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Spatial and temporal patterns of alkaloid variation in the poison frog *Oophaga pumilio* in Costa Rica and Panama over 30 years ☆

Ralph A. Saporito[a], Maureen A. Donnelly[a], Poonam Jain[b], H. Martin Garraffo[b], Thomas F. Spande[b], John W. Daly[b,*]

[a]*Department of Biological Sciences, Florida International University, Miami, FL, 33199, USA*
[b]*Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, 20892, USA*

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Abstract

A total of 232 alkaloids, representing 21 structural classes were detected in skin extracts from the dendrobatid poison frog *Oophaga pumilio*, collected from 53 different populations from over 30 years of research. The highly toxic pumiliotoxins and allopumiliotoxins, along with 5,8-disubstitiuted and 5,6,8-trisubstituted indolizidines, all of which are proposed to be of dietary mite origin, were common constituents in most extracts. One decahydroquinoline (DHQ), previously shown be of ant origin, occurred in many extracts often as a major alkaloid, while other DHQs occurred rather infrequently. Histrionicotoxins, thought to be of ant origin, did not appear to possess a specific pattern of occurrence among the populations, but when present, were usually found as major components. Certain 3,5-disubstituted pyrrolizidines and indolizidines, known to be of ant origin, did occur in extracts, but infrequently. Alkaloid composition differed with regard to geographic location of frog populations, and for populations that were sampled two or more times during the 30-year period significant changes in alkaloid profiles sometimes occurred. The results of this study indicate that chemical defense in a dendrobatid poison frog is dependent on geographic location and habitat type, which presumably controls the abundance and nature of alkaloid-containing arthropods.

Keywords: Ants; Beetles; Chemical defense; Decahydroquinolines; Dendrobatid frogs; Histrionicotoxins; Indolizidines; Mantellid frogs; Mites; Pumiliotoxins

1. Introduction

Animals that are chemically defended from predation either produce their own defensive compounds or acquire them from external sources. Pathways of chemical acquisition include symbiotic relationships with other chemically defended organisms or dietary sequestration of chemical defenses (Termonia et al., 2001). The ecological and evolutionary implications of dietary sequestration have been well studied among invertebrates, particularly among phytophagous arthropods (e.g., Bowers, 1990; Futuyma and Keese, 1992; Hartmann and Ober, 2000; Nishida, 2002; Fordyce et al., 2005), but

☆ *Ethical statement*: No animal experiments were conducted.

*Corresponding author. NIH, Bldg. 8, Room 1A-17, Bethesda, Maryland 20892-0820, USA. Tel.: +1 301 496 4024; fax: +1 301 402 0008.
E-mail address: jdaly@nih.gov (J.W. Daly).

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remain a relatively understudied phenomenon in vertebrates. Poison frogs are a group of chemically defended vertebrates dependent on diet for their alkaloid-based chemical defense (Daly, 2003), and thus represent a unique vertebrate system in which to study this trophic-based defense strategy.

Alkaloid-containing poison frogs are comprised of some of the dendrobatids from Central and South America, certain bufonids (*Melanophryniscus*) from South America, mantellids (*Mantella*) from Madagascar, and certain myobatrachids (*Pseudophryne*) from Australia (Daly, 2003). Poison frogs are generally brightly colored, which is thought to serve as an aposematic signal to potential predators (Daly and Myers, 1967; Myers and Daly, 1983; Summers and Clough, 2001; Santos et al., 2003; Vences et al., 2003; Darst et al., 2006; Darst and Cummings, 2006; Saporito et al., 2007a). Collectively, more than 800 lipophilic alkaloids, organized into over 20 different structural classes, have been identified from poison frogs (Daly et al., 2005). These alkaloids are stored in skin glands (i.e., granular or poison glands) located mainly on the dorsum (Neuwirth et al., 1979), and on secretion appear to function as a chemical defense against predation (Daly and Myers, 1967; Silverstone, 1975, 1976; Myers and Daly, 1976; Brodie and Tumbarello, 1978; Fritz et al., 1981; Szelistowski, 1985) and possibly ectoparasites (Weldon et al., 2006) and microorganisms (Macfoy et al., 2005). The pumiliotoxin group of alkaloids is highly toxic (Daly and Myers, 1967; Daly et al., 2003), while many of the other alkaloids are much less toxic, but would be noxious because of a bitter taste (Myers and Daly, 1976; Daly et al., 1987).

Alkaloids are not produced by most lineages of poison frogs, but instead appear to be accumulated from a natural diet of alkaloid-containing arthropods, which include certain mites, ants, millipedes, and beetles (Daly et al., 1994a, 2000; Jones et al., 1999; Saporito et al., 2003, 2004, 2007b; Dumbacher et al., 2004; Clark et al., 2005; Takada et al., 2005). Accumulation of alkaloids from the diet has been experimentally demonstrated for certain dendrobatids (*Dendrobates*, *Adelphobates* (formerly *Dendrobates;* Grant et al., 2006), *Phyllobates*, and *Epipedobates*) (Daly et al., 1994a, b; Daly, 2003) and for mantellids (*Mantella*) (Daly et al., 1997), and it is likely that bufonids (*Melanophryniscus*) share a similar ability (Daly et al., 2007). Myobatrachids (*Pseudophryne*) accumulate pumiliotoxin alkaloids through diet, but are also able to produce unique pseudophrynamine alkaloids and appear to be the only known poison frogs capable of synthesizing alkaloids (Smith et al., 2002). The ability to modify alkaloids obtained from diet is known only from certain frogs in the genera *Dendrobates* and *Adelphobates*, which have been shown to efficiently and stereoselectively hydroxylate dietary pumiliotoxin (+)-**251D** to a more toxic allopumiliotoxin (+)-**267A** (Daly et al., 2003), and one frog in the genus *Pseudophryne*, which has been shown to reduce/hydroxylate dietary pumiliotoxin **307A** to a limited extent (Smith et al., 2002).

Studies of variation in alkaloid composition (i.e., the type, number, and amount of alkaloids) within and among species of poison frogs have illustrated marked geographic and temporal differences in composition (Myers and Daly, 1976, 1980; Daly et al., 1978, 1987, 1992, 1994a, 1996, 2002, and unpublished data; Garraffo et al., 1993a, b; Myers et al., 1995; Mebs et al., 2005; Saporito et al., 2006; Clark et al., 2006 and references within). In general, many of these studies were conducted with only a few frog populations/species, over a relatively narrow geographic range, and during short time periods (i.e., months or a few years). The dendrobatid poison frog *Oophaga* (formerly *Dendrobates*; Grant et al., 2006) *pumilio* ranges from the Caribbean lowlands of southern Nicaragua through Costa Rica and into the northwestern portions of Panama (Savage, 2002). In 1967, the extreme variation in alkaloid content and toxicity of extracts of seven populations of *Oophaga pumilio* from Bocas del Toro, Panama was reported (Daly and Myers, 1967). The frogs of that inaugural study are presented in Fig. 1. There was no correlation of toxicity with bright aposematic coloration. Frogs from two populations were highly toxic (see Fig. 1A–C), while frogs from a third population (see Fig. 1D) were about four-fold less toxic. Frogs from the remaining four populations all had low toxicity (see Fig. 1E–H). Alkaloid profiles were later reported for more than 20 populations of *O. pumilio* from Bocas del Toro, Panama (Daly et al., 1987, 2002; Saporito et al., 2006). In the current study, we present more than 30 years of data on alkaloid composition among 53 populations of *O. pumilio*, throughout Costa Rica and Panama. Both small- and large-scale geographic and temporal differences in alkaloid composition of *O. pumilio* from these populations were found. We discuss this variation in alkaloid content in this dendrobatid poison frog with respect to the possible distribution, abundance, and potential targeting by the frog of alkaloid-containing mites, ants, millipedes, and beetles.

2. Methods and materials

2.1. Collection of *Oophaga pumilio*

Adult specimens of *O. pumilio* were collected from Costa Rica and Panama between October 1972 and August 2003. Frogs were collected from a total of 53 different populations, of which 22 were sampled at least twice during the study. The locations of the sites are shown in Figs. 2 and 3. All frogs were sacrificed in the field and skins were stored together in methanol for subsequent alkaloid analyses. Voucher specimens of *O. pumilio* are located at the University of Kansas, Lawrence, KS, the American Museum of Natural History, New York, NY, and Florida International University, Miami, FL.

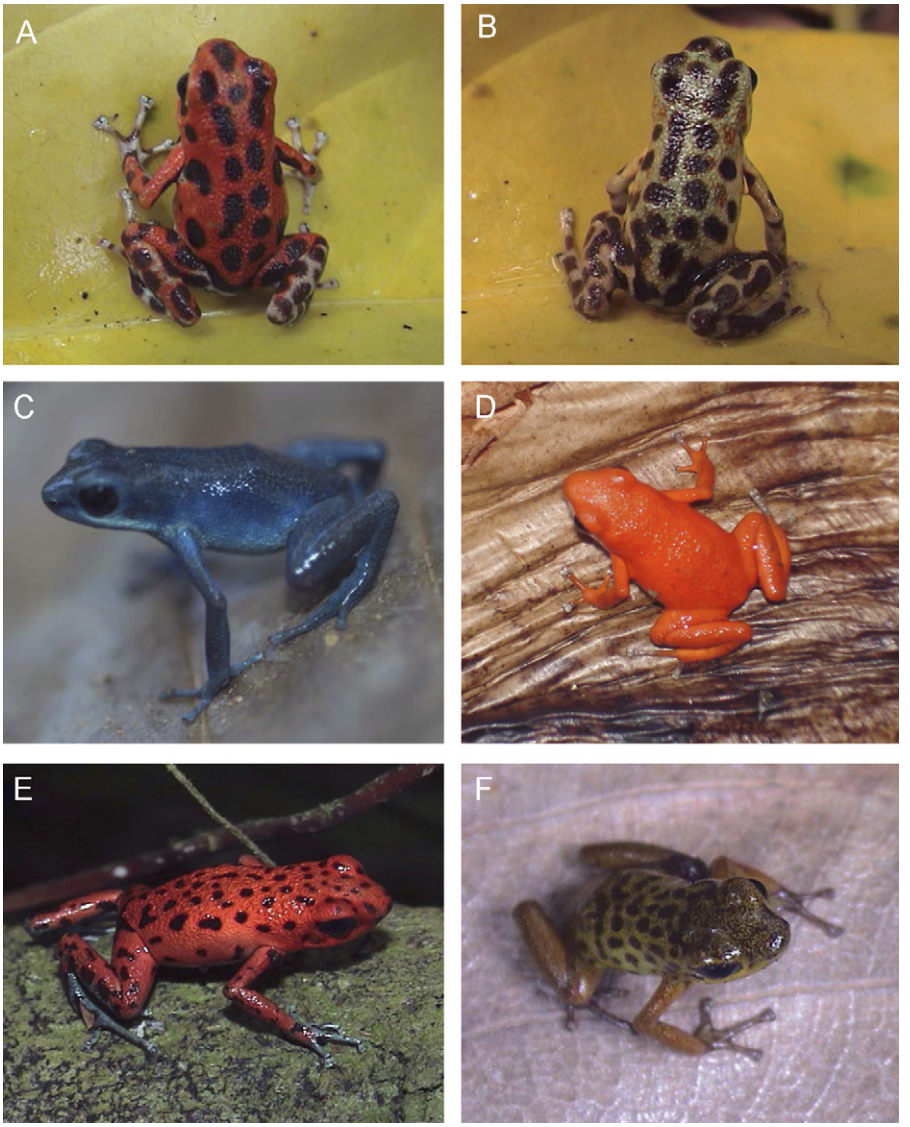


Fig. 1. Poison frogs (*Oophaga pumilio*) of 13 populations from Panamá and Costa Rica. (A and B) Red and green color morphs from Isla Bastimentos (Site 24), (C) Mainland near Isla Split Hill (site 13), (D) Cayo Nancy (site 15), (E) Isla Cristobal (site 35), (F) Isla Pastores (site 36), (G) Almirante (site 37), (H) Isla Colon, Northeast Coast (Site 30), (I) Isla Popa (Site 12), (J) Cayo Agua (Site 10), (K) Isla Escuda (Site 1), (L) Rio Sandbox (Site 42), and (M) Tortuguero (Site 51). See Fig. 2 for site locations and see Supplementary Material for GC-FID traces and for alkaloid profiles in Appendix 1 of the Supplementary Material. The first seven (A–H) are from the Bocas, Panamá populations for which toxicity to mice did not correlate with bright coloration (Daly and Myers, 1967). The last two (L, M) represent Costa Rican populations.

Fig. 1. (*Continued*)

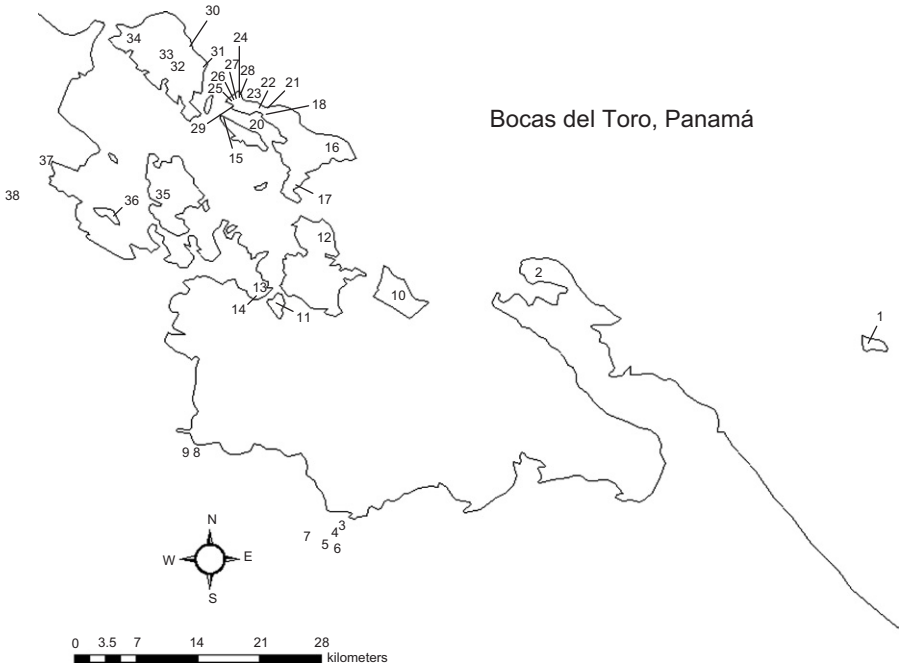


Fig. 2. Map of collection sites for the dendrobatid poison frog *Oophaga pumilio* in Bocas del Toro Panamá. Sites 39 & 40 are located on Fig. 3.

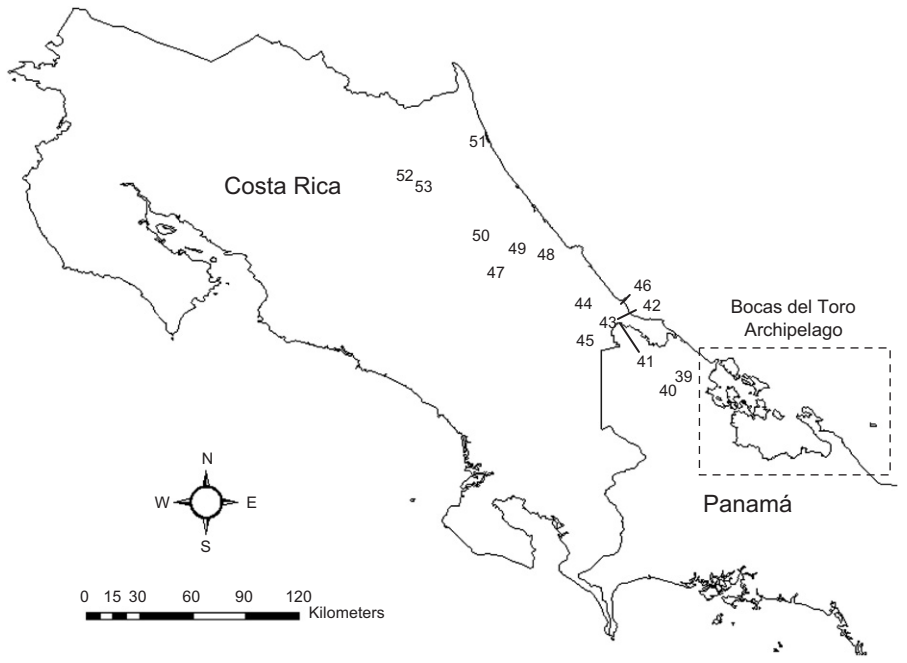


Fig. 3. Map of collection sites for the dendrobatid poison frog *Oophaga pumilio* in Costa Rica and northwest Panamá.

2.2. Alkaloid analysis of Oophaga pumilio

Alkaloid profiles were generated for populations of *O. pumilio* by analysis of alkaloid fractions that were prepared from the methanol extract of each population using the partition methodology described in Daly et al. (1978) and later as it was modified slightly in 1994 (Daly et al., 1994b).

The analyzed collections consisted of 1–66 frogs with most being comprised of 5–10 frogs. Alkaloids within each fraction were characterized using gas chromatography in combination with mass spectrometry (GC–MS) and sometimes vapor-phase Fourier-transformed infrared spectroscopy as described in prior studies (Daly et al., 1992, 2005; Garraffo et al., 1993a, b). Identification of individual alkaloids was based on the comparison of spectral properties, deuterium exchange data, and GC retention times with those of previously described anuran alkaloids (for the most recent tabulation of properties for over 800 anuran alkaloids, see the Supporting Information of Daly et al., 2005). Anuran alkaloids have been assigned a code name, consisting of a bold-faced number corresponding to the nominal mass and a bold-faced letter for identification of individual alkaloids with the same nominal mass (Daly et al., 1987, 2005). During the course of these studies no evidence for transformation products has been found, but there have been instances where a trace alkaloid could no longer be detected on later reanalysis of an extract. The sensitivity of mass spectrometers has increased over the years and thus in recent years more of the trace alkaloids may have been detected.

A semi-quantitative profile of the alkaloids within most fractions was obtained using GC separation with a flame-ionization detector (GC-FID). The GC-FID quantitation has over the years been performed on six-foot 1.5% OV-1 packed columns (2 mm i.d.) as described (Daly et al., 1987). Representative GC-FID traces for most of the alkaloid fractions of *O. pumilio* of the current study are presented in the Supplementary Material. Previously, GC-FID traces have been published for *O. pumilio* (ten skins) from Isla Bastimentos (site 24), October 1972 (Daly et al., 1978), and for skins from one male, one female, and 30 pooled frogs from Rio Sand Box (site 42), June 1990 (Myers et al., 1995). GC-FID traces for other dendrobatid species also have been published (Daly et al., 1978, 1986, 1987, 1992, 1994a, b; Edwards et al., 1988; Myers and Daly, 1979, 1980; Myers et al., 1984, 1995; Tokuyama et al., 1986). The GC-FID traces have been kept comparable from 1972 to date by using a standard extract to normalize responses. In order to estimate the amounts of individual alkaloids in such GC-FID traces, a calibration curve for GC-FID was constructed using measured amounts of the alkaloid decahydroquinoline (DHQ) **195A**. DHQ **195A** is a common alkaloid found in many populations of *O. pumilio*. Each alkaloid within a frog fraction then was estimated to belong to one of the following quantitative categories based on the calibration curve for **195A**: (1) major alkaloid (present in an amount greater than or equal to 50 μg per 100 mg frog skin), (2) minor alkaloid (present in an amount between 5 and 50 μg per 100 mg frog skin), or (3) trace alkaloid (present in an amount less than 5 μg per 100 mg frog skin). The alkaloid quantification scheme allows for comparison of data from the current GC-FID report to that of previous and on-going studies conducted on other alkaloid-containing anurans.

2.3. Statistical analyses

To graphically illustrate variation in alkaloid composition among populations of *O. pumilio*, we used non-metric multidimensional scaling (nMDS). Multidimensional scaling is an ordination technique based on a similarity (or dissimilarity) data matrix among samples (in this case, alkaloid profiles among populations) that is used to create a plot of the relative similarity (or dissimilarity) of these samples. The distance between samples in an nMDS plot is proportional to the similarity of these samples in the original data matrix (i.e., the closer two points are in a plot, the more similar they are to each other). Therefore, in nMDS plots, populations that have greater similarity in alkaloid composition will be plotted closer to each other than populations with very different alkaloid composition. In order to examine spatial variation in alkaloid composition, we used only data from populations that were sampled during the same time period. We examined 16 populations that were sampled from Panama during 1981 and seven populations sampled from Costa Rica between 1988 and 1992. To examine temporal variation in alkaloid composition, only data from populations that were sampled repeatedly through time and in which the initial samples were collected during the same time period were used. When appropriate, we used a one-way analysis of similarity (ANOSIM) to detect statistical differences in alkaloid composition among populations. Both nMDS plots and ANOSIM results are based on Bray–Curtis dissimilarity matrices. To examine the relationship between geographic distance and variation in alkaloid composition we used Mantel Tests. The statistical program PRIMER (version 5) was used to create all nMDS plots and ANOSIM results and the PopTools add-in function in Microsoft

Excel (written by Hood, G.; available at: http://www.cse.csiro.au/poptools) was used for Mantel Tests.

The number of frogs collected at each population during the 30 years covered in the current study ranged from 1 to 66 frogs. To determine if alkaloids vary with the number of frogs collected, we regressed the number and amount of alkaloids for each population with the number of frogs from each of these same populations. To examine the relationship between the number and amount of alkaloids for each population, we used correlation analysis. An estimate of the relative total amount of alkaloids per frog for each population was obtained by summing for each population the area for alkaloid peaks of the GC-FID traces shown in the Supplementary Material. The statistical program SPSS (version 11.5 for Microsoft Windows) was used to perform these statistical analyses.

3. Results

A total of 232 alkaloids, which represent 21 structural classes, were detected and identified in skin extracts of the *O. pumilio* examined in the current study. In addition, there often was more than one isomer of certain alkaloids. The occurrence and amount (major, minor, trace) of alkaloids and of isomers in each extract are presented in tabular form in Appendix 1 of the Supplementary Material. The tabular form is patterned after that used in prior reports on alkaloid profiles from dendrobatid (Daly et al., 1987) and mantellid (Daly et al., 1996) frogs. A summary of all of the alkaloids, listed by structural class, that were identified in extracts of *O. pumilio* is provided in Table 1. It should be noted that in some extracts, alkaloids were detected in trace amounts that were inadequate for definitive characterization and hence are not reported. Structures of many of the most common alkaloids of each structural class are depicted in Fig. 4. The relative amount of alkaloids in extracts is provided by the GC-FID traces depicted in the Supplementary Material.

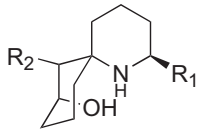
The majority of alkaloids found among populations of *O. pumilio* were 5,8-disubstituted and 5,6,8-trisubstituted indolizidines (5,8-Is and 5,6,8-Is), which together accounted for approximately 25% of all alkaloids. The most common and widespread of these alkaloids were the 5,8-I's **203A**, **205A**, **207A**, and **235B** and the 5,6,8-I's **223A** and **231B**, which were found in more than 25 different populations (Fig. 4). The next most abundant alkaloids belonged to the pumiliotoxin group (which includes the pumiliotoxin (PTX), allopumiliotoxin (aPTX), homopumiliotoxin (hPTX), and deoxy-homopumiliotoxin (d-hPTX) classes) that accounted for 15%, and the DHQs that accounted for 7% of all alkaloids found in *O. pumilio*. The most common and widespread of these alkaloids were the PTXs **251D**, **307A**, and **323A**, the aPTXs **267A** and **323B**, and the DHQs **195A** and **269AB** (Fig. 4). Other relatively widespread alkaloids that were found in more than 20 populations include the histrionicotoxins (HTXs) **283A**, **285A**, and **285C**, the 3,5-disubstituted indolizidine (3,5-I) **195B**, the 3,5-disubstituted pyrrolizidine (3,5-P) **223H**, the piperidine (Pip) **241D**, the spiropyrrolizidine (Spiro) **236**, and the tricyclic (Tri) **205B**. The hPTX **223G**, dehydro-5,8-I **201A**, 4,6-disubstituted quinolizidine (4,6-Q) **195C**, 1,4-Q **257D**, lehmizidine (Lehm) **275A**, and pyrrolidine (Pyr) **197B** were detected in five or more extracts, but represent alkaloids (and their respective classes) that are relatively uncommon in the 53 extracts of *O. pumilio*. The presence of the common alkaloids, depicted in Fig. 4, as major/minor constituents in the 41 extracts from Panama and in the 12 extracts from Costa Rica is presented in Table 2. It is clear that HTXs occurred more commonly in the Costa Rican extracts, while the PTXs and aPTXs occurred more commonly as major/minor constituents in the Panamanian extracts. Trace alkaloids were not considered in this analysis. A proposed dietary origin of each alkaloid class is included in Table 2.

Although 232 alkaloids were identified from *O. pumilio*, many of these alkaloids were detected from a small number of populations and many in only trace amounts. When we focus only on alkaloids that were found in at least five different populations over time, a total of 71 alkaloids, representing 18 structural classes are predominant in *O. pumilio* (Table 3). As expected, the indolizidine alkaloids account for the majority of the common alkaloids (38%), followed by the pumiliotoxin group (17%), the DHQs (10%), and the histrionicotoxins (9%). When analyzing all 232 of the alkaloids, the izidines, other than the common 5,8-I and 5,6,8-I classes, the Pyrs, Pips, tricyclics, and unclassified alkaloids collectively accounted for 39% of all alkaloids. These alkaloids account for 18% of all alkaloids when examining only the alkaloids found in five or more populations.

Table 1
Alkaloids detected in *Oophaga pumilio* arranged by structural class

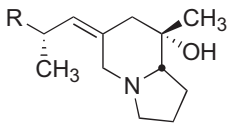
1 HTX	2 PTX	3 aPTX	4 hPTX	5 Deoxy-hPTX	6 DHQ	7 OHQ	8 3,5-P	9 3,5-I	10 5,8-I	11 Dehydro-5,8-I	12 5,6,8-I	13 Lehm	14 4,6-Q	15 1,4-Q	22 CPQ	16 Izidine	17 Pyr	18 Pip	19 Spiro	20 Tri	21 Uncl
283A	209F	225E	223G	193F	181D	193D	195F	167E	167A	201A	195D	275A	195C	207I	247A	155	183B	183A	151B	191B	153B
285A	237A	241H		207O	195A		209K	195B	195I	207E	195G	277A	237I	217A		205F	197B	197E	222	193B	167C
285B	251D	251I		281B	211A		223B	223AB	197C	221J	207C	293F		219B		211B	225C	197F	236	203B	181C
285C	253F	253A			211K		223H	223R	203A	233E	209C			231A		211F	225H	211I	252A	205B	207F
287A	277B	267A			219A		249I	275C	205A	243F	209E			233A		215	235F	211J		205E	207H
287B	281A	293N			219C		251K		207A	269D	219N			257D		219E	253I	225I		205H	207N
291A	289C	305A			219D		251O		209B		223A			263H		223E	279G	237J		207GH	209G
	305B	307C			221B		265J		209I		231B			277H		223I		239L		207K	211G
	307A	309D			223F				217B		235E			279E		225G		239O		207P	211H
	307B	323B			223Q				219F		237C					241E		241D		219I	231F
	307F	325A			231E				223D		237L					265C		241G		221G	235D
	309A	339A			243A				223J		249C					291B		253J		223P	235P
	309C	341A			269A				225D		249H							267K		247H	241C
	"321"				269AB				231C		251M							269C		253S	253N
	323A				269B				233D		251S									261F	263B
	"325"				271D				235B		251T										265R
	325B				275B				237D		253H										267G
					293A				239C		259C										267I
									239D		263A										269G
									243B		265O										271C
									245B		267J										279A
									247E		275E										279I
									249O		277E										281C
									251B		279F										281G
									251U		293C										291I
									253B		353B										305H
									261D												309F
									263F												323I
									271A												339F
									275F												
									279D												
Total 7	17	13	1	3	18	1	8	5	31	6	26	3	2	9	1	12	7	14	4	15	29 232

All alkaloids, even those occurring only once and in trace amounts, are listed. For occurrence of alkaloids in each extract, see Appendix 1 in Supporting Material.Abbreviations for alkaloid classes are as follows: HTX, histrionicotoxin; PTX, pumiliotoxin; aPTX, allopumiliotoxin; hPTX, homopumiliotoxin; Deoxy-hPTX, deoxy-homopumiliotoxin; DHQ, decahydroquinoline; OHQ, octahydroquinoline; 3;5-P, 3;5-disubstituted pyrrolizidine; 3;5-I, 3;5-disubstituted indolizidine; 5,8-I, 5,8-disubstituted indolizidine; Dehydro-5,8,-I, dehydro-5,8-disubstituted indolizidine; 5,6,8-I, 5,6,8-trisubstituted indolizidine; Lehm, lehmizidine; 4,6-Q, 4,6-disubstituted quinolizidine; 1,4-Q, 1,4-disubstituted quinolizidine; CPQ, cyclopentaquinazoline; Pyr, pyrrolidine; Pip, piperidine; Spiro, spiropyrrolizidine; Tri, Tricyclic; and Uncl, unclassified. Two of the probable PTX's are in quotes, since further characterization is needed before documentation as new alkaloids. They occurred only in population 20b.



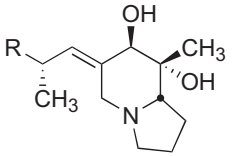
HTX

283A $R_1 = CH_2CH{=}CHC{\equiv}CH$ $R_2 = CH{=}CHC{\equiv}CH$
285A $R_1 = (CH_2)_2CH{=}C{=}CH_2$ $R_2 = CH{=}CHC{\equiv}CH$
285C $R_1 = (CH_2)_3C{\equiv}CH$ $R_2 = CH{=}CHC{\equiv}CH$
291A $R_1 = (CH_2)_3CH{=}CH_2$ $R_2 = (CH_2)_2CH{=}CH_2$



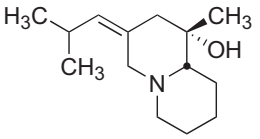
PTX

251D $R = (CH_2)_3CH_3$
307A $R = CH_2CH{=}C(CH_3)CHOHCH_2CH_3$
323A $R = CH_2CH{=}C(CH_3)CHOHCHOHCH_3$



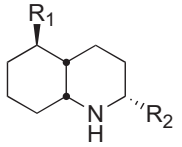
aPTX

267A $R = (CH_2)_3CH_3$
323B $R = CH_2CH{=}C(CH_3)CHOHCH_2CH_3$



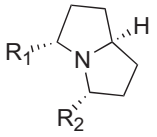
hPTX

223G



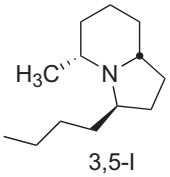
DHQ

195A $R_1 = CH_3$ $R_2 = CH_2CH_2CH_3$
269AB $R_1 = CH_2CH{=}CHC{\equiv}CH$ $R_2 = (CH_2)_3C{\equiv}CH$



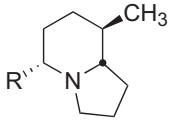
3,5-P

223H $R_1 = CH_3$ $R_2 = (CH_2)_6CH_3$
251K $R_1 = (CH_2)_3CH_3$ $R_2 = (CH_2)_5CH_3$



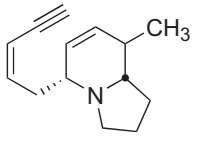
3,5-I

195B



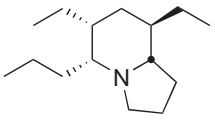
5,8-I

205A $R = (CH_2)_3C{\equiv}CH$
207A $R = (CH_2)_3CH{=}CH_2$
235B" $R = (CH_2)_3CH{=}CHCH_2CH_3$



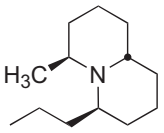
Dehydro-5,8-I

201A



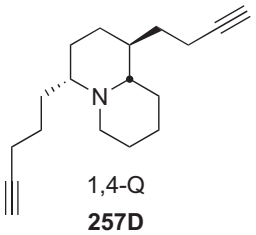
5,6,8-I

223A



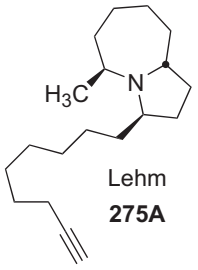
4,6-Q

195C



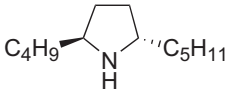
1,4-Q

257D



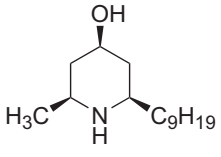
Lehm

275A



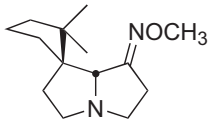
Pyr

197B



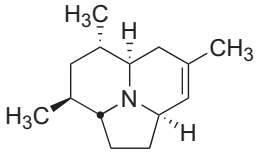
Pip

241D



Spiro

236



Tri

205B

Fig. 4. Structures of the more common alkaloids of 17 structural classes found in extracts of the poison frog *Oophaga pumilio*. In some cases more than one isomer occurs (see Daly et al., 2005), but the identity of the isomer is not certain in many of the extracts. The position of the ring double bond in dehydro-5,8-Is is currently under investigation. For abbreviations of alkaloid classes see the legend of Table 1.

Table 2
Occurrence of alkaloids as major/minor component in skin extracts of *Oophaga pumilio* from each site: 41 sites in Panamá and 12 sites in Costa Rica

Structural class alkaloid	Panama	Costa Rica	Structural class alkaloid	Panama	Costa Rica
HTX (Ant[a])			5,8-I (Mite)		
283A	10	6	205A	23	4
285A	10	6	207A	23	5
285C	10	6	235B	16	3
291A	1	3	Dehydro-5,8-I (Mite)		
PTX (Mite/Ant)			201A	1	1
251D	13	0			
307A	17	1	5,6,8-I (Mite)		
323A	13	2	223A	22	7
aPTX (Frog metabolite/Mite[a])			4,6-Q (Ant)		
267A	21	1	195C	1	0
323B	19	2	1,4-Q (Mite)		
hPTX (Mite)			257D	2	0
223G	1	0	Lehm (Ant[a])		
Deoxy-hPTX (Mite[a])			275A	0	3
193F	1	2	Pyr (Ant)		
DHQ (Ant)			197B	2	4
195A	35	1			
269AB	8	6	Pip (Ant)		
			241D	2	3
3,5-P (Ant)					
223H	9	4	Spiro (Millipede/Mite)		
251K	3	4	236	5	1
3,5-I (Ant)			Tri (Beetle/Mite)		
195B	6	8	205B	7	1

Only the most common alkaloids from each class are tabulated. A probable dietary origin of alkaloids of that class is indicated (see also Table 3). Structures of representative alkaloids of each class are depicted in Fig. 4.

[a]Tentative: A frog alkaloid of this structural class has not been detected in an arthropod. However, a monosubstituted lehmizidine occurs in a myrmicine ant (Jones et al., 2007).

In Table 4, the sites and the number of alkaloids of each class present as major or minor constituents are documented (trace alkaloids are excluded). Alkaloids of presumed dietary mite origin, namely the PTX, aPTX, 5,8-I, 5,6,8-I, and 1,4-Q classes (Saporito et al., 2007b), were predominant in number in about 28 of the 53 extracts. Alkaloids of presumed dietary ant origin, namely the HTX, DHQ, 3,5-P, 3,5-I, 4,6-Q, Lehm, Pyr, and Pip classes (Jones et al., 1999; Daly, 2003), were predominant in number in about 14 of the 53 extracts, mainly due to the presence of HTXs and accompanying DHQ **269AB** and congeners.

Alkaloid composition varied with geographic location and over time among populations of *O. pumilio* examined in the current study (see Table 4 and the GC-FID traces and complete tabulation in Appendix 1 of the Supplementary Material). Alkaloid composition was significantly different among populations in Panama compared to populations in Costa Rica (Fig. 5, Global $R = 0.42$; $P \leqslant 0.001$). Alkaloid composition varied among the 16 populations of frogs sampled from Panama in 1981; however, similarity in composition was not correlated with geographic distance (Fig. 6, $R = 0.01$). Alkaloid composition also varied among the seven populations of frogs sampled from Costa Rica between 1988 and 1992, and was positively correlated with geographic distance (Fig. 7, $R = 0.56$; $P \leqslant 0.01$). Populations of frogs sampled through time also varied in alkaloid composition (see Table 4 and the complete tabulation in Appendix 1 of the Supplementary Material), but the degree of variation through time differed among populations (see nMDS plots of Fig. 8). In some cases, there were great changes with time, while in others the changes with time were minimal (see GC-FID traces in the Supplementary Material).

The average number of alkaloids per population of *O. pumilio* was 17 and ranged from 4 to 37 alkaloids. The number and amount of alkaloids per population was not related to the number of frogs

Table 3
Alkaloids detected in *Oophaga pumilio* arranged by structural class

	1 HTX (A)	2 PTX (M/A)	3 aPTX (M)	4 hPTX (M)	5 DHQ (A)	6 3,5-P (A)	7 3,5-I (A)	8 5,8-I (M)	9 Dehydro-5,8-I (M)	10 5,6,8-I (M)	11 Lehm (A)	12 4,6-Q (A)	13 1,4-Q (M)	14 Pyr (A)	15 Pip (A)	16 Spiro (Mil./M)	17 Tri (M/B)	18 Uncl (M)	
	283A	251D	253A	223G	195A	195F	195B	167A	201A	223A	275A	195C	257D	197B	239L	222	193B	167C	
	285A	277B	267A		211A	223H	275C	203A	221J	231B					241D	236	205B	209G	
	285C	307A	309D		223F	251K		205A	221J	237C					267K	252A	207GH		
	287A	307F	323B		269AB			207A	233E	263A					269C				
	287B	309A	325A		269B			209B		267J									
	291A	323A			275B			209I		275E									
					293A			217B		277E									
								223D		293C									
								223J											
								225D											
								233D											
								235B											
								251B											
								271A											
Total	6	6	5	1	7	3	2	14	3	8	1	1	1	1	4	3	3	2	71

Only alkaloids that were found in five or more populations are included. Probable dietary origin is indicated under each structural class. A = ant; M = mite; Mil = millipede; B = beetle. A probable source is indicated for HTXs, aPTXs, and Lehms, but frog alkaloids of these structural classes have not yet been detected in an arthropod. A monosubstituted lehmizidine occurs in a myrmicine ant (Jones et al., 2007). Abbreviations for alkaloid classes are in the legend to Table 1.

sampled at each population (Fig. 9A and B in the Supplementary Material, $F_{1,81} = 0.537$, $P = 0.466$; $F_{1,81} = 0.079$, $P = 0.780$, respectively), and thus alkaloid composition for populations appeared relatively independent of frog sample size. However, it should be noted that there is variation among individuals within populations of *O. pumilio* (see Saporito et al., 2006). The amount of alkaloids per population was positively correlated to the number of alkaloids per population (Fig. 9C in the Supplementary Material, $F_{1,81} = 114.9$, $P \leqslant 0.001$, $R^2 = 0.581$), and in general, populations with larger quantities of alkaloids tended to have multiple alkaloids rather than larger amounts of only a few alkaloids.

4. Discussion

Over 30 years of research with the dendrobatid poison frog *O. pumilio* throughout Costa Rica and Panama has resulted in the detection of 232 alkaloids that are organized into 21 different structural classes (Table 1). The most common alkaloids (in number, abundance, and geographic distribution) that were detected in skin extracts of *O. pumilio* were the 5,8- and 5,6,8-indolizidines, 1,4-quinolizidines (5,8-Is, 5,6,8-Is, and 1,4-Qs: total number 72), the pumiliotoxin group (PTXs, aPTXs, and hPTXs: total number 35), the DHQs (total number 16), and the histrionicotoxins (HTXs: total number 7). These alkaloids represented more than 55% of the alkaloids found in populations of *O. pumilio*, which suggests that the arthropods containing these compounds are equally abundant and widespread throughout the region. However, alkaloid composition often differed markedly for populations of *O. pumilio* separated by only a few kilometers. This is strikingly illustrated in the GC-FID traces in the Supplementary Material. Clearly, the species of arthropods providing such alkaloids are not uniformly distributed throughout Costa Rica and Panama. Alkaloids of the classes 5,8-I and 5,6,8-I have been identified in oribatid mites, as have 1,4-Qs (Takada et al., 2005; Saporito et al., 2007b). It seems likely that such izidines with branched-carbon skeletons in poison frog skin are derived from dietary mites. Pumiliotoxins also have been identified in oribatid mites (Takada et al., 2005; Saporito et al., 2007b). The PTXs, aPTXs, and hPTXs all have branched-carbon skeletons and it now seems likely that mites are the main dietary source of the pumiliotoxin group. However, it should be noted that a 5,8-I has been reported from an ant of the myrmicine genus *Tetramorium* (Clark

Table 4
Alkaloid classes present as major/minor constituents in populations of *Oophaga pumilio*

Site	Site name	Year	Mite/Ant	Alkaloid class (total number of alkaloids)
Panamanian sites:				
1	Isla Escudo	1987	7/1	PTX (3), aPTX (1), 5,8-I (1), Pip (1)
2a	Punta Valiente	1981	7/1	PTX (2), aPTX (1), DHQ (1), 5,8-I (3), 5,6,8-I (1)
2b	Punta Valiente	1984	6/1	PTX (2), aPTX (1), DHQ (1), 5,8-I (2), 1,4-Q (1)
3	Chiriquí Grande (Red Population)	1981	4/1	aPTX (1), DHQ (1), 5,8-I (1), 5,6,8-I (2), Unclass (1)
4a	Chiriquí Grande (Green Population)	1981	5/0	aPTX (1), 5,8-I (2), 5,6,8-I (2)
4b	Chiriquí Grande (Green Population)	1992	2/1	DHQ (1), 5,6,8-I (2), Unclass (1)
5	Rumpala (Red Population)	1981	2/1	PTX (1), aPTX (1), DHQ (1)
6	Rumpala (Green Population)	1981	1/1	aPTX (1), DHQ (1), Spiro (2)
7	Rumpala (Yellow Population)	1981	6/1	PTX (1), aPTX (2), DHQ (1), 5,8-I (3), 5,6,8-I (1)
8	Rio Gloria	1992	5/8	HTX (4), PTX (1), aPTX (1), DHQ (2), 3,5-P (2), 5,8-I (2), 5,6,8-I (1)
9a	Cerro Miramar	1981	7/5	HTX (3), PTX (1), aPTX (1), DHQ (2), 5,8-I (3), 5,6,8-I (2)
9b	Cerro Miramar	1983	6/6	HTX (3), PTX (1), aPTX (1), DHQ (2), 3,5-P (1), 5,8-I (3), 5,6,8-I (1)
10a	Isla Cayo Agua	1981	7/1	PTX (2), aPTX (2), DHQ (1), 5,8-I (3)
10b	Isla Cayo Agua	1983	5/1	PTX (2), aPTX (1), DHQ (1), 5,8-I (2)
11a	Isla Split Hill	1981	4/4	HTX (3), PTX (2), aPTX (1), DHQ (1), 5,8-I (1)
11b	Isla Split Hill	1983	6/6	HTX (3), PTX (2), aPTX (1), DHQ (2), 3,5-P (1), 5,8-I (1), 5,6,8-I (1), Izidine (1)
11c	Isla Split Hill	1992	5/1	DHQ (1), 5,8-I (3), 1,4-Q (2)
12a	Isla Popa	1981	4/1	PTX (1), aPTX (1), DHQ (1), 5,8-I (1), 1,4-Q (1)
12b	Isla Popa	1983	6/1	PTX (1), aPTX (1), DHQ (1), 5,8-I (2), 5,6,8-I (1), 1,4-Q (1)
13a	Mainland NW Isla Split Hill	1981	6/2	PTX (3), aPTX (1), DHQ (1), 5,8-I (2), Pyr (1)
13b	Mainland NW Isla Split Hill	1983	5/1	PTX (3), aPTX (1), DHQ (1), 5,8-I (2)
13c	Mainland NW Isla Split Hill	1986	5/2	PTX (2), aPTX (1), DHQ (1), 5,8-I (2), Pyr (1)
14	Aguacate	1992	3/6	HTX (4), aPTX(1), DHQ (1), 3,5-P (1), 5,8-I (2), Tri (2)
15a	Cayo Nancy	1981	2/1	aPTX (1), DHQ (1), 5,8-I (1)
15b	Cayo Nancy	1983	1/1	PTX (1), DHQ (1)
16a	Isla Bastimentos, Sol Creek	1987	8/1	PTX (2), aPTX (2), DHQ (1), 5,8-I (4)
16b	Isla Bastimentos, Sol Creek	1992	5/2	PTX (1), DHQ (1), 3,5-P (1), 5,8-I (3), 5,6,8-I (1)
17a	Isla Bastimentos, Macca Bite	1981	5/1	3,5-I (1), 5,8-I (4), 5,6,8-I (1), Tri (1)
17b	Isla Bastimentos, Macca Bite	1992	3/3	3,5-P (2), 3,5-I (1), 5,6,8-I (3), Tri (1)
18	Isla Bastimentos, New Guinea	1986	4/1	PTX (1), aPTX (1), DHQ (1), 5,8-I (1), 1,4-Q (1), Tri (1)
19	Isla Bastimentos, New Guinea	2000	3/3	aPTX (1), DHQ (1), OHQ (1), 3,5-P (1), 5,8-I (1), 5,6,8-I (1)
20a	Isla Bastimentos, Short Cut	2000	4/2	DHQ (1), 3,5-I (1), 5,8-I (2), 5,6,8-I (2)
20b	Isla Bastimentos, Short Cut	2003	12/1	PTX (4), DHQ (1), 5,8-I (5), 5,6,8-I (3), Unclass (1)
20c	Isla Bastimentos, N. Short Cut	2000	4/1	DHQ (1), 5,6,8-I (3), Izidine (1), Tri (2)
21	Isla Bastimentos, Red Frog Beach	2003	4/2	PTX (1), DHQ (1), 5,8-I (2), 5,6,8-I (1), Pip (1)
22	Isla Bastimentos, Wizard Beach	2000	2/1	PTX (1). DHQ (1), 5,8-I (1), Tri (1)
23	Isla Bastimentos, Wizard Beach Trail	2000	0/1	DHQ (1), Tri (1)
24a	Isla Bastimentos, NW Coast	1972	2/1	PTX (2), DHQ (1)
24b	Isla Bastimentos, NW Coast	1980	3/1	PTX (2), DHQ (1), 5,8-I (1), Spiro (2)
24c	Isla Bastimentos, NW Coast	1980	3/1	PTX (2), DHQ (1), 5,8-I (1), Spiro (2)
24d	Isla Bastimentos, NW Coast	1981	4/1	PTX (2), DHQ (1), 5,8-I (2), Spiro (2)
24e	Isla Bastimentos, NW Coast	1987	4/1	PTX (2), DHQ (2), 5,8-I (2), Spiro (2)
24f	Isla Bastimentos, NW Coast	1992	4/1	PTX (2), DHQ (1), 5,8-I (2), Spiro (2)

Table 4 (*continued*)

Site	Site name	Year	Mite/Ant	Alkaloid class (total number of alkaloids)
25a	Isla Bastimentos, NW Coast	2000	6/1	PTX (2), aPTX (1), DHQ (1), 5,8-I (2), 5,6,8-I (1), Spiro (2)
25b	Isla Bastimentos, NW Coast	2003	3/1	PTX (2), DHQ (1), 5,8-I (1), Tri (1)
26a	Isla Bastimentos, NW Coast, Heliconia	2000	7/2	PTX (2), aPTX (1), DHQ (1), 3,5-P (1), 5,8-I (3), 5,6,8-I (1)
26b	Isla Bastimentos, NW Coast, Heliconia	2003	5/1	PTX (2), aPTX (1), DHQ (1), 5,8-I (2), Tri (2)
27a	Isla Bastimentos, NW Coast, Cyclanthus	2000	5/1	PTX (2), aPTX (1), DHQ (1), 5,8-I (2), Spiro (1), Tri (1)
27b	Isla Bastimentos, NW Coast, Cyclanthus	2003	6/2	PTX (2), DHQ (1), 3,5-P (1), 5,8-I (4), Dehydro-5,8-I (1), Spiro (2), Tri (2)
28a	Isla Bastimentos, NW Coast, Faro	1986	5/3	PTX (2), aPTX (1), DHQ (2), 3,5-I (1), 5,8-I (2)
28b	Isla Bastimentos, NW Coast, Faro	2000	7/1	PTX (3), aPTX (1), DHQ (1), 5,8-I (3), 5,6,8-I (1), Tri (1)
28c	Isla Bastimentos, NW Coast, Faro	2003	4/1	DHQ (1), 5,8-I (3), 5,6,8-I (1)
29a	Isla Bastimentos, Punta Juan Brown	1987	10/1	PTX (3), aPTX (3), hPTX (1), DHQ (1), 5,8-I (3)
29b	Isla Bastimentos, Punta Juan Brown	2000	9/1	PTX (2), aPTX (1), DHQ (1), 5,8-I (4), 5,6,8-I (2), Tri (1)
30	Isla Colon, NE Coast	1977	3/4	HTX (3), aPTX (1), 3,5-I (1), 5,8-I (1), 1,4-Q (1), Pip (1), Unclass (1)
31	Isla Colon, Rocky Bluff	2000	2/1	DHQ (1), 5,6,8-I (2), Spiro (1)
32a	Isla Colon, La Gruta	1983	0/4	DHQ (2), Pip (2)
32b	Isla Colon, La Gruta	1986	2/1	3,5-I (1), 5,8-I (2)
32c	Isla Colon, La Gruta	1992	2/5	HTX (3), DHQ (1), 5,8-I (2), Pyr (1), Unclass (1)
33	Isla Colon, N. La Gruta	1992	5/3	PTX (2), aPTX (2), DHQ (1), 3,5-P (1), 3,5-I (1), 5,8-I (1)
34	Isla Colon, Boca del Drago	1986	6/2	PTX (1), aPTX (2), DHQ (1), 5,8-I (2), 5,6,8-I (1), Pip (1), Unclass (2)
35a	Isla Cristobal	1981	5/1	PTX (1), aPTX (1), 3,5-I (1), 5,8-I (1), 5,6,8-I (2), Unclass (1)
35b	Isla Cristobal	1983	6/1	PTX (1), aPTX (1), DHQ (1), 5,8-I (1), 5,6,8-I (3)
36a	Isla Pastores	1981	4/4	HTX (3), PTX (1), aPTX (1), DHQ (1), 5,6,8-I (2)
36b	Isla Pastores	1983	5/2	HTX (1), DHQ (1), 5,8-I (2), 5,6,8-I (3)
37	Almirante	1983	3/2	PTX (1), aPTX (1), DHQ (1), 3,5-P (1), 5,6,8-I (1)
38	Ojo de Agua	1983	5/6	HTX (3), PTX (1), DHQ (2), 3,5-I (1), 5,8-I (3), 5,6,8-I (1), Tri (1)
39	Dos Bocas	1980	7/4	HTX (3), PTX (1), aPTX (2), DHQ (1), 5,8-I (1), 5,6,8-I (2), 1,4-Q (1)
40	Quebrada El Guabo	1980	6/5	HTX (3), PTX (2), aPTX (1), 5,8-I (1), 5,6,8-I (2), 4,6-Q (1), Pip (1)
41	Cerro de Pierda Grande	1989	5/2	Deoxy-hPTX (2), DHQ (1), 3,5-P (1), 5,8-I (1), 5,6,8-I (2)
Costa Rican sites:				
42a	Río Sand Box	1989	2/4	HTX (3), DHQ (1), 5,8-I (2)
42b	Río Sand Box	1990	5/11	HTX (4), DHQ (2), 3,5-P (1), 3,5-I (2), 5,6,8-I (5), Lehm (1), Pyr (1), Tri (2)
43	W Bribrí	1990	4/5	HTX (3), aPTX (1), 3,5-P (1), 3,5-I (1), 5,8-I (2), 5,6,8-I (1), Tri (1)
44	Carbón	1990	5/7	HTX (3), Deoxy-hPTX (1), DHQ (1), 3,5-P (1), 3,5-I (1), 5,6,8-I (4), Lehm (1), Tri (1)
45	Amubri	1995	10/4	aPTX (1), 3,5-P (1), 3,5-I (1), 5,8-I (6), Dehydro-5,8-I (1), 5,6,8-I (2), Pyr (1), Pip (1)
46	Cahuita	1995	6/8	HTX (3), aPTX (1), Deoxy-hPTX (1), DHQ (1), 5,8-I (3), 5,6,8-I (1), Lehm (2), Pyr (1), Pip (1)
47	Río Chitaría	1990	3/12	HTX (3), DHQ (5), 3,5-P (2), 3,5-I (1), 5,6,8-I (3), Pyr (1)
48a	28 Millas	1990	7/2	

Table 4 (*continued*)

Site	Site name	Year	Mite/Ant	Alkaloid class (total number of alkaloids)
				aPTX (2), 3,5-I (1), 5,8-I (1), Dehydro-5,8-I (1), 5,6,8-I (3), Pyr (1)
48b	28 Millas	1995	7/6	aPTX (1), 3,5-P (2), 3,5-I (1), 5,8-I (3), 5,6,8-I (3), Pyr (1), Pip (2)
49	Siquierres	1995	7/2	PTX (3), aPTX (1), DHQ (1), 3,5-I (1), 5,8-I (3), Tri (1)
50	Pocora	1995	5/10	HTX (4), PTX (1), aPTX (1), DHQ (2), 3,5-P (2), 3,5-I (1), 5,8-I (2), 5,6,8-I (1), Pip (1), Spiro (2)
51	Tortuguero	1988	3/1	aPTX (1), 5,8-I (2), Pip (1)
52	Near La Selva	1984	1/1	HTX (1), 5,8-I (1)
53	Río Sarapiqui	1989	2/3	HTX (1), 3,5-I (1), 5,8-I (2), Pyr (1)

Data are from tabulation in Appendix 1 of the Supplementary Material. A ratio of putative mite-derived to ant-derived alkaloids is indicated. Abbreviations are as in the legend to Table 1. For temporal changes in alkaloids for a certain site, see GC-FID traces in Supplementary Material.

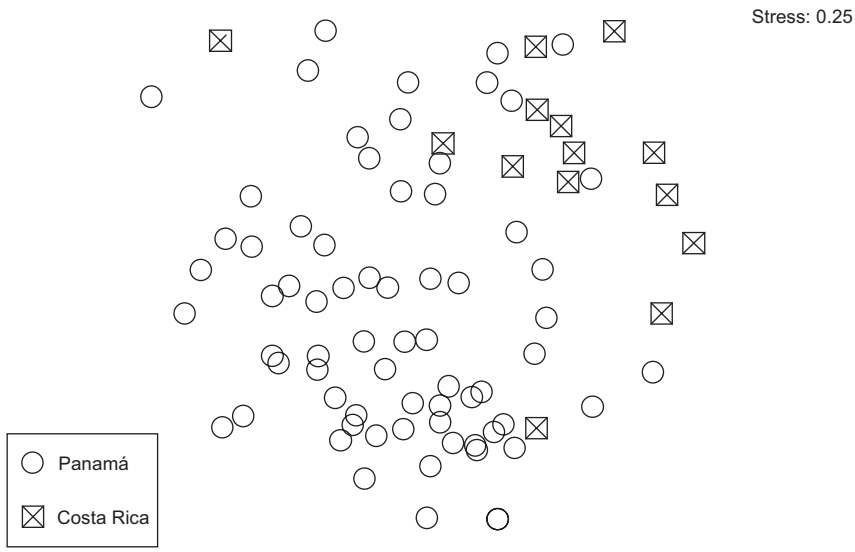


Fig. 5. nMDS plot of alkaloid composition among populations of *Oophaga pumilio* between Costa Rica and Panamá. Each symbol represents an individual population. The distance between symbols represents the difference in alkaloid composition.

et al., 2005), and two PTXs were detected from some but not all collections of two species of formicine ants (Saporito et al., 2004).

A number of the alkaloids found in poison frog skin have been presumed to originate from dietary myrmicine ants, based in part on the detection of members of the alkaloid class in such ants (Jones et al., 1999; Spande et al., 1999; Daly et al., 2002). These include the DHQs, the 3,5-Ps, the 3,5-Is, the 4,6-Qs, the Pyrs, and the Pips, all of which have linear, unbranched-carbon skeletons. The histrionicotoxins and the lehmizidines, both with unbranched-carbon skeletons, are also considered (Daly, 2003) as likely to originate from dietary myrmicine ants, but have as yet not been detected in arthropods. Feeding of leaf-litter arthropods to the dendrobatid poison frog *Dendrobates auratus* did result in accumulation of histrionicotoxins into the frog's skin (Daly et al., 1994a). A monosubstituted lehmizidine now has been found in an ant of the myrmicine genus *Myrmicaria* (Jones et al., 2007). It would appear that the vast majority of alkaloids present in skin extracts of *O. pumilio* are of oribatid mite and myrmicine ant origin, both of which are

extremely common and widespread throughout the tropics. The fact that most of the skin alkaloids appear to be of mite and ant origin is also consistent with the natural diet of *O. pumilio*, which is composed mainly of mites and ants (Donnelly, 1991; Saporito et al., unpublished data). The spiropyrrolizidines, such as spiropyrrolizidine **236**, are of millipede origin (Saporito et al., 2003) and the tricyclic alkaloids, such as **205B**, are thought to be of beetle origin, based on the occurrence of certain such tricycles in coccinellid beetles (Daloze et al., 1995). However, recently the spiropyrrolizidine **236** and the so-called coccinellid beetle alkaloid precoccinelline were detected in oribatid mites (Takada et al., 2005; Saporito et al., 2007b).

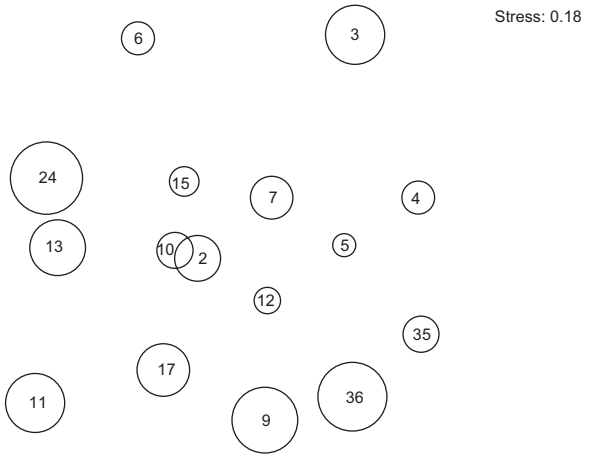


Fig. 6. nMDS plot of alkaloid composition among 16 populations of *Oophaga pumilio* in Panama sampled during 1981. Each circle represents an individual population with the number of that site in the circle. For site locations see Figs. 2 and 3. The size of each circle is proportional to the number of alkaloids in that population. The number of alkaloids is correlated to the amount of alkaloids ($R2 = 0.58$). The distance between symbols reflects the difference in alkaloid composition.

The PTXs and/or aPTXs occurred in almost all of the extracts. PTX **251D** and aPTX **267A** (C16 alkaloids) occurred together as major or minor alkaloids in 20 extracts. However, only aPTX **267A** was detected in 10 extracts. In only one extract was PTX **251D** detected alone. It is likely that dietary PTX **251D** can be hydroxylated by *O. pumilio* to form aPTX **267A**, as has been reported for three other species of dendrobatid frogs (Daly et al., 2003). The hydroxylation increases the toxicity of this pumiliotoxin by five-fold. At present, the extent to which aPTX **267A** comes directly from a dietary source in *O. pumilio* or is formed by hydroxylation of dietary PTX **251D** remains an unanswered question (see Daly et al., 2003). As yet, an aPTX has not been detected in an arthropod. The relatively common PTXs **307A** and **323A** (C19 alkaloids) were found together in 28 of the 66

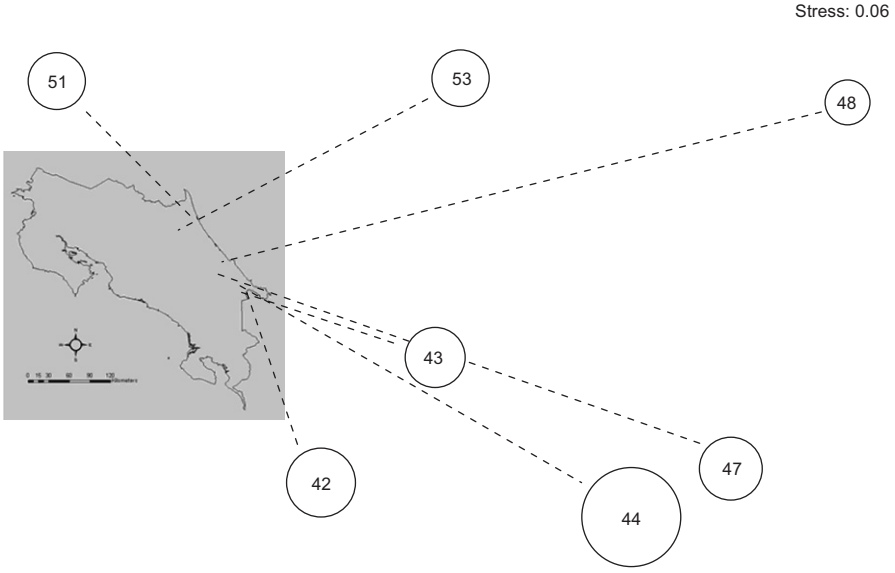


Fig. 7. nMDS plot of alkaloid composition among seven populations of *Oophaga pumilio* in Costa Rica sampled between 1988 and 1992. Each circle represents an individual population with the number of that site in the circle. The dotted lines serve to indicate the site locations (see also Fig. 3 for site locations). The size of each circle is proportional to the number of alkaloids in that population. The number of alkaloids is correlated to the amount of alkaloids ($R2 = 0.58$). The distance between symbols reflects the difference in alkaloid composition.

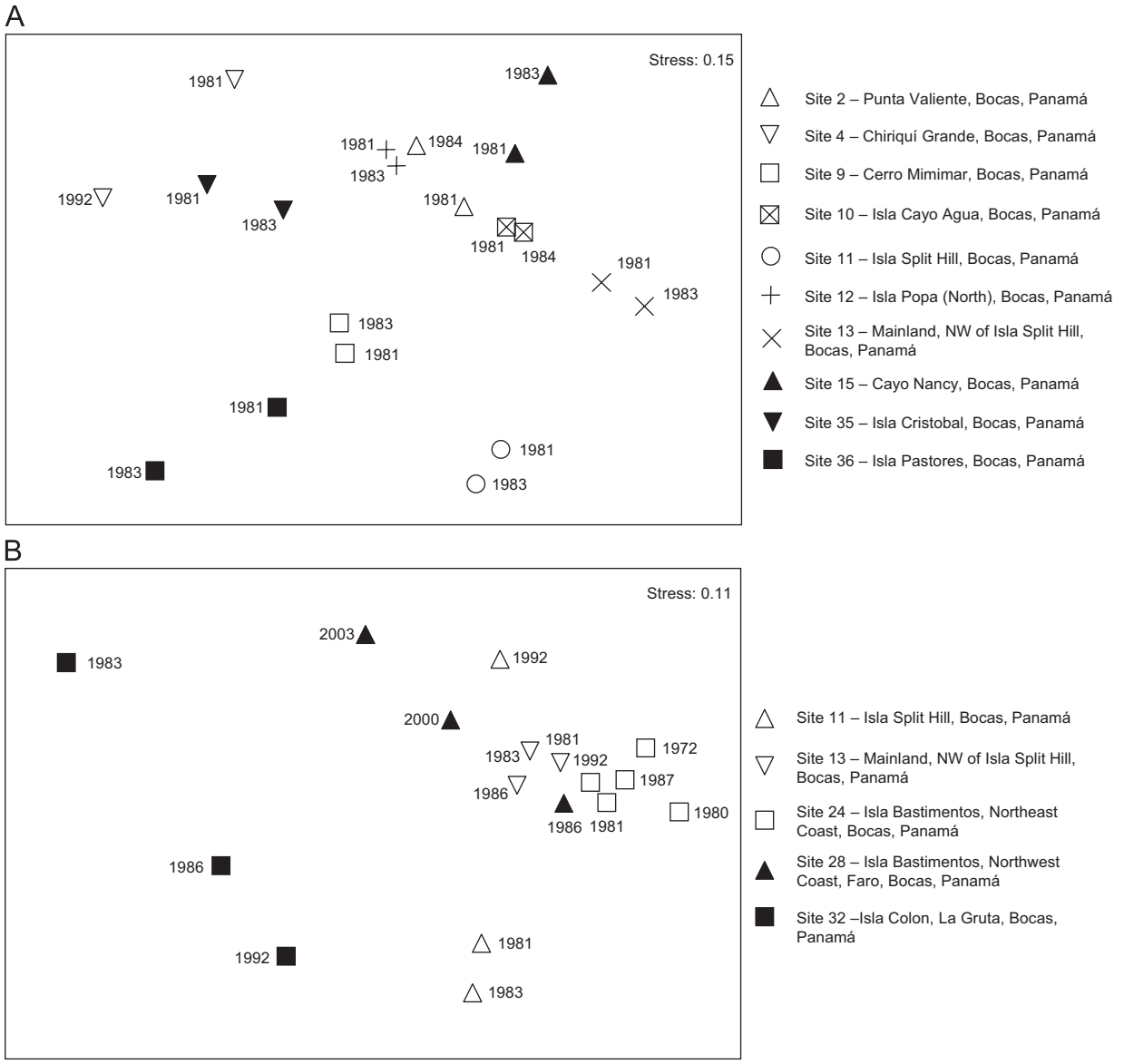


Fig. 8. nMDS plot of alkaloid composition within populations of *Oophaga pumilio*. (A) Sampled over two time periods in Panamá. (B) Sampled over three time periods in Panamá. Each symbol represents a site sampled at a different point in time. Site numbers and names correspond to map in Fig. 2 and Appendix 1 of the Supplementary Material. The distance between symbols reflects the difference in alkaloid composition.

extracts. PTX **307A** was detected without **323A** in nine extracts, while the converse was true in only four extracts. In many, but not all extracts, both C19 and C16 PTXs were present. PTX **237A**, **251D**, **307A**, and **307F** have been detected in oribatid mites (Takada et al., 2005; Saporito et al., 2007b), while PTX **307A** and **323A** have been detected in formicine ants (Saporito et al., 2003).

In many of the extracts where the alkaloid profile seemed dominated by putative mite alkaloids, the only ant alkaloid present (in other than trace amounts) was the DHQ **195A**. DHQ **195A** (a C-13 alkaloid) occurred as a major or minor alkaloid in 61 of the 69 extracts from Panamanian sites. It was much less common in extracts from the Costa Rican sites, where it occurred in only five of the 14. The other relatively common DHQ, DHQ **269AB** (a C-19 alkaloid), occurred in 13 extracts from Panama and Costa Rica, but only in extracts where C-19 HTXs also were present. DHQ **269AB** has been

detected in a Brazilian myrmicine ant (currently unidentified) along with closely related congeners (T.F. Spande et al., unpublished results; see also Spande et al. (1999) for related C-19 DHQs present in a Puerto Rican myrmicine ant). Although HTXs occur frequently as major alkaloids in dendrobatid poison frogs (Daly et al., 1987), the arthropod source as yet has not been detected (see Daly et al., 1994b). It should be noted that HTXs have only been reported from dendrobatid poison frogs collected in the Neotropics. In most cases, either a group of HTXs (**283A**, **285A**, **285C** and minor congeners) all with highly unsaturated side chains, or only one HTX (**291A**) with terminal double bonds in the side chains is detected (see Daly et al. (1987) and GC-FID traces and Appendix 1 in Supplementary Material).

4.1. General patterns of alkaloid composition in *Oophaga pumilio*

Alkaloid composition differs among populations of *O. pumilio* and varies with geographic location and over time throughout Costa Rica and Panama (Table 4). Although geo-political borders are usually not biologically meaningful, variation in alkaloid composition between populations in Costa Rica and Panama clearly illustrates the large-scale differences in composition between geographic locations (Fig. 5). On a smaller geographic scale, alkaloid composition among populations within Costa Rica and Panama is also variable (Figs. 6 and 7). In Costa Rica, variation is related to geographic distance, and populations that are closer to each other have more similar compositions than populations that are more distant. In Panama, variation was not related to geographic distance; however, this is likely due to differences in spatial scale and geographic arrangement of populations in Panama as compared to Costa Rica. The majority of populations examined in Costa Rica were further apart from each other and arranged in a longitudinal manner, whereas in Panama, populations were not arranged longitudinally and were spatially closer together. Alkaloid compositions for a population do not remain the same over time, and populations that were sampled at different time periods exhibited temporal changes in composition. Interestingly, changes in composition appear independent of the amount of time between sampling, where some populations change noticeably in composition and others remain relatively similar over the same time period (see Fig. 8).

Variation in alkaloid composition among populations has been reported for other dendrobatids (Myers and Daly, 1976, 1980; Daly et al., 1987, 1992, 1994a, 1996 and unpublished data; Myers et al., 1995) as well as for poison frogs of the mantellid genus *Mantella* (Garraffo et al., 1993a; Daly et al., 1996; Clark et al., 2005, 2006), the bufonid genus *Melanophryniscus* (Garraffo et al., 1993b; Mebs et al., 2005; Daly et al., 2007) and the myobatrachid genus *Pseudophryne* (Daly et al., 1990; Smith et al., 2002). In some of these studies, alkaloid composition has been shown to be related to geographic distance. For instance, geographically close populations of *Oophaga histrionica* and *O. sylvatica* (formerly *Dendrobates histrionicus*; see Grant et al., 2006) have more alkaloids in common with each other than that of more distant populations (Myers and Daly, 1976). Introduced populations of *D. auratus* in Hawaii have alkaloid compositions more similar with each other than those of their ancestral population from Panama (Daly et al., 1992). Although on a smaller spatial scale, Saporito et al. (2006) demonstrated that alkaloid composition is related to geographic distance among certain populations of *O. pumilio* on Isla Bastimentos, Panama. The relationship between geographic distance and alkaloid composition is further illustrated between different species of poison frogs. For example, sympatric populations of *O. pumilio* and *Oophaga* (formerly *Dendrobates*; Grant et al., 2006) *granulifera* share more alkaloids in common with each other than when geographically distant populations of *O. granulifera* are compared (Myers et al., 1995). Alkaloid composition has also been shown to change with time among different species of dendrobatids, including possible seasonal changes in composition among populations of *O. pumilio* from the Bocas del Toro region of Panama (Daly et al., 2002; Saporito et al., 2006). We suggest that variation in forest dynamics may play a significant role in alkaloid variation, wherein secondary forest regeneration drives differences in arthropod communities that in turn affect frog alkaloid profiles.

Given the large amount of spatial and temporal variation in alkaloid composition observed in the present study with *O. pumilio* as well as the high degree of variation within and among other species of poison frogs, it is of interest to ask the question: why is there so much variation in alkaloid composition? Alkaloid composition in *O. pumilio*

(as well as all other poison frogs) is due to the uptake and storage of alkaloids from a diet of alkaloid-containing arthropods. Arthropod abundances are known to vary spatially and temporally in tropical regions, due to a variety of factors which include (but are not limited to) forest and vegetation type, forest succession, frequency and type of disturbance, and season (Janzen and Schoener, 1968; Janzen, 1973; Janzen et al., 1976; Toft and Levings, 1977; Lieberman and Dock, 1982; Levings and Windsor, 1982, 1984; Levings, 1983). Variation in the abundance of alkaloid-containing arthropods may lead to differences in the spatial and temporal availability of these arthropods to poison frogs. These differences in availability may cause the majority of observed variation in alkaloid composition of *O. pumilio* and other poison frogs. There is also some evidence of alkaloid variation within the same species of arthropods (Deslippe and Guo, 2000; Torres et al., 2001; Saporito et al., 2004, 2007b), which might play an additional role in chemical defense variation of poison frogs.

Although difference in availability of alkaloid-containing arthropods is likely the most important factor in explaining variation in alkaloid composition of poison frogs, including *O. pumilio*, there are other factors that may also be important. Although it does not appear to be a widespread phenomenon, certain poison frogs possess an ability to modify specific dietary alkaloids (Smith et al., 2002; Daly et al., 2003). This ability is undoubtedly an enzyme-mediated process and thus contains a genetic component. It follows that a differential ability to modify certain alkaloids may influence variability in alkaloid composition. A difference in the ability to modify alkaloids probably is not of major importance within a species, but may be more important in explaining variation among species. The ability to take up and sequester certain alkaloids and not others (Daly et al., 1994b) is certainly under genetic control and therefore differences within and among poison frog species in such systems may also contribute to variation. Differences in diet between sexes and ages have been reported for *O. pumilio* (Donnelly, 1991), and therefore some of the variation in composition may be due to differences in feeding behavior between sexes (see also Saporito et al., 2006). It is also possible that prey 'electivity' differs within and among species of poison frogs. Finally, alkaloid composition is the direct result of alkaloid accumulation over time. Therefore, differences in composition should also be expected among individuals of different ages, and therefore variation may be partly dependent on the age make-up and history of a certain population and of individuals in that population.

4.2. Variation in dendrobatid chemical defenses—palatability spectra?

Variation in chemical defense is common among organisms that are dependent on sequestering defenses from dietary sources. For example, phytophagous arthropods are well known for their ability to sequester chemical defenses from plant sources (i.e., host plants), and can exhibit geographic, temporal, sexual, and age-related variation in both the amount and type of chemical defenses they possess (e.g., Boppre, 1990; Hartmann and Witte, 1995; Klitzke and Trigo, 2000; Hartmann and Ober, 2000; Fordyce et al., 2005). In general, such variation in chemical defenses of arthropods is attributed to geographic and temporal variation in host plant chemistry, shifts in the usage and availability of host plants (geographically and temporally), and differences in sequestration ability between sexes and ages (Bernays and Chapman, 1994; Bowers and Williams, 1995; Moranz and Brower, 1998; Fordyce et al., 2005). Whether such an explanation applies to the trophic relationships of alkaloid-containing arthropods and alkaloid-sequestering anurans will require further study. We suggest that an understanding of the remarkable variation in poison frog chemical defenses must be linked to an understanding of the arthropods that harbor these chemicals.

The large variation in chemical defense observed throughout the natural geographic range of *O. pumilio* may have important implications with regard to predator–prey interactions. In certain species of phytophagous arthropods, chemical variation can result in 'palatability spectra', in which individual arthropods of the same species differ in their palatability to potential predators (Brower et al., 1967, 1968; Bowers, 1980). Palatability spectra can lead to behavioral differences of predators towards prey and influence the survival of prey (Bowers, 1980; Fink and Brower, 1981; Moranz and Brower, 1998). Whether dendrobatid frogs exhibit palatability spectra is currently unknown, but alkaloid variation within populations of dendrobatid frogs is common (including in *O. pumilio*; see Saporito et al., 2006), and differences in

alkaloid composition could translate into differences in palatability.

Differences in amounts (and probably nature) of alkaloids were reported to correspond to differences in 'toxicity' among populations of *O. pumiilo* from Bocas, Panama (Daly and Myers, 1967). Toxicity, measured on injection in mice, correlated with high levels of pumiliotoxin A (**307A**) and pumiliotoxin B (**323A**). Pumiliotoxins and allopumiliotoxins are highly toxic (Daly and Myers, 1967; Daly et al., 1978, 2003), while DHQs and histrionicotoxins are much less toxic (Daly et al., 1978). Recently, 'toxicity' of skin extracts from three species of dendrobatid frogs was assayed by observing a prolongation of wakefulness in mice after injection of alkaloid extracts (Darst and Cummings, 2006; Darst et al., 2006). Further studies are needed to verify the reproducibility of such a 'toxicity' assay. In addition, the time course for 1-month-old domestic chickens to learn not to attack dendrobatid frogs was used to determine the level of 'toxicity' of three different species (Darst et al., 2006). Handling of poison frogs with high levels of pumiliotoxins more often than not results in unpleasant burning and irritation to buccal and nasal tissue (Hagman, 2006; J.W.D., personal observations). The unpleasant nature of secretions from dendrobatid frogs, eliciting bitter, burning sensations, has been noted by Silverstone (1975), and Myers and Daly (1976) mention using a taste test for assessing the noxiousness of a frog in the field. Such effects, and those reported by Silverstone (1975), would likely relate to a palatability spectrum to natural predators. At present there is far too little information regarding the identity of natural predators, the effectiveness of different classes of alkaloids in defending against these predators, and the ability of these predators to detect differences in chemical defense. Clearly, there are many aspects of the trophic-based chemical defense in dendrobatid frogs that will represent challenges for further research.

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Appendix A. Supplementary Material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxicon.2007.06.022.

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