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RESEARCH PAPER

Variation in alkaloid-based microbial defenses of the dendrobatid poison frog *Oophaga pumilio*

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Abstract Conspicuously colored dendrobatid frogs possess alkaloid-based antipredator defenses that are sequestered from a diet of arthropods. The type and quantity of alkaloids in dendrobatids vary substantially with geographic location, mainly due to differences in arthropod availability. It has been experimentally demonstrated that some individual alkaloids inhibit the growth of certain microbes, and that different alkaloids vary in their antimicrobial efficacy. We further tested this hypothesis by examining the antimicrobial effectiveness of naturally occurring mixtures of alkaloids (i.e., alkaloid cocktails) isolated from the dendrobatid frog *Oophaga pumilio* from five different locations in Costa Rica and Panama. Alkaloid cocktails in frogs from these locations varied significantly in their alkaloid composition. Bacterial cultures of *Escherichia coli* and *Bacillus subtilis*, and the fungus *Candida albicans* were subjected to alkaloid cocktails from individual frogs. These antimicrobial susceptibility tests demonstrated significant inhibition of bacterial and fungal growth of cultures incubated with these alkaloids, suggesting that the mixture of alkaloids present naturally in *O. pumilio* has the potential to defend frogs against natural microbes. Furthermore, there were significant differences in the degree of microbial inhibition among alkaloid cocktails, suggesting that frogs from different locations vary in their defense against microbes.

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Introduction

Chemical defenses are widespread among amphibians and represent complex defensive adaptations aimed at protection from predators, parasites, and/or microbes (Toledo and Jared 1995; Rivas et al. 2009; Conlon 2011a, b; Savitzky et al. 2012). Amphibian skin secretions contain a diversity of defensive chemicals that include biogenic amines of the serotonin-, tryptamine-, histamine-, and tyramine-classes (Daly et al. 1987; Erspamer 1994; McClean et al. 2002), numerous peptides and proteins (Conlon 2011a, b; Rollins-Smith et al. 2005), steroidal bufadienolides (Erspamer 1994; Daly et al. 2004), tetrodotoxins (Cardall et al. 2004; Daly 2004), a saxitoxin analog (Yotsu-Yamashita et al. 2004), indolic alkaloids of the pseudophrynamine class (Smith et al. 2002), and a variety of lipophilic alkaloids (Daly et al. 2005; Saporito et al. 2012). Amphibians appear to synthesize most of these defensive chemicals, but certain peptides are derived from symbiotic bacteria (Harris et al. 2009; Becker and Harris 2010; Loudon et al. 2014) and lipophilic alkaloids are sequestered from dietary arthropods (Saporito et al. 2009, 2012). The dependence of some amphibians on an exogenous and potentially variable environmental source for their chemical defenses may have significant implications in their protection from predators and pathogens.

Approximately 150 species of anurans collectively known as poison frogs sequester alkaloids from their diet. This diverse group of frogs is divided among eight lineages in five families, and includes members of Bufonidae (*Melanophryniscus*), Dendrobatidae (independently derived

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in *Epipedobates*, *Ameerega*, and Dendrobatinae), Eleutherodactylidae (*Eleutherodactylus*), Mantellidae (*Mantella*), and Myobatrachidae (*Pseudophryne*) (for review, see Saporito et al. 2012). Feeding experiments with most lineages have revealed that alkaloids are accumulated into dermal skin glands by consuming alkaloid-containing arthropods, predominantly brachypiline mites and myrmicine ants (for review, see Saporito et al. 2009; Hantak et al. 2013). Poison frog alkaloids are generally considered unpalatable or noxious to various invertebrate and vertebrate predators (Brodie and Tumbarello 1978; Fritz et al. 1981; Szelistowski 1985; Gray et al. 2010; Weldon et al. 2013; Stynoski et al. 2014), but recent research suggests that these same alkaloids may also function in protection against parasites and/or microbes (MacFoy et al. 2005; Weldon et al. 2006; Grant et al. 2012). The pumiliotoxin alkaloid **251D** is an effective defense against the ectoparasitic mosquito *Aedes aegypti* (Weldon et al. 2006). A diversity of alkaloids was recently identified in muscle and liver of the bufonid poison frog *Melanophryniscus simplex*, suggesting a possible role in protection against internal parasites (Grant et al. 2012). Certain individual alkaloids have been found to inhibit the growth of the bacteria *Bacillus subtilis* and *Escherichia coli*, and the fungus *Candida albicans* (Macfoy et al. 2005).

Collectively, more than 850 alkaloids (organized into ca. 24 structural classes) have been identified in skin secretions of poison frogs (Daly et al. 2005; Garraffo et al. 2012; Saporito et al. 2012), with individual frogs containing between one and more than 40 different alkaloids (see Daly et al. 1987; Saporito et al. 2006, 2007). MacFoy et al. (2005) examined the antimicrobial activity for several individual alkaloids of the pyrrolidine, piperidine, decahydroquinoline, indolizidine, histrionicotoxin, and pumiliotoxin classes. Most of the individual alkaloids were synthetic enantiomers related to common poison frog alkaloids, but a few represented naturally occurring frog alkaloids (see Fig. 1 in MacFoy et al. 2005). Using the paper disc method and measurements of growth zone inhibition, individual alkaloids were generally found to be most effective against the gram-positive bacterium *B. subtilis* and the fungus *C. albicans*, and were least effective against the gram-negative bacterium *E. coli* (MacFoy et al. 2005). Alkaloid structural class was an important determinant of microbial inhibition, with certain pyrrolidine, piperidine, and pumiliotoxin alkaloids being most inhibitory (MacFoy et al. 2005). Although these individual frog alkaloids appear to provide defense against microbes, nothing is known about the effectiveness of alkaloid combinations (i.e., mixtures of individual alkaloids, hereafter referred to as alkaloid cocktails) that are naturally found in wild-caught poison frogs. Research on antimicrobial peptides in other frog species has found that natural combinations are more effective against microbes than are individual peptides (Rollins-Smith et al. 2002a, b; Rosenfeld et al. 2006). It has been hypothesized that peptide combinations allow for protection against a wide diversity of microorganisms and/or there may be synergistic effects between individual peptides that increase their overall effectiveness (Rollins-Smith et al. 2005; Rosenfeld et al. 2006; Conlon 2011b), and it is possible that the same is true for poison frog alkaloids.

Alkaloid defenses are extremely variable among poison frogs, and different species, individuals, and populations can exhibit marked differences in the composition (type and quantity) of alkaloids present in their skin (e.g., Clark et al. 2006; Saporito et al. 2007; Garraffo et al. 2012). These differences in alkaloids are generally associated with geographic location, and frogs that are found close together typically share more similar alkaloids when compared to frogs that are geographically distant (e.g., Saporito et al. 2006, 2007; Daly et al. 2008; Grant et al. 2012). Although numerous factors are likely involved in explaining alkaloid variation (e.g., differences in age, sex, species, etc.), changes in the local availability of alkaloid-containing arthropods appear to be largely responsible for geographic differences in alkaloid defenses (for review, see Saporito et al. 2009, 2012). Given that individual alkaloids differ in their effectiveness against certain microbes (MacFoy et al. 2005), it is possible that environmentally driven alkaloid variation leads to differences in microbial defense among poison frogs.

The dendrobatid frog *Oophaga pumilio* represents a well-studied poison frog with particularly variable alkaloid defenses (Daly et al. 1987; Saporito et al. 2006, 2007, 2010), making it one of the foremost candidates to examine the link between alkaloid variation and microbial inhibition. The geographic range of *O. pumilio* extends from southern Nicaragua through the Caribbean Slope of Costa Rica and into the northwest region of Panama (Savage 2002), and frogs from different locations are known to vary considerably in their alkaloid defenses (Daly et al. 1987; Saporito et al. 2006, 2007, 2010). The present study aims to examine differences in the antimicrobial properties of alkaloid cocktails extracted from geographically isolated populations of *O. pumilio* to evaluate the impact of naturally occurring alkaloid variation on microbial growth of the bacteria *E. coli* and *B. subtilis* and the fungus *C. albicans*.

Methods and materials

Frog collections

A total of 15 adult *O. pumilio* were collected from five different locations in Costa Rica and Panama from June–

August of 2005 and 2006. Three individual frogs were obtained from each of the following locations: (1) Tortuguero, Limon, Costa Rica (1 male and 2 females); (2) La Selva Biological Station, Heredia, Costa Rica (2 males and 1 female); (3) Puerto Viejo de Talamanca, Limon, Costa Rica (2 males and 1 female); (4) Isla Solarte, Bocas del Toro, Panama (1 male and 2 females); and (5) Isla San Cristobal, Bocas del Toro, Panama (2 males and 1 female; Fig. 1). All frogs were collected within a single 45 × 45 m plot, measured for snout-to-vent length, euthanized by freezing at −20 °C, and skins were removed. Frog skins were individually stored in glass vials with 4 mL of 100 % methanol and sealed with Teflon lined caps (hereafter, referred to as a methanol extract).

Alkaloid extraction and fractionation

Alkaloids were extracted from frog skins using the procedure outlined in Saporito et al. (2010), which is described here briefly. One milliliter of each methanol extract was transferred into a 10-mL conical vial and 50 μL 1 N hydrochloric acid was added. Each sample was then mixed and evaporated with nitrogen gas to a volume of 100 μL. Subsequently, each sample was diluted with 200 μL of deionized water. The samples were then extracted with four 300 μL portions of hexane. The resulting hexane (organic) layer was disposed of and the remaining aqueous layer was basified with saturated sodium bicarbonate. Basicity was tested with pH paper. Once basic, each sample was extracted with three 300 μL portions of ethyl acetate. Anhydrous sodium sulfate was added to the newly extracted mixture to remove any excess water. The remaining samples were carefully evaporated to dryness with nitrogen gas. Alkaloid fractions were resuspended in 100 μL methanol and stored at −20 °C.

Alkaloid analysis

Gas chromatography–mass spectrometry (GC–MS) was performed for each individual frog on a Varian Saturn 2100T ion trap MS instrument, which was coupled to a Varian 3900 GC with a 30 m × 0.25 mm i.d. Varian Factor Four VF-5 ms fused silica column. GC separation of alkaloids was achieved using a temperature program from 100 to 280 °C at a rate of 10 °C per minute with helium as the carrier gas (1 mL/min). Each alkaloid fraction was analyzed with both electron impact MS and chemical ionization (CI) MS with methanol as the CI reagent.

Individual alkaloids of *O. pumilio* were identified based on comparison of mass spectrometry properties and GC retention times with those of previously reported alkaloids in dendrobatid frogs (Daly et al. 2005; Saporito et al. 2007). Alkaloids in dendrobatid frogs have been assigned a series of code names that consists of a boldfaced number indicating the alkaloids' nominal mass, and a boldfaced letter to distinguish those alkaloids with the same nominal mass (Daly et al. 2005). The relative quantity of alkaloids present in *O. pumilio* was determined by comparing the observed areas of individual alkaloid peaks using a Varian MS Workstation v.6.9 SPI.

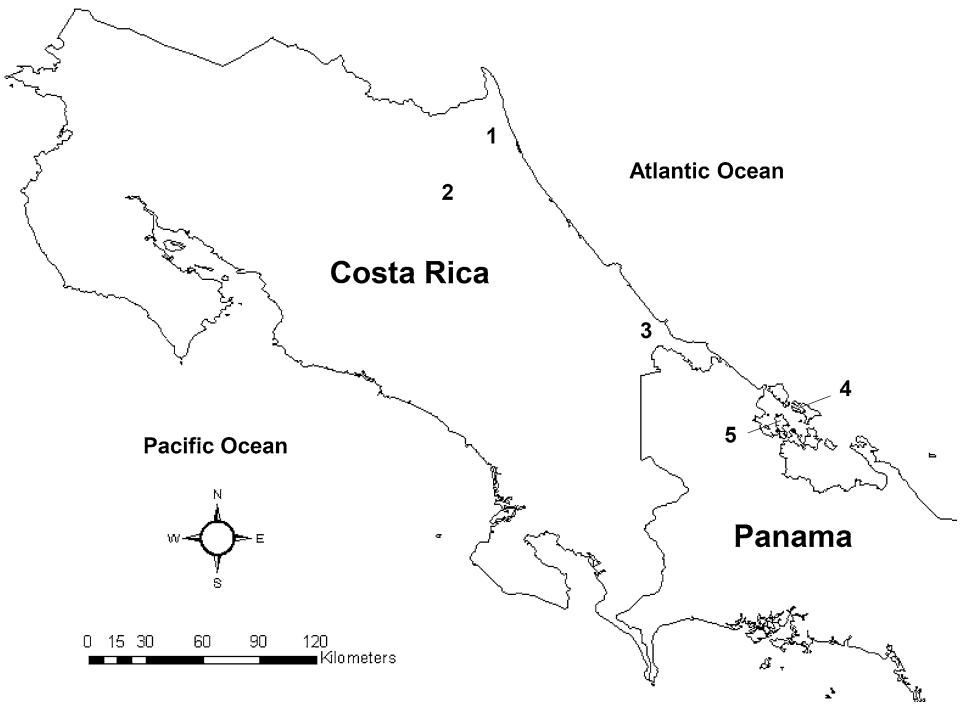

Fig. 1 Map of relative geographic locations of *Oophaga pumilio* in Costa Rica and Panama. (*1*) Tortuguero, Limon, Costa Rica; (*2*) La Selva Biological Station, Heredia, Costa Rica; (*3*) Puerto Viejo de Talamanca, Limon, Costa Rica; (*4*) Isla Solarte, Bocas del Toro, Panama; and (*5*) Isla San Cristobal, Bocas del Toro, Panama

Microbial growth

Bacillus subtilis (154921), *Escherichia coli* (155065), and *Candida albicans* (155965) were purchased from Carolina Biological Supply Company. Liquid cultures of *B. subtilis*, *E. coli*, and *C. albicans* were grown in Bacto nutrient broth (Difco), Luria–Bertani (LB) broth (Miller, Difco), and Sabouraud dextrose broth (Difco), respectively. Cultures were incubated at 37 °C with agitation at 200 rpm, except *B. subtilis* which was incubated at 30 °C. All cultures were grown to the mid-log phase prior to dilution for the antimicrobial susceptibility assay.

Antimicrobial susceptibility assays

Once cultures reached the mid-log phase, they were either Gram stained (*B. subtilis*, *E. coli*) or stained with methylene blue (*C. albicans*) to ensure purity. Cultures were diluted in their respective media to 1.5×10^8 colony forming units/mL according to the McFarland turbidity standard No. 0.5 (Hardy Diagnostics). An equal volume (200 µL) of each microbial suspension was pipetted in a 96-well flat-bottom plate (Falcon 1172, Becton–Dickinson Labware). Previous work has demonstrated a threshold for the antimicrobial activity of individual synthetic alkaloids (MacFoy et al. 2005). The specific threshold of activity for each alkaloid fraction (i.e., individual frog) was not determined in the present study; however, prior to the utilization of alkaloid fractions in the microbial assays, a pilot study with the synthetic alkaloid decahydroquinoline (DHQ) (Acros Organics) was carried out to determine the optimal dose range for ensuring that the threshold was met (data not shown). Based on this pilot, aliquots of each alkaloid fraction from individual frogs in the amount of 1.6, 3.2 and 4.8 µL were added to the wells containing the microbial suspensions. Methanol alone served as the vehicle control. Previous studies using methanol in this capacity have demonstrated growth suppression for several microbes when methanol concentrations were greater than 4 % of the total culture volume (Wadhwani et al. 2008). Preliminary experiments with the microorganisms used in the present study, showed no growth inhibition at methanol concentrations of up to 10 % of the total volume (data not shown). Based on these data, all of the experiments reported in the present study were conducted at 2.4 % methanol and all growth curves were compared to cells treated only with 'methanol' as they were not significantly different from 'no treatment'. The baseline optical density 600 (OD_{600}) was assessed using a Fisher Scientific Multiskan FC plate reader and plates were returned to the incubator. The OD_{600} measurements were taken every 6 h for 24 h (*C. albicans*), 36 h (*E. coli*), or 48 h (*B. subtilis*) to ensure that antimicrobial susceptibility was assessed during log phase/rapid growth of each organism.

Statistical analyses

Non-metric multidimensional scaling (nMDS) was used to graphically visualize differences in alkaloid composition among locations. Alkaloid composition is a combined measure of the number, type, and quantity of alkaloids present within an individual frog skin. A one-way analysis of similarity (ANOSIM) was used to identify differences in alkaloid composition among locations. nMDS and ANOSIM results are based on Bray–Curtis dissimilarity matrices. All multivariate statistical analyses were performed using PRIMER-E version 5.

The effects of alkaloids on microbial growth were evaluated using one-way analysis of variance (ANOVA), by comparing final optical densities of frog alkaloids and methanol controls among each location and for each microbe. Multiple comparisons (Tukey's HSD test) were used to detect differences in antimicrobial inhibition between frog locations. Growth curves were constructed for each microbe relative to a methanol control, and the point at which lag growth began was chosen as the final optical density for comparison in the statistical analyses (growth curves not included). Lag growth began at 30 h for *E. coli*, 36 h for *B. subtilis*, and 24 h for *C. albicans*. All parametric statistical analyses were performed using SPSS version 17.0 for Mac (SPSS, Inc.).

Results

Alkaloid analysis

GC–MS analysis of 15 *Oophaga pumilio* led to the detection of 230 alkaloids (including isomers), which are classified into 18 different structural classes and are derived largely from mites and ants (see Table 1 for most common alkaloids). The average number of alkaloids (including isomers) present in *O. pumilio* is 42 alkaloids (average for Tortuguero: 37 alkaloids; La Selva: 30 alkaloids; Puerto Viejo: 69 alkaloids; Isla Solarte: 35 alkaloids; Isla San Cristobal: 40 alkaloids). Alkaloid composition of *O. pumilio* differed significantly among the five locations (Global $R = 0.91$; $p \leq 0.001$), and frogs from Puerto Viejo contained the largest number and quantity of alkaloids, whereas frogs from La Selva contained the least (Fig. 2).

Optical density assays

Escherichia coli

Mean optical density differed significantly among the five locations of *O. pumilio* and methanol controls at the 4.8 µL

Table 1 The most common alkaloids identified in the skin of *Oophaga pumilio* from each location

Geographic location	Arthropod source of alkaloids			
	Mites		Ants	
	Structural class:	Alkaloid	Structural class:	Alkaloid
Tortuguero, Costa Rica	5,8-I:	**205A**, **207A**, **237D**, **247E**, **249O**, **251N**, **259B**	Pip:	**213A**, **241D**
	5,6,8-I:	**251T**, **253H**, **265V**		
	PTX:	**251D**, **323A**, **323F**		
	aPTX:	**267A**, **341A**		
	Unclass:	**207F**, **235S**, **249N**		
La Selva Biological Station, Costa Rica	5,8-I:	**205A**, **207A**, **209S**	DHQ:	**269AB**, **275B**
	5,6,8-I:	**251T**, **265L**		
	1,4-Q:	**257D**		
Puerto Viejo de Talamanca, Costa Rica	5,8-I:	**205A**	Pyr:	**277D**
	5,6,8-I:	**231B**, **249C**, **251M**, **259C**, **263A**, **265L**	Pip:	**213A**, **225B**, **241D**
	Deo-hPTX:	**207O**	3,5-P:	**223B**, **223H**, **251K**
	Tri:	**205H**, **207J**, **235I**, **253G**	DHQ:	**269A**, **269AB**, **269B**, **271D**
	Unclass:	**207N**	Lehm:	**275A**
			HTX:	**283A**, **285A**, **287A**, **287B**
Isla Solarte, Bocas del Toro, Panama	5,8-I:	**235B**, **257C**	3,5-I:	**195B**
	5,6,8-I:	**237C**	DHQ:	**195A**, **211A**
	PTX:	**307A**, **309C**, **323A**		
	Spiro:	**252A**		
Isla San Cristobal, Bocas del Toro, Panama	5,8-I:	**205A**, **207A**	Pyr:	**225C**
	5,6,8-I:	**223A**, **223X**, **231B**	Pip:	**241D**
	PTX:	**251D**, **323A**	3,5-P:	**223B**
	aPTX:	**267A**, **323B**	DHQ:	**223F**
	1,4-Q:	**257D**	Lehm:	**275A**
	Tri:	**235I**		

Common alkaloids were present in all individuals of *O. pumilio* from each location. Alkaloids are arranged by presumed dietary arthropod source

Tricyclic alkaloids have also been identified in beetles, and spiropyrrolizidine alkaloids have also been identified in millipedes (Saporito et al. 2012)

Underlined alkaloids are the 5 most abundant in terms of realtive quantity for each location. In all cases, these alkaloids collectively represented at least 60% of the total quantity of alkaloids present within a location

5,8-I 5,8-disubstituted indolizidine, *5,6,8-I* 5,6,8-trisubstituted indolizidine, *1,4-Q* 1,4-disubstituted quinolizidine, *PTX* pumiliotoxin, *aPTX* allopumiliotoxin, *Deo-hPTX* deoxy homopumiliotoxin, *Tri* tricylcic, *Spiro* spiropyrrolizidine, *Unclass* unclassified, *Pyr* pyrrolidine, *Pip* piperidine, *3,5-P* 3,5-disubstituted pyrrolizidine, *3,5-I* 3,5-disubstituted indolizidine, *DHQ* 2,5-disubstituted decahydroquinoline, *Lehm* lehmizidine, *HTX* histrionicotoxin

volume (30-h growth measurement) for *E. coli* ($F_{5,45} = 13.66$; $p \leq 0.001$; Fig. 3a). The mean optical density of *E. coli* treated with alkaloids was significantly less than the methanol control for all five locations ($p \leq 0.006$ in all cases). Differences in mean optical density varied among the five locations of *O. pumilio* (see Table 2 for results of pairwise comparisons). Mean optical density was not significantly different among locations at the 1.6 or 3.2 μL alkaloid volumes (data not included).

Bacillus subtilis

Mean optical density differed significantly among the five locations of *O. pumilio* and methanol controls at the 4.8 μL volume (36-h growth measurement) for *B. subtilis* ($F_{5,45} = 29.78$; $p \leq 0.001$; Fig. 3b). The mean optical density of *B. subtilis* treated with alkaloids was significantly less than the methanol control for all five locations ($p \leq 0.001$ in all cases). Mean optical density of Puerto

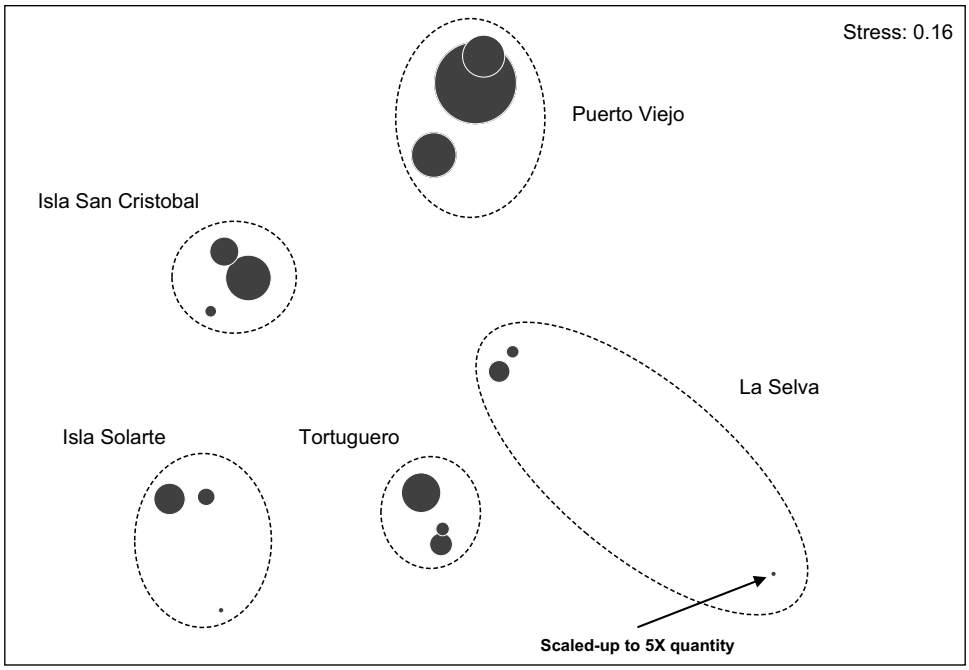

Fig. 2 nMDS plot of alkaloid composition among five locations of *Oophaga pumilio* from Costa Rica and Panama. Each *circle* represents an individual frog from a specific location (3 individuals from each location). The diameter of each circle is equivalent to the relative quantity of alkaloid present in each frog. One individual from La Selva was scaled to 5× its quantity, due to the low amount of alkaloids present in that individual. The distance between any two symbols represents differences in alkaloid composition (number, type, and quantity of alkaloids). Points that are closer together are more similar in alkaloid composition, whereas points that are further apart are less similar in alkaloid composition. *Dotted lines* are present only to indicate individual frogs from the same location

Viejo was significantly less than each of the four other locations of *O. pumilio* ($p \leq 0.001$ in all cases; see Table 2 for results of pairwise comparisons). Mean optical density was not significantly different among locations at the 1.6 or 3.2 µL alkaloid volumes (data not included).

Candida albicans

Mean optical density differed significantly among the five locations of *O. pumilio* and methanol controls at the 4.8 µL alkaloid volume (24-h growth measurement) for *C. albicans* ($F_{5,45} = 3.67$; $p = 0.007$; Fig. 3c). The mean optical density of *C. albicans* treated with alkaloids from Isla Solarte was significantly less than the methanol control ($p = 0.039$), whereas the mean optical density of *C. albicans* treated with alkaloids from Puerto Viejo was marginally less than the methanol control ($p = 0.052$). There were no differences in mean optical density among the five locations of *O. pumilio* (see Table 2 for results of pairwise comparisons). Mean optical density was not significantly different among locations at the 1.6 or 3.2 µL alkaloid volumes (data not included).

Discussion

The alkaloid-based chemicals present in poison frog skin secretions are unpalatable to a number of animals and are largely considered to function as a defense against potential predators (e.g., Brodie and Tumbarello 1978; Szelistowski 1985; Stynoski et al. 2014). It has recently been demonstrated, however, that certain individual alkaloids have the potential to also serve as a defense against certain microbial pathogens (MacFoy et al. 2005). The present study indicates that natural alkaloid cocktails from different populations of the dendrobatid poison frog *O. pumilio* inhibit the growth of certain microbes, which provides further support for the hypothesis that skin alkaloids function as both a deterrent to predators and pathogens.

Alkaloid cocktails were more effective against growth of the gram-positive bacteria *B. subtilis* and gram-negative *E. coli* compared to the fungus *C. albicans* (Fig. 3), indicating that alkaloid inhibition was largely dependent on microbe type. The growth of both bacterial species was significantly inhibited by alkaloid treatments from all five populations of *O. pumilio*, whereas only the Puerto Viejo population inhibited the growth of *C. albicans*. Macfoy

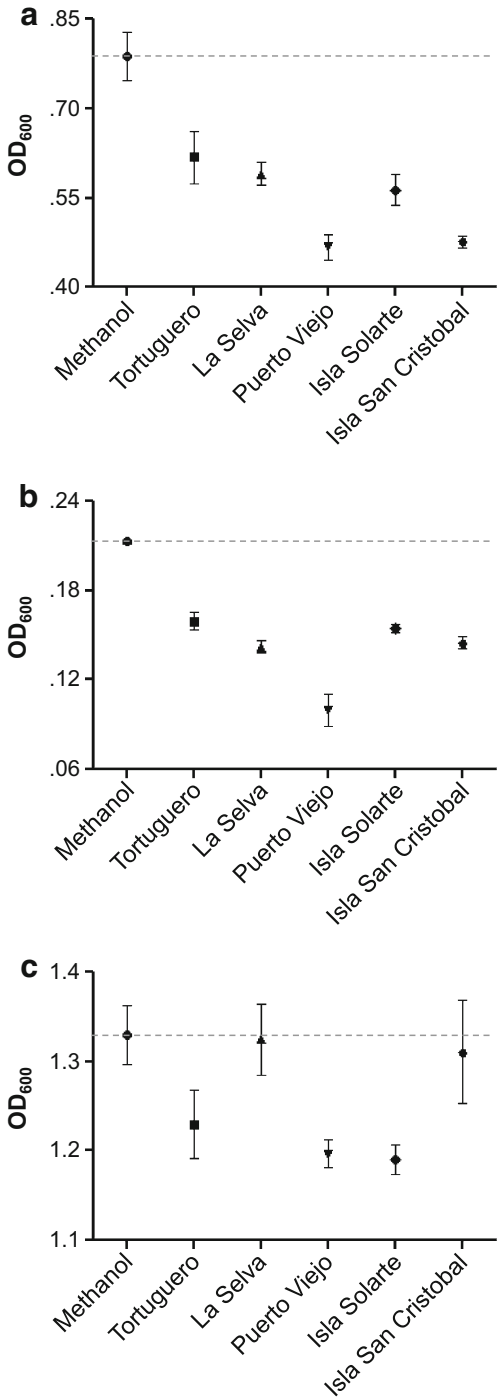

Fig. 3 Mean optical densities (OD_{600}) of alkaloid cocktails extracted from *Oophaga pumilio* for each location and microbe type **a** *Escherichia coli*; **b** *Bacillus subtilis*; **c** *Candida albicans*. The *dotted line* represents the mean OD_{600} for the methanol control

et al. (2005) also reported differences in alkaloid inhibition between microbe types, but instead found that most individual alkaloids inhibited the growth of *B. subtilis* and *C. albicans*, whereas only one piperidine inhibited *E. coli*. Although piperidine alkaloids are present in three of the five populations examined in the present study (Table 1), they occur only in small amounts and it is therefore more likely that alkaloids other than piperidines or a combination of different alkaloids are responsible for inhibition of *E. coli*. MacFoy et al. (2005) also reported that specific pyrrolidine, piperidine, decahydroquinoline, and pumiliotoxin alkaloids were the best at inhibiting *C. albicans*. In the present study, alkaloid cocktails from Isla Solarte significantly inhibited growth of *C. albicans* (Fig. 3), and frogs from this location contained mainly decahydroquinoline and pumiliotoxin alkaloids, but did not contain pyrrolidines or piperidines (Table 1). Furthermore, alkaloid cocktails from Puerto Viejo were marginally significant ($p = 0.052$) in their inhibition of *C. albicans*, and frogs from this location contained large quantities of decahydroquinolines and histrionicotoxins (Table 1). On the basis of our findings, it appears that decahydroquinoline, pumiliotoxins, and possibly histrionicotoxins are most effective at inhibiting the growth of the fungus *C. albicans*. While MacFoy et al. (2005) did not find histrionicotoxins to be effective at inhibiting *C. albicans*, these alkaloids did inhibit growth of the bacteria *B. subtilis*. It should also be noted that frogs from Puerto Viejo also contained the largest number and quantity of alkaloids, and therefore marginal inhibition of *C. albicans* could be due to the large amount of alkaloids present in this population of frogs or an interaction between multiple alkaloid types. Although most alkaloid cocktails in *O. pumilio* appear to inhibit the growth of bacteria, most of these same cocktails are less effective at inhibiting fungal growth, which may suggest that certain populations of *O. pumilio* are more susceptible to fungal pathogens. Furthermore, the same alkaloid cocktails that significantly inhibited the growth of the bacteria did not always inhibit growth of the fungus *C. albicans*. For example, alkaloids from La Selva and Isla San Cristobal were effective against both species of bacteria, but were not effective against *C. albicans*. On the basis of the present study and that of MacFoy et al. (2005), certain individual alkaloids and alkaloid cocktails differ in their activity against specific microbes.

Alkaloid composition differed significantly among the five populations of *O. pumilio* (Fig. 2; Table 1), which is likely due to differences in the type and availability of arthropods among geographic locations (Saporito et al. 2007, 2009, 2012). Alkaloid-based microbial inhibition also differed significantly among these same five populations (Fig. 3; Table 2), suggesting that variation in the alkaloid defense of *O. pumilio* is responsible for the observed differences in microbial inhibition. Although alkaloids from each population were effective at inhibiting growth of both bacterial species, some populations were more or less effective. For example, frog alkaloids from Puerto Viejo were significantly more effective at inhibiting

Table 2 *P* values for Tukey's pairwise comparisons of alkaloid optical density among the five geographic locations of *O. pumilio* for each of the three microbes

Microbe	Location	*P* value				
Escherichia coli		Tortuguero	La Selva	Puerto Viejo	Isla Solarte	Isla San Cristobal
	Tortuguero	*				
	La Selva	0.977	*			
	Puerto Viejo	**0.004**	**0.034**	*		
	Isla Solarte	0.720	0.984	0.160	*	
	Isla San Cristobal	**0.009**	0.059	0.999	0.245	*
Bacillus subtilus		Tortuguero	La Selva	Puerto Viejo	Isla Solarte	Isla San Cristobal
	Tortuguero	*				
	La Selva	0.274	*			
	Puerto Viejo	**0.001**	**0.001**	*		
	Isla Solarte	0.984	0.673	**0.001**	*	
	Isla San Cristobal	0.501	0.998	**0.001**	0.886	*
Candida albicans		Tortuguero	La Selva	Puerto Viejo	Isla Solarte	Isla San Cristobal
	Tortuguero	*				
	La Selva	0.453	*			
	Puerto Viejo	0.984	0.144	*		
	Isla Solarte	0.966	0.109	0.999	*	
	Isla San Cristobal	0.617	0.999	0.237	0.186	*

* Significant *P* values are indicated in bold

growth of both bacterial species when compared with frog alkaloids from La Selva (see Fig. 3). Although both populations have relatively large quantities of decahydroquinolines, most alkaloids are unique to each population. The presence of specific alkaloids is likely responsible for many of the differences in microbial inhibition between populations, but the number and quantity of alkaloids as well as potential synergistic effects between alkaloids may also be important. Alkaloids from Puerto Viejo were the most effective against all microbes, representing the only population to significantly inhibit the growth of the gram-positive bacteria *B. subtilis*, gram-negative *E. coli*, and fungus *C. albicans*. Large relative quantities of histrionicotoxins are unique to the Puerto Viejo population, suggesting that they may be important to microbial inhibition; however, it should be noted that MacFoy et al. (2005) found individual histrionicotoxins to be some of the least effective alkaloids against the same microbes. Frogs from Puerto Viejo also have the highest diversity of decahydroquinoline and piperidine alkaloids, both of which represent classes that MacFoy et al. (2005) reported to be particularly effective at inhibiting microbes. Furthermore, frogs from Puerto Viejo have the highest amount of alkaloids when compared with all of the populations, suggesting that increases in alkaloid diversity and quantity may also be important to inhibiting microbial growth. Overall, these results suggest that frog alkaloid potency varies among geographic locations, which could lead to differential inhibition against microbial infection. Geographic variation in frog alkaloids is thought to play an important role in defense against predators (Saporito et al. 2006, 2007, 2009), and the findings of the present study suggest that this same variation may also be an important determinant in defense against microbes.

The proximate factors that drive differences in microbial inhibition among populations of *O. pumilio*, while undoubtedly complex, likely depend on the specific microbes and their response to certain alkaloids, overall alkaloid diversity and quantity, and possibly, synergistic effects between individual alkaloids. Natural combinations of peptides in some frog species are more effective against microbes when compared with individual peptides (Rollins-Smith et al. 2005; Rosenfeld et al. 2006), and it has been suggested that a varied assemblage of peptides allow for an effective defense against a diversity of microorganisms (Nicolas and Mor 1995; Simaco et al. 1998; Zasloff 2002). Given the tremendous diversity of alkaloids present in poison frogs, it is possible that variable alkaloid defenses provide poison frogs with protection from a wide range of microorganisms.

The results of our study demonstrate that the alkaloid cocktails present in the poison frog *O. pumilio* inhibit microbial growth, suggesting that these alkaloids serve as both a defense against predators and microbes. Alkaloids in *O. pumilio* are most effective against bacteria and only moderately effective against a fungus, suggesting that

microbial inhibition is highly dependent upon microbe type. The degree of microbial inhibition varies significantly among geographic locations, which is consistent with differences in frog alkaloids on similar scales. These findings illustrate that differences in arthropod-derived alkaloids in *O. pumilio* may play a significant role in a poison frog's ability to defend itself from certain microbial infections. Furthermore, variable alkaloid defenses may protect frogs from a diversity of potentially pathogenic microbes. The microbes used in the present study, however, represent common, relatively non-pathogenic strains of bacteria and fungi that are not likely to infect frogs. Future studies should aim to examine the effectiveness of poison frog alkaloids against biologically relevant microbes, such as the gram-negative bacterium *Aeromonas hydrophila*, the microbial cause of red leg syndrome in amphibians, and ranaviruses and the chytrid fungus *Batrachochytrium dendrobatidis*, which have been shown to be the cause of certain amphibian declines worldwide (e.g., Pessier et al. 1999, 2002; Pianetti et al. 2005; Lips et al. 2006; Miller et al. 2008; Whitfield et al. 2013).

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