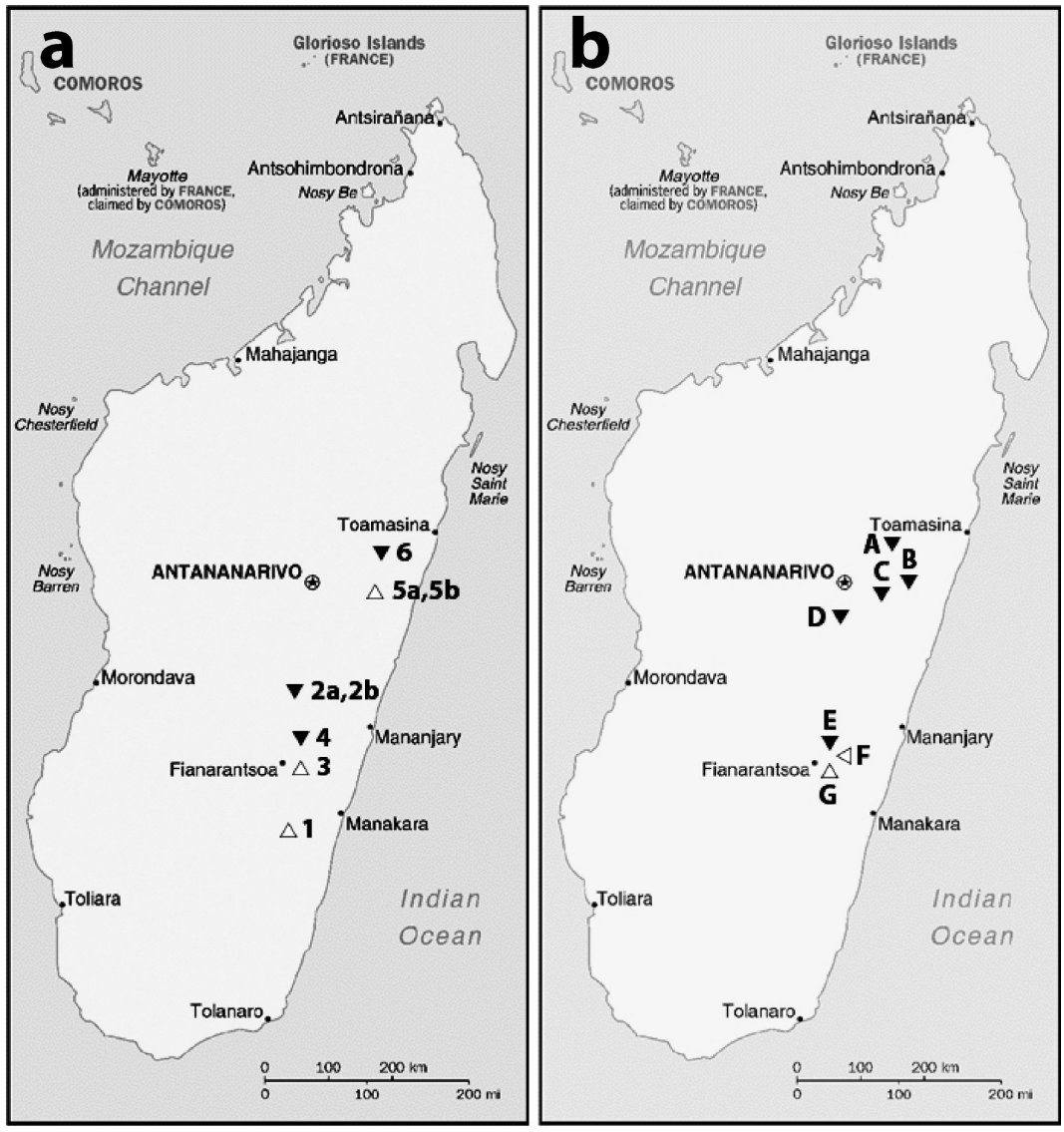

Figure 2. (a) Sites collected for the frog *M. baroni* in the present work. Disturbed sites are indicated by filled triangles and are **2a/2b**, Ampasimpotsy; **4**, Vohiparara; and **6**, Andohan'i Sity An'ala. Undisturbed sites are indicated by open triangles and are **1**, Korokoto; **3**, Mangevo, and **5a/5b**, Fanjavala. (b) Sites collected for *M. baroni* in earlier work. Disturbed sites are indicated by filled triangles and are **A**, Andriabe; **B**, Vohidrazana; **C**, Besariaka; **D**, Tsinjoarivo (all from ref 8); and **E**, Sahavondrona (ref 7). Undisturbed sites, indicated by open triangles, are **F**, Vatoharanana (ref 7); and **G**, Mangevo (ref 8). Sites **E**, **F**, and **G** are all near or within Ranomafana National Park.

Table 1. Collection Data and Site Descriptions

site, collection date(s)	habitat description	elevations and GPS coordinates
Korokoto, Andringitra **1**, March 24, 2004; end of rainy season	Undisturbed forest	848 m 22° 11′ 45.4″ S 47° 1′ 55.3″ E
North Ampasimpotsy, Antoetra **2a**, December 5, 2003 **2b**, February 25, 2004	Disturbed: collected from a cornfield (also with beans) bordering a gallery forest. The site contained ashes from burning off forest (**2a**) and later (**2b**) had higher corn and less ash (see Figure 3 photo)	1332 m 20° 50′ 2.4″ S 47° 19′ 59.5″ E
Mangevo (Menavava River), Ranomafana **3**, December 10, 2003	Relatively undisturbed forest	501 m 21° 23′ 14.8″ S 47° 27′ 22.8″ E
Vohiparara, Ranomafana **4**, March 6, 2004	Disturbed: second growth forest along a relatively large stream	1190 m 21° 15′ 27.5″ S 47° 21′ 41.5″ E
Fanjavala **5a**, January 15, 2004 **5b**, April 15, 2004	Undisturbed forest (see Figure 3 photo)	974 m 19° 4′ 1.1″ S 48° 17′ 41.1″ E
Andohan'i Sity An'ala **6**, January 12, 2004	Disturbed: collected near meandering streams in a clearing surrounded by second growth forest	813 m 18° 55′ 8.5″ S 48° 29′ 15.4″ E

Table 2. Habitat Status and Alkaloid Numbers in Skins of the Madagascan Poison Frog *M. baroni*

		undisturbed collection sites (n = 5 for each)				disturbed collection sites (n = 5 for each)				
alkaloid	structural type (see abbrev[a])	**1** 3−24−04	**3** 12−10−03	**5a** 1−15−04	**5b** 4−15−04	**2a** 12−5−03	**2b** 2−25−04	**4** 3−6−04	**6** 4−24−04	total occurrences including diastereomers
193B	Tri	•								1
195B	3,5-I			•	•					2
195F	3,5-P		•							1
197E*	Pip	•						•		2
203A	5,8-I	•	•			•	•			4
205J	Tri							•		1
209C	5,6,8-I					•	•			2
209K	3,5-P		•							1
211F*	Izidine		•							1
211O	Unclass			•	•					2
217A	1,4-Q	•	•	•	•	•	• (1)	•	• (1)	8 (2)
217B	5,8-I	•	•			•	•	•	•	6
217G	5,6,8-I			•	•					2
219B	1,4-Q	•								1
219L	5,8-I							•		1
219O	Tri							•		1
221F	5,8-I	•								1
221Y	5,8-I		•					•		2
223AA	5,8-I	•								1
223AB	3,5-I	•				•	•			3
223M	3,5-P								•	1
223X	5,6,8-I		• (1)							1 (1)
223Y	Unclass					•				1
225A	Izidine								•	1
225C*	Pyr						•			1
231A	1,4-Q	• (1)	•	•	•	• (1)	• (1)	• (1)	• (1)	8 (5)
233A	1,4-Q	•						•	•	3
233B*	Izidine							•		1
233F	Dehydro-desMe-PTX			•	•					2
235E	5,6,8-I					•	•			2
236	Spiro							•		1
237A	PTX	•	•	•	•					4
237E*	3,5-I	•								1
237P	De5,8-I			•						1
237R	3,5-P	•	•							2
237T	1,4-Q								•	1
239N	Unclass								•	1
241F	5,8-I	• (1)	•	•	•	•			•	6 (1)
243B	5,8-I								•	1
243C	5,8-I		•	•	•				•	4
243G	Tri						•			1
245B	5,8-I								•	1
245C	5,8-I	•								1
245F	De5,8-I			•	•				•	3
245H	De5,8-I		•							1
245J	Tri						•			1
245N	5,8-I								•	1
247E	5,8-I					•			•	2
247F	5,8-I			•	•				•	3
247J	Izidine		•							1
247K	Unclass			•	•					2
249A	3,5-I	•				•	•		•	4
249F	Unclass					•	•			2
249O	5,8-I					•				1
249W	De5,8-I			•	•		•			3
249X	3,5-P							•		1
249Z	Tri								•	1
251D	PTX	•	•	•	•	•	•	•	•	8
251N	5,8-I					•				1
251O	3,5-P		•					•	•	3
251P	De5,8-I								• (1)	1 (1)
251R	hPTX	•						•		2
252A	Spiro							•		1
252B	Spiro							•		1
255B	1,4-Q		•							1
257D	1,4-Q		•			•	•		•	4
259E	1,4-Q								• (1)	1 (1)
261E	Tri					•	•		•	3
265F	De5,8-I			•	•			•		3
265N	hPTX							•		1
265T	De5,8-I					•				1
265Z	Tri	•					•		•	3

Table 2. Continued

		undisturbed collection sites (n = 5 for each)				disturbed collection sites (n = 5 for each)				
alkaloid	structural type (see abbrev[a])	**1** 3−24−04	**3** 12−10−03	**5a** 1−15−04	**5b** 4−15−04	**2a** 12−5−03	**2b** 2−25−04	**4** 3−6−04	**6** 4−24−04	total occurrences including diastereomers
267E	5,8-I					•				1
267N	desMe-hPTX			•	•				•	3
273A	5,6,8-I			•	•			• (1)	•	4 (1)
275C	3,5-I	•				• (1)	• (1)	•		4 (2)
275I*	4,6-Q					• (1)	• (1)			2 (2)
279D	5,8-I								•	1
279F	5,6,8-I		•							1
281A	PTX	•								1
281F	diH PTX							•	•	2
281I	5,8-I								•	1
281K	hPTX							•		1
281N	deoxyPTX							•	•	2
289F	1,4-Q							• (1)		1 (1)
291E	deoxyPTX		•					•	•	3
293D	deoxyPTX		• (1)				•	•	• (1)	4 (2)
293E	PTX	•								1
293 L	1,4-Q							•		1
295C	deoxyPTX						•			1
307A	PTX		•			•			•	3
307B	PTX	•						•		2
307C*	aPTX			•	•					2
307D*	PTX								•	1
307F	PTX	•	•	•	•					4
307F′	PTX								•	1
307F″	PTX								•	1
307G	PTX	•	•	•	•	•	•	•	•	8
307G′	PTX								•	1
309A	PTX	•	•	•	•	•	•	•	•	8
323A	PTX					•	•		•	3
323B	aPTX	•	•	•	•	•	• (1)		•	7 (1)
323E	hPTX	•					•	•	•	4
323G	Unclass				•					1
325A	aPTX	•	•	•	•			•	•	6
325B	PTX					•	•			2
335	hPTX								•	1
337A	hPTX								•	1
total at each site including diastereomers		32 (2)	30 (2)	24 (0)	24 (0)	30 (3)	32(5)	36 (3)	51 (5)	total alkaloids = 121 (13)
major and minor alkaloids		6	8	3	3	13	11	7	5	
major and minor alkaloids: mean ± SD		5 ± 2.4				9 ± 3.7				
percent of total alkaloid numbers represented by diastereomers		6.3%	6.3%	0%	0%	10%	15,6%	8.3%	9.8%	13/121 = 10.7%
percent of diastereomers per site: mean ± SD		3.2 ± 3.6				10.9 ± 3.2				
alkaloid numbers per site: mean ± SD		27.5 ± 4.1				37.3 ± 12.0				

[a] Abbreviations used: Asterisks indicate an alkaloid not previously seen in mantellid frogs. Straight-chain-derived structures (S): Pyr = 2,5-disubstituted pyrrolidine; Pip = 2,6-disubstituted piperidine; 3,5-P = 3,5-disubstituted pyrrolizidine; 3,5-I = 3,5-disubstituted indolizidine; 4,6-Q = 4,6-disubstituted quinolizidine; branched-chain-derived structures (B): 5,8-I = 5,8-disubstituted indolizidine; de-5,8-I = dehydro 5,8-disubstituted indolizidine; 5,6,8-I = 5,6,8-trisubstituted indolizidine; 1,4-Q = 1,4-disubstituted quinolizidine; PTX = pumiliotoxin; hPTX = homopumiliotoxin; aPTX = allopumiliotoxin; Spiro = spiropyrrolizidine; Tri = tricyclic. Unclass and Izidine could be either S or B. Number of diastereomers is in parentheses and also included in the totals at each site; e.g., 32 (2) indicates 32 total alkaloids, including 2 diastereomers.

51 alkaloids. Table 2 and Figure 4 indicate that frogs from Andohan'i Sity An'ala had more skin alkaloids than frogs from any of the undisturbed areas. The same trend holds for the number of major and minor alkaloids (ignoring trace alkaloids). Two of the disturbed habitats (**2a/2b**) had frogs with more major/minor alkaloids (12 average) than frogs from any of the undisturbed areas. In particular, frogs from one undisturbed site (**1**), while having an overall alkaloid number similar to the disturbed sites (**2a/2b**), had only six major/minor alkaloids. The frogs from the two undisturbed sites **5a** and **5b** had only one major and two minor alkaloids each (see Table 2).

Table 3 expresses as a percentage the number of times a given alkaloid occurs in *M. baroni* frogs from just one of the eight collection sites, from two of the sites, three of the sites, etc. Table 4 further refines these data and is stratified by habitat type (disturbed/undisturbed). Remarkably, 54% (65/121) of all the alkaloids were found in frogs from a single site only. This result indicates that, in addition to the difference in the number of frog-skin alkaloids observed between disturbed and undisturbed locations, the specific alkaloids within sites were often different. A similar observation of differences in alkaloid diversity has been reported by Saporito et al.[22] The unique alkaloids of Table 4 were more than twice as likely (45 vs 20; see first entry of Table 4) to be found in frog extracts from one of the four disturbed sites. In fact, it can also be observed that the doubly and triply occurring alkaloids were slightly more likely to be found in frogs from the disturbed sites. The greater number of alkaloids, including diastereomers, in disturbed sites is undoubtedly due to these more infrequently occurring, usually trace alkaloids. The erratic occurrence of trace alkaloids in extracts makes it difficult to assign a defensive role for these alkaloids in the frogs that harbor them. As noted above, however, by ignoring the trace alkaloids, the difference in the number of alkaloids between disturbed and undisturbed sites becomes even more evident.

Figure 3. Photographs showing the disturbed collection site **2a/2b** and the undisturbed collection site **5a/5b** where the frog *M. baroni* was collected three months apart at each site.

Examination of the published data[7] upon which the "habitat hypothesis" of Clark et al. rests reveals that *three single skins only* of *M. baroni* from an undisturbed site were compared with 12 single-skin samples of *M. baroni* from two disturbed sites. The undisturbed site was Vatoharanana, within Ranomafana National Park, while the disturbed sites were Vohiparara (where two sides of a stream were sampled) and nearby Sahavondrona (see Figure 2a, site **4**; Figure 2b, sites **F**, **E**). While the numbers of frogs analyzed from the disturbed sites of the 2006 Clark et al. study[7] do not appreciably differ from our study (12 vs 15, respectively; if our repeated collection from site **2** is omitted), the number of frogs analyzed from their and our undisturbed site collections *do* differ appreciably (3 vs 15 frogs, respectively; even omitting our repeated collection from site **5**).

Table 3. Occurrence of a Given Alkaloid at Sites **1**−**6**[a]

at only one site	54% (65/121)
at only two sites	36% (2 × 22/121)
at only three sites	32% (3 × 13/121)
at only four sites	33% (4 × 10/121)
at only five sites	4% (1 × 5/121)
at only six sites	15% (6 × 3/121)
at only seven sites	6% (7 × 1/121)
at all eight sites	33% (8 × 5/121)

[a] In these tabulations, diastereomers are counted. The number 121 includes 108 alkaloids and 13 diastereomers, the latter occurring randomly.

The analysis of Clark et al.[7] was complicated by their use of a device called a trans-cutaneous amphibian stimulator (TAS) to obtain alkaloids from some of their samples. Clark et al.[7] used the TAS for one-third of the individual *M. baroni* frogs of the 12 total that were collected in the two disturbed "Vohi" and "Saha" habitats. We have argued elsewhere[8] that the TAS procedure gave erroneously incomplete alkaloid profiles from those four frogs, especially in view of our earlier work that showed incomplete removal of alkaloids using the TAS technique. One study by Daly (unpublished data) estimated that only 25% of the total alkaloids were obtained by the TAS method, in comparison with those obtained by the usual protocol: skinning, macerating the cut-up skins, followed by conventional alkaloid fractionation using acid/base extractions. The incomplete removal of alkaloids using the TAS method may result in a reduction of the minor alkaloid amounts to trace level and cause trace alkaloids to disappear altogether in the chemical analysis. This consequence is likely seen in comparing sample #57 in Table 1 of Clark et al.[7] versus the other three single-skin samples they collected from the "Saha" disturbed habitat. They state that samples for which the TAS was used showed *more* alkaloids (yet only two additional trace alkaloids were reported)[7] when compared to the three samples obtained by conventional extract preparation. However, that same TAS extract lacked roughly a dozen alkaloids, most of which occurred in trace amounts and were detected in the

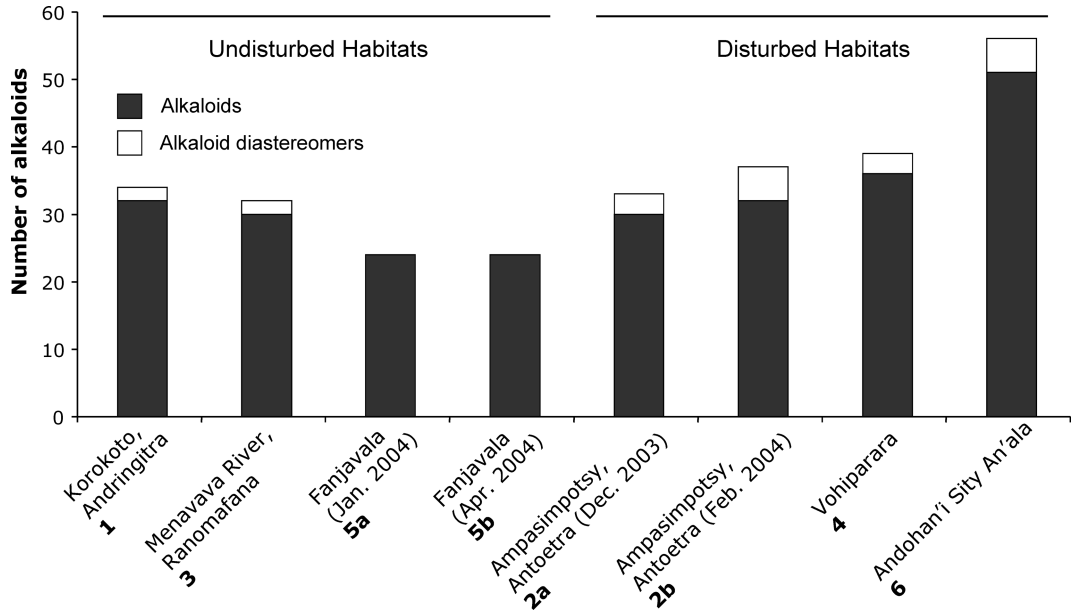

Figure 4. Bar graph indicating numbers of alkaloids and diastereomers found in skins of *M. baroni* collected at sites **1**−**6**.

Table 4. Alkaloid Numbers in Undisturbed and Disturbed Habitats

number of occurrences	undisturbed habitats					disturbed habitats					
	1	**3**	**5a**	**5b**	total	**2a**	**2b**	**4**	**6**	total	%B[a]
one (65 alk.)	9 (1)	9 (1)	1	1	20 (2)	5	4	15 (3)	21 (3)	45 (6)	78
two (44 alk.)	4	2	6	6	18	8 (2)	8 (3)	6	4 (1)	26 (6)	59
three (39 alk.)	3	3	5	5	16	4	5	4	10	23	85
four (40 alk.)	6	6	4	4	20	4	6	4	6	20	80
five (5 alk.)	1	1	1	1	1	1	4	100			
six (18 alk.)	3	3	2	2	10	2	1	2	3	8	100
seven (7 alk.)	1	1	1	1	4	1	1	1	3	100	
eight (40 alk.)	5	5	5	5	20	5	5	5	5	20	100
total diastereomers					2					12	

[a] The percentage of branched-chain alkaloids (%B) in all habitats is obtained from the number of alkaloids of that type (see Figure 1 for branched structural types) divided by occurrences (*n*), e.g., double/2; triple/3, etc. The difference from 100% would be mostly straight-chain alkaloids, presumably ant-derived, although a few are unclassified or unknown izidines. Diasteromers are counted as they occur in one, two, three, etc. sites, so alkaloid **231A**, which occurs in eight sites, but five having diastereomers, is counted separately as eight alkaloids and five alkaloids. Number of diastereomers are in parentheses and also included in the totals at each site; e.g., 32 (2) indicates 32 total alkaloids, including 2 diastereomers.

most alkaloid-rich extract (#84) from the same disturbed habitat. The authors *did not* use the TAS on any of the three frogs from the "Vato" habitat they describe as "pristine". Despite being aware of the incomplete alkaloid removal from frog skin using the TAS procedure, Clark et al. concluded[7] that "future investigations on alkaloid profiles should rely on the non-lethal TAS ...". If the TAS procedure is preferred (e.g., studying endangered/protected species (of which *M. baroni* is not) or for conservation reasons), the investigators should indicate that alkaloids, both in numbers and quantities, are not being fully detected using this method. Clark et al. also used a different extraction method[7] (total immersion of the frog in alcohol and no skinning) for another one of the frogs from a disturbed site. This appears to have resulted in the presence of many fatty acid esters and apparent sterols, along with alkaloids, in the sample, which probably obscured some of the trace alkaloids, resulting in a lower number of alkaloids, and may have erroneously lent support for the "habitat hypothesis". Consequently, interpretation of the results of Clark et al.[7] is complicated because three different isolation procedures were used for the 12 skins from the disturbed habitats, which may have reduced, impaired, or obscured detection of trace alkaloids in five of the samples. In the disturbed "Vohi" area, the three TAS extracts and one whole-frog extract led to the four fewest numbers of alkaloids reported[7] in frogs from that site.

The Daly publication, which included the first habitat study[3] of *M. baroni* with respect to skin alkaloids, was cited by Clark et al.,[7] and two of Daly's extracts reported in that study with their site characterizations were used as reference (!) samples by Clark et al. In addition, these reference samples gave the authors the principal means of identifying the alkaloids they found. Otherwise, the other results of the Daly et al. study[3] were ignored. One of these reference samples, a combined 17-skin extract of *M. baroni* from January 1993 described by Daly et al.[3] as from a disturbed habitat ("Saha"), and the other reference sample, a 10-skin extract from an undisturbed site ("Vato", November 1989), were used by Clark et al.[7] An error is seen in the fact that alkaloid data from a one-skin sample of *M. madagascariensis*, obtained and identified by Clark et al.,[7] were combined unintentionally with the data from four skins collected at the same disturbed "Vohi" site (a site near that of the disturbed site **4** of our present study). The combining of a single skin from another mantellid species, slightly higher in alkaloid numbers than the *M. baroni* from the disturbed "Vohi" site, actually inflated the number of alkaloids found by Clark et al.[7] from that site to 59 (from what should have been 49) and consequently decreased the percentage of alkaloids shared with Daly's 1989 sample from the "Vato" site to 28% (from the correct 35%). Incidentally, note in the data of Clark et al.[7] that Daly's 17-skin extract from the disturbed "Saha" habitat contains three more alkaloids (not a fewer number as predicted by the "habitat hypothesis") than his extract from the undisturbed "Vato" site of 1989. Furthermore, their own collection of 2003[7] indicated that one frog skin from their undisturbed "Vato" site gave the same number of alkaloids (26) as a skin from one of the disturbed "Vohi" sites. These numbers are statistically equivalent, but here the disturbed sites do not have frogs with a substantially smaller number of alkaloids as predicted by Clark et al.

We consider that the "habitat hypothesis" proposed[7] by Clark et al. has a fundamental weakness in that their arguments rely on a comparison of 12 single skins from two disturbed sites, with a small sample of only three single skins from one undisturbed site, where the extracts were also prepared with three different techniques. It is a slender reed upon which to base a hypothesis of such importance for the ecology and conservation of mantellid frogs.

Although limited data are available for population numbers of *M. baroni* in disturbed versus undisturbed habitats, one survey of which we are aware does not indicate any major differences in the number of frogs with habitat quality.[23] In fact, a comparison of frog populations from the undisturbed site **5** compared with the disturbed site **2** indicated a 31% higher density of frogs in the disturbed habitat.

The "Constancy Hypothesis". It is informative to compare the collection pairs **5a/5b** and **2a/2b** (Tables 1, 2) that were made in the same rainy season from undisturbed and disturbed habitats, respectively. As seen in Table 2, the former pair, collected 3 months apart, differed only by the presence of the dehydro 5,8-disubstituted indolizidine, **237P** in collection **5a**, and the unclassified alkaloid, **323G**, in collection **5b**. These two sites had an an overall alkaloid similarity of 96% between sampling periods. The presence of a different unique alkaloid in each of the **5a/5b** collections suggests that the number and type of alkaloids are highly "constant" in frogs from this undisturbed site. Nearly all of the alkaloids in **5a/5b**, with the exception of **209K**, **243G**, **245J**, **265N**, and **279D**, were trace alkaloids (i.e., < 8% total mass spectrometric ion current). Alkaloid **209K**, detected in minor amounts, is a 3,5-disubstituted pyrrolizidine and is likely of ant origin; the other trace alkaloids were all of branched-chain structures and are likely derived from mites. Conversely, the **2a/2b** pair from the disturbed habitat, which was sampled also in two collections separated by ca. 3 months, differed markedly. Site **2a** had eight alkaloids, many of which were of the 5,8-I class that were not found in **2b**, whereas **2b** had eight alkaloids of more varied structural types that did not occur in **2a**. These two sites had an overall alkaloid similarity of 74% between sampling periods. Alternatively, the average percentage of *unshared* alkaloids would be 4% (2/48) in the undisturbed site (**5a/5b**) versus 26% (16/62) in the disturbed sites (**2a/2b**). The general constancy of alkaloid composition over time in an undisturbed environment has been suggested earlier,[7] albeit on the basis of comparisons between a combined 10-skin sample of *M. baroni* collected by Daly et al.[3] in 1989 and composite alkaloid numbers from only three skins collected and analyzed separately by Clark et al. in 2003.[7] This "constancy hypothesis" [our emphasis] is supported by our data

from the undisturbed Fanjavala site (**5a/5b**). Additional support is also found in a collection of *M. baroni* ($n = 6$) from the Mangevo site **3** in January−February 2003[8] (i.e., 2 months later than the collection ($n = 5$) of the present work), where 12/17 (71%) of the alkaloids are shared.

As an extension of the constancy hypothesis, we have also found that alkaloids in the skin of *M. baroni* are more similar among undisturbed sites that are remote from each other when compared to disturbed sites that are, in some cases, nearer to each other. In our present study, we find that the undisturbed habitats, Korokoto (**1**) and Mangevo (**3**), quite distant from one another, still had 42% and 50% of their alkaloids in common with *M. baroni* from the undisturbed Fanjavala site (**5**). Between sites **1** and **3**, 43% of the alkaloids are found in common, only one of which (**237R**) likely is of ant origin.

Complicating this simple picture is the observation that *M. baroni* collected at a *disturbed* site still showed significant constancy in alkaloid compositions. For example we found that a single skin of *M. baroni* collected[8] from a "Vohi" site in January−February of 2003 (rainy season) shared 18 of its 20 alkaloids (90%) with the collection of Clark et al. in March−April (end of rainy season) at the same site. Here a disturbed site sampled roughly within the same time interval as that of our **2a/2b** collections did *not* show a significant difference in alkaloid composition with time, as would have been predicted by the "constancy hypothesis" as we observed for site **2**.

It might be argued that frogs collected from disturbed sites that are in close proximity will likely have more alkaloids in common, and this is suggested by preliminary data, but only in some cases. For example, collections[8] of *M. baroni* from close by disturbed areas such as Andriabe ($n = 2$; Figure 2b, **A**) and Vohidrazana ($n = 2$; Figure 2b, **B**) had 24 and 30 skin alkaloids, respectively, and did share 12 alkaloids with one another and 10 alkaloids with the 24 alkaloids (44%) of the nearby undisturbed Fanjavala (**5**) site. However, *M. baroni* collected from site **5** and the nearby disturbed site **6** shared only 13/51 (25%) skin alkaloids, interestingly all of which were branched-chain structures. Frog-skin alkaloids shared between disturbed sites in our present study range from 22% for **2** and **6** to 31% for **4** and **6**. Such inconsistencies make it difficult at the moment to accept as a general rule that alkaloid profiles from the same species of frog collected at various times at an undisturbed site will resemble one another but those from frogs collected at disturbed sites will not. The "constancy hypothesis" will provide a first approximation only until additional critical variables are identified.

It is possible that the availability of certain alkaloid-containing arthropods is more variable in disturbed habitats, because these habitats can vary from their "time from disturbance" and thus successional stage. As disturbed habitats are expected to shift in species composition (plants, animals, etc.) more frequently than undisturbed habitats, these shifts may translate into changes in arthropod communities. This would result in variable frog alkaloid profiles. Conversely, in undisturbed habitats, a lack of changes in arthropod communities may result in limited dietary variation in frogs, which would be reflected in relatively "constant" frog alkaloid profiles over time. Unfortunately, we have very little data on how alkaloid-containing arthropods vary with habitat and over time. Since the alkaloid types that are found among frogs from the majority of our sites **1**−**6** are overwhelmingly (ca. 78%) branched-chain in structure, which appear to arise largely from mites, we believe that studies on the occurrence, distribution, and availability of alkaloid-containing mites are necessary to better understand the role of habitat on alkaloid profiles in mantellid poison frogs.

Table 5 summarizes the alkaloid structural types (see Figure 1) found in the undisturbed and disturbed habitats. Examination of the results indicates some differences in the appearance of various alkaloid structural types, including diastereomers, with habitats, particularly between five of the branched-chain structural classes, which include 5,8-disubstituted I, 1,4-disubstituted Q, and deoxy-PTX classes, in which frogs from the disturbed sites contain more of these alkaloids, although frogs from the undisturbed sites have more allopumiliotoxins than do those from the disturbed sites (10 vs six, respectively). Other classes that differed in overall numbers of alkaloids between undisturbed and disturbed sites are the tricyclics (two vs 10, respectively) and homopumiliotoxins (two vs eight, respectively). In the numbers of straight-chain structures, believed to be ant-derived, there was little difference in frogs from disturbed and undisturbed sites. For example, in the distribution of 3,5-disubstituted indolizidines, the undisturbed sites have seven versus 10 in the disturbed sites; of the 3,5-disubstituted pyrrolizidines, four versus four are observed, respectively. This result suggests that ants, with their more complex social organization and living arrangements, may be more permanent tenants of a given habitat, whether disturbed or not. It could also suggest that the distribution of certain alkaloid-containing ants may not differ much spatially between disturbed and undisturbed sites or may not differ much temporally before and after a disturbance of the site.

Table 5. Alkaloid Structural Classes in Undisturbed and Disturbed Habitats

alkaloid class	undisturbed habitats				disturbed habitats			
straight-chain classes[a]	**1**	**3**	**5a**	**5b**	**2a**	**2b**	**4**	**6**
Pyr						1		
Pip	1	1						
3,5-P	1	3					2	2
3,5-I	4	1	1	1	4 (1)	4 (1)	1	1
4,6-Q	2 (1)	2 (1)						
total	6	4	1	1	6 (2)	7 (2)	4	3
mean ± SD		3 ± 2.5				5 ± 1.8		
branched-chain[a] classes[a]	**1**	**3**	**5a**	**5b**	**2a**	**2b**	**4**	**6**
5,8-I	7 (1)	5	4	4	7	2	5	10
De5,8-I		1	3	2	1	1	2	
5,6,8-I		3 (1)	2	2	2	2	2 (1)	1
1,4-Q	5 (1)	4	2	2	4 (1)	5 (2)	7 (2)	10 (3)
Tri	2	1	4	2	3			
Spiro							3	
PTX	8	6	6	6	6	5	4	9
DiH-PTX							1	1
a-PTX	2	2	3	3	1	2 (1)	1	2
DeoxyPTX	3 (1)					2	3	4 (1)
Dehydro desMe-PTX			1	1				
h-PTX	2					1	3	4
DesMe-hPTX			1	1				1
total	26 (2)	24 (2)	22	21	22 (1)	24 (3)	31 (3)	47 (4)
mean ± SD		23.3 ± 2.2				31.0 ± 11.3		
Izidine		2					1	1
Unclass			2	4 (1)	2	1		1

[a] See Figure 1 for representational structures and Table 2 footnote for abbreviations.

Conclusion

In the present study, we have provided evidence that casts doubt on the "habitat hypothesis"[7] put forward by Clark and co-workers in 2006, which stated that *M. baroni* frogs (and other poison frogs) from an undisturbed habitat will have a greater number or quantity of skin alkaloids than frogs from a disturbed habitat. On the basis of the present study, the converse appears true, wherein the number and amount of skin alkaloids in *M. baroni* frogs are either similar or increased in clearly disturbed habitats. Another hypothesis put forward by Clark and co-workers, which predicted that frogs sampled over time from an undisturbed area will have more alkaloids in common than frogs sampled from a disturbed habitat, seems consistent with our limited data presented here, but lacks generality. The complexity of variables, such as the availability of alkaloid-containing arthropods, the multiple sources of some of the

frog-skin alkaloids, and the sex and ages of the frogs studied (see Saporito et al. in this issue), makes any conclusions as to ecological drivers responsible for the observed differences in mantellid alkaloid profiles between habitats open to uncertainty. While the "habitat hypothesis" has a certain *a priori* appeal, it is not supported by the present work and other published studies, and appears to be incorrect, at least, for *M. baroni*. At this point, we believe that the habitat hypothesis is too simplistic to provide a reliable prediction on the effect of habitat quality on the chemical ecology of sequestered frog-skin alkaloids.

Experimental Section

Frog Collections. A total of eight five-skin collections of *Mantella baroni* were made from the sites indicated in Table 1 (see also Figure 2a) in Madagascar and, unless indicated otherwise, were taken during the rainy season (December−February), when reproduction takes place. Collection areas were 40 m × 40 m, except along streams, when 80 m × 20 m areas were used. The skins were placed in vials with 3 mL of methanol for storage and transportation.

Voucher specimens are deposited with the Université d'Antananarivo, Département de Biologie Animale, Madagascar.

Frog Alkaloid Extract Preparation. The skins were weighed, cut into small pieces, placed with the original methanol in a small mortar, and triturated twice each time with 3 mL of MeOH. The extract was filtered and concentrated with nitrogen to a final volume of 0.5 mL, to which an equal volume of water was added, and the aqueous methanol was extracted with three 1 mL portions of $CHCl_3$. The chloroform layer was concentrated to ca. 0.5 mL, then diluted to 3 mL with *n*-hexane. The hexane−$CHCl_3$ solution was extracted three times with 1 mL of 0.1 N HCl and the hexane layer discarded. The acid extract was basified with NH_4OH to pH > 8 and extracted three times with 2 mL portions of $CHCl_3$, and the chloroform extracts were combined and dried with anhydrous Na_2SO_4. The dried solution was carefully evaporated with nitrogen to a final volume of 0.5 mL. Since the original five skins weighed ca. 0.5 g, 1 μL of this extract was equivalent to 1 mg of the original wet weight of skins.

Spectrometric Analyses. Mass spectral data [EIMS, CIMS (NH_3), CIMS (ND_3)] were obtained with a Thermo Electron Polaris Q mass spectrometer interfaced with a Thermo Electron Focus GC. The GC was fitted with a Restek RTX-5MS capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness), and a temperature program from 100 °C (1 min) to 280 °C at a rate of 10 °C/min was used. An approximate quantitation of alkaloids was performed using total ion current intensities.

Statistical Analyses. Differences between the number and amount of alkaloids in frogs from undisturbed and disturbed habitats were determined using a *t*-test. In order to meet the assumptions of normality and equality of sample variances, all of our raw data were $\log_{10}$ transformed. All statistical analyses were performed using SPPS version 17.0 for Mac (SPSS, Inc., Chicago, IL).

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