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Sequestered Alkaloid Defenses in the Dendrobatid Poison Frog *Oophaga pumilio* Provide Variable Protection from Microbial Pathogens

Kyle J. Hovey¹ · Emily M. Seiter¹ · Erin E. Johnson¹ · Ralph A. Saporito¹

Abstract

Most amphibians produce their own defensive chemicals; however, poison frogs sequester their alkaloid-based defenses from dietary arthropods. Alkaloids function as a defense against predators, and certain types appear to inhibit microbial growth. Alkaloid defenses vary considerably among populations of poison frogs, reflecting geographic differences in availability of dietary arthropods. Consequently, environmentally driven differences in frog defenses may have significant implications regarding their protection against pathogens. While natural alkaloid mixtures in dendrobatid poison frogs have recently been shown to inhibit growth of non-pathogenic microbes, no studies have examined the effectiveness of alkaloids against microbes that infect these frogs. Herein, we examined how alkaloid defenses in the dendrobatid poison frog, *Oophaga pumilio*, affect growth of the known anuran pathogens *Aeromonas hydrophila* and *Klebsiella pneumoniae*. Frogs were collected from five locations through-out Costa Rica that are known to vary in their alkaloid profiles. Alkaloids were isolated from individual skins, and extracts were assayed against both pathogens. Microbe subcultures were inoculated with extracted alkaloids to create dose-response curves. Subsequent spectrophotometry and cell counting assays were used to assess growth inhibition. GC-MS was used to characterize and quantify alkaloids in frog extracts, and our results suggest that variation in alkaloid defenses lead to differences in inhibition of these pathogens. The present study provides the first evidence that alkaloid variation in a dendrobatid poison frog is associated with differences in inhibition of anuran pathogens, and offers further support that alkaloid defenses in poison frogs confer protection against both pathogens and predators.

Keywords *Aeromonas* · Chemical defense · Colony-forming unit · *Klebsiella* · Optical density

Introduction

Predation and disease are important selective pressures that affect individuals, populations, and communities, thereby driving the evolution of highly diverse defensive strategies (Edmunds 1974). The use of unpalatable or toxic compounds as a defense mechanism is a common adaptation present in a variety of microorganisms, plants, invertebrates, and vertebrates (reviewed in Mebs 2001). Among

vertebrates, chemical defenses are widely known for protecting against predators, but they also play a critical role in defending against pathogenic microorganisms (Nicolas and Mor 1995). Amphibians secrete a variety of chemicals from dermal granular glands, including biogenic amines (Daly et al. 1987; Erspamer 1994; McClean et al. 2002), peptides and proteins (Rollins-Smith 2005; Conlon 2011a, b), steroidal bufadienolides (Daly 2004; Erspamer 1994), tetrodotoxin and related analogs (Yotsu-Yamashita et al. 2004; Cardall et al. 2004), indolic alkaloids (Daly and Garraffo 1990; Jeckel et al. 2015a), and an array of lipophilic alkaloids (Daly et al. 2005; Saporito et al. 2012). Amphibians synthesize the majority of these chemical defenses (Toledo and Jared 1995; Daly 1995), but some antimicrobial peptides and metabolites are provided by microbial symbionts (Brucker et al. 2008; Harris et al. 2009; Becker and Harris 2010; Loudon et al. 2014), and lipophilic alkaloids are acquired through diet (Saporito et al. 2009).

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Poison frogs are a polyphyletic group of anurans that have evolved to accumulate and secrete lipophilic alkaloids sequestered from dietary arthropods as a defense. This globally-distributed assemblage is represented by more than 150 species from seven lineages in the families Bufonidae (*Melanophryniscus*), Dendrobatidae, Eleutherodactylidae (*Eleutherodactylus*), Mantellidae (*Mantella*), and Myobatrachidae (*Pseudophryne*) (reviewed in Saporito et al. 2012). Experimental evidence of alkaloid sequestration in bufonids (Hantak et al. 2013), dendrobatids (Daly et al. 1994), mantellids (Daly et al. 1997), and myobatrachids (Smith et al. 2002) suggests that poison frogs uptake and store an assortment of lipophilic alkaloids (i.e., alkaloid cocktails) obtained from alkaloid-containing ants, mites, beetles, and millipedes (Daly et al. 2002; Saporito et al. 2007a, b, 2009).

Alkaloids in poison frogs are considered an effective defense against predators as a result of their unpalatability and/or toxicity (Hantak et al. 2016; Murray et al. 2016; Bolton et al. 2017), but they may also provide protection from microorganisms including parasites, bacteria, and fungi (Macfoy et al. 2005; Weldon et al. 2006; Grant et al. 2012; Mina et al. 2015). Alkaloids in the bufonid poison frog, *Melanophryniscus simplex*, occupy muscle and liver tissues, as well as oocytes, in the same relative proportions as the skin, and may deter internal parasites (Grant et al. 2012). Pumiliotoxin **251D**, a common alkaloid among dendrobatid poison frogs, appears to be a highly effective deterrent of a tropical mosquito known to parasitize anurans (Weldon et al. 2006). Individual synthetic and natural alkaloids inhibit the growth of the common bacteria *Bacillus subtilis* (Gram-positive) and *Escherichia coli* (Gram-negative), and the fungus *Candida albicans* (Macfoy et al. 2005). Most recently, natural alkaloid cocktails isolated from the dendrobatid poison frog, *Oophaga pumilio*, were found to inhibit growth of those same common microbes to varying degrees (Mina et al. 2015).

Over 1200 unique alkaloids in 28 structural classes have been identified from poison frog skin secretions (Daly et al. 2005; Garraffo et al. 2012; Saporito et al. 2012; Jeckel et al. 2015a; Santos et al. 2016). Poison frogs accumulate between one and 69 of these individual alkaloids in varying quantities (Daly et al. 2005; Saporito et al. 2006, 2007a; Mina et al. 2015). Dendrobatid alkaloid defenses vary considerably in diversity and quantity within and among populations primarily as a result of differences in the local availability of dietary arthropods (Myers and Daly 1976; Daly et al. 1987, 2008a; Saporito et al. 2006, 2007a), although factors such as species, sex, age, and life stage are also important determinants of variation (Saporito et al. 2010a, b, 2012; Stynoski et al. 2014; Jeckel et al. 2015b). Given that antimicrobial activity differs among individual alkaloids (Macfoy et al. 2005) and among natural alkaloid cocktails (Mina et al. 2015),

environmentally-driven differences in alkaloid defenses may influence protection against natural pathogens at the population level. Specifically, populations with greater quantities of alkaloids and more diverse alkaloid cocktails likely benefit from enhanced protection from pathogenic microorganisms, although this has not yet been tested.

Numerous pathogenic microorganisms infect amphibians, including opportunistic Gram-negative bacteria, the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), mycobacteria, ranaviruses, water molds, trematodes, and other various parasites (Taylor et al. 2001; Green et al. 2002; Schadich and Cole 2010). Opportunistic Gram-negative bacteria have been frequently implicated in bacterial dermatosepticemia, a fatal systemic infectious disease in frogs that has been linked to population declines and extirpations in the wild (Nyman 1986; Bradford 1991; Sherman and Morton 1993) and mass mortalities in captivity (Green et al. 1999). Although numerous Gram-negative bacilli may cause dermatosepticemia, bacteria such as *Aeromonas hydrophila* have been most frequently implicated as the etiological agent, and host susceptibility depends on the amphibian species as well as environmental, stress, dietary, health, and immune status factors (Schadich and Cole 2010). For instance, Schadich and Cole (2010) isolated the Gram-negative *A. hydrophila*, *Klebsiella pneumoniae*, and *Proteus mirabilis* from infected brown tree frogs (*Litoria ewingii*) during a dermatosepticemia epizootic, and determined that *K. pneumoniae* was the most virulent in healthy frogs. Although antimicrobial peptide defenses are thought to have evolved as a secondary, broad-spectrum immune defense (Nicolas and Mor 1995; Roelants et al. 2013) and are effective in that role (Simmaco et al. 1998a, b; Zasloff 2002; Rollins-Smith 2005), there is a fundamental gap in our understanding of the role that sequestered alkaloid defenses serve in defending poison frogs from infection.

Research on the antimicrobial activity of alkaloid defenses in frogs (Macfoy et al. 2005; Mina et al. 2015) has focused on the dendrobatid poison frog, *O. pumilio*, a terrestrial dendrobatid frog whose alkaloid defenses are relatively well-characterized and vary considerably with geographic location at both small and large spatial scales (Saporito et al. 2006, 2007a, 2010a). The substantial variability in *O. pumilio* alkaloid defenses, in addition to an abundance of previously-identified alkaloids, makes *O. pumilio* an ideal study organism in which to examine ecologically relevant antimicrobial activity of sequestered alkaloid defenses. The aim of the present study is to examine the extent to which the variable alkaloid defenses in a dendrobatid poison frog, *O. pumilio*, inhibit the growth of the anuran pathogens *A. hydrophila* and *K. pneumoniae*.

Methods and Materials

Study Sites and Frog Collections A total of 70 adult *O. pumilio* were collected from five lowland tropical rainforest sites throughout eastern Costa Rica ($n = 14$ per site), which were selected based on known differences in the alkaloid defenses (i.e., number and quantity of unique alkaloids and alkaloid structural classes) of *O. pumilio* from each site (alkaloid data from Saporito et al. 2007a). Frogs were collected from each of the following locations: La Selva Biological Station (10°26' N, 83°59' W; 7 males and 7 females), Finca los Nacientes (10°21'53" N, 84°8'6" W; 8 males and 6 females), Tortuguero (10°35'14" N, 83°31'34" W; 7 males and 7 females), Río Palmas (10°10'16" N, 83°36'26" W; 8 males and 6 females), and Gandoca (9°35'03" N, 82°37'13" W; 7 males and 7 females). Frogs were collected within a single 45 m × 45 m collection plot at each site, sexed based on gular skin patches (Donnelly 1989), measured for snout-to-vent length (SVL), and euthanized by freezing at -20 °C. Frog skins were stored individually in 4 mL of 100% methanol contained in glass vials sealed with Teflon-lined caps ("methanol extract").

Alkaloid Fractionations Alkaloids were extracted from frog skins using the procedure outlined in Saporito et al. (2010a), which is briefly described here. One mL of each methanol extract was transferred to a 10 mL conical vial and acidified with 50 µL hydrochloric acid (1 N). Each sample was then mixed and evaporated to a volume of 100 µL using nitrogen gas, followed by dilution with 200 µL deionized water. Samples were then extracted with four 300 µL portions of hexane. The organic hexane layer was then disposed of, and the remaining aqueous solution was basified with sodium bicarbonate. Once basicity was verified with pH paper, each sample was extracted with three portions of 300 µL ethyl acetate. Anhydrous sodium sulfate was added to remove remaining trace amounts of water. The remaining samples were carefully evaporated to dryness with nitrogen gas. Alkaloid fractions were resuspended in 100 µL of methanol and stored at -20 °C.

Alkaloid Analysis To determine which unique alkaloids were present in extracts, and in what quantities (µg), samples of individual frog extracts containing a nicotine standard (1 µg/10 µL; (-)-nicotine ≥99%, Sigma-Aldrich) were analyzed using gas chromatography-mass spectrometry (GC-MS). Analyses were performed on a Varian Saturn 2100 T ion trap MS coupled to a Varian 3900 GC with a 30 m × 0.25 mm ID Varian FactorFour VF-5 ms fused silica column. The temperature program increased from 100 °C to 280 °C at a rate of 10 °C/min and helium was the carrier gas (flow rate of 1 mL/min). Samples

of alkaloid fractions were analyzed using both electron impact (EI) MS and chemical ionization (CI) MS, with methanol as the CI reagent. EI-MS for alkaloid identification and quantification was conducted in triplicate for each frog and CI-MS for alkaloid identification was performed once. In total, four 1 µL samples from each of 70 frogs' alkaloid extract were analyzed ($n = 280$ GC-MS runs).

Unique alkaloids were identified by comparing and matching retention times and mass spectral data of individual peaks, largely with data from the alkaloid library presented in Daly et al. 2005 (but also Daly et al. 2007, 2008a, b, 2009; Saporito et al. 2007a; Garraffo et al. 2012; Grant et al. 2012; Fitch et al. 2013; Andriamaharavo et al. 2015), which includes alkaloid assignment data and confirmed/proposed alkaloid structures. Synthetic standards for DHQ 195A, DHQ 211A, and 5,8-I 235B (N. Toyooka, University of Toyama) were used in identification, and to calibrate the retention times for other alkaloids when comparing them to previously identified alkaloids. The quantity of unique alkaloids present in each frog was calculated by comparing each alkaloid's peak to an internal nicotine standard (1 µg/10 µL; (-)-nicotine ≥99%, Sigma-Aldrich) using Varian MS Workstation v.6.9 SPI; however, it should be noted that this type of quantitation should be considered semi-quantitation, given that ionization likely differs between nicotine and frog alkaloids as well as among different alkaloids (for further discussion, see Andriamaharavo et al. 2015).

Culture and Maintenance of Microbes Liquid cultures of *Aeromonas hydrophila* (ATCC 35654) and *Klebsiella pneumoniae subsp. pneumoniae* (ATCC 13883) were incubated in tryptic soy broth (TSB; Remel) for 18 hr at 30 °C (*A. hydrophila*) or 37 °C (*K. pneumoniae*) with 200 rpm agitation. All cultures were grown to the mid-log phase prior to dilution for the growth inhibition assays.

Microbial Growth Inhibition Assays Optical density (OD) assays were adapted from an assay described by Mina et al. (2015). Based on pilot growth experiments, cultures were diluted with TSB to a ratio of 1:10 (*A. hydrophila*) or 1:5 (*K. pneumoniae*) and equal volumes (200 µL; "subcultures") were immediately aliquotted into 96-well flat-bottom microplate (Falcon). Aliquots of alkaloids from individual frogs and pure methanol were added in the amount of 2.4 and 4.8 µL to four replicate wells (subsamples) each. Methanol alone served as the vehicle control given that alkaloid extracts were dissolved in methanol. Treatment volumes were selected based on pilot experiments which indicated that 9.6 µL of methanol in 200 µL of *A. hydrophila* was highly inhibitory and would likely obscure any inhibition due to natural alkaloid extracts; while treatments with 2.4 µL and 4.8 µL of methanol were

inhibitory compared to negative controls, differences between methanol and alkaloid treatments were still evident at these volumes. Moreover, the treatment volumes are considered ecologically relevant for what microbes may be exposed to on frog skin, representing 0.6% and 1.2%, respectively, of the total quantity of alkaloids present in each individual frog skin.

Immediately following inoculation, the microplate was placed in a Fisher Scientific Multiskan FC microplate reader inside an incubator (ThermoForma) at the microbe's corresponding growth temperature. A baseline measurement of each subculture's OD at 620 nm (OD_{620}) was recorded prior to incubation to verify that all subcultures began at an OD similar to those in pilot experiments ($\pm 0.02 OD_{620}$) to account for random variation. A 24-hr program that recorded OD_{620} at 15-min intervals after shaking the plate at medium speed for five seconds was used to create 24-hr growth curves for each treatment. The final OD_{620} readings of subsamples per treatment were averaged for use in statistical analyses of OD assays.

Colony-forming unit (CFU) assays were used to assess the viability of the aforementioned subcultures after a 24-hr incubation period with natural alkaloid extracts and methanol treatments. Immediately following the final measurement of OD assays, three randomly-selected subsamples from each treatment were serially-diluted in tenfold increments up to 10^{-7} using microplates. Ten microliters of each serial dilution were then plated on TSA in square 6×6 gridded petri dishes (Fisherbrand) and incubated until single colonies (i.e., colony-forming units) formed. Once CFUs were visible, the number of CFUs at each dilution ($10^{-1} - 10^{-7}$) for each subsample was recorded. When CFU counts exceeded 30, colonies tended to grow into one another and it became difficult to accurately quantify CFUs. Therefore, the serial dilution that grew CFUs nearest to 30 in number, while still being equal to or below 30, was used to calculate the original cell density (OCD; CFU/mL; equation below) of each subsample. The average of each treatment's three subsample OCD values was used in statistical analyses.

Original cell density (CFU/mL)

$$= \frac{(\text{Colony-forming units})}{(\text{Dilution factor}) \times (\text{Volume of aliquot in mL})}$$

In the OCD equation, dilution factor refers to the magnitude of dilution of the quantified aliquot (e.g., 10^{-7}), and volume of aliquot in mL refers to the volume of each subsample's dilutions that were plated (10 μL in this case, or 0.01 mL).

Statistical Analyses Since alkaloid composition is a collective measure of the number, type, and quantity of alkaloids within individual frog skins, nonmetric multidimensional scaling (NMDS) was used to visualize differences in alkaloid

composition among populations. Analysis of similarity (ANOSIM) was used to detect statistical differences in alkaloid composition among these populations. Multivariate analyses were based on Bray-Curtis similarity matrices and performed using PRIMER-E version 5.

A nested one-way analysis of variance (ANOVA) was used to examine variation of growth inhibition within individual frog alkaloid treatments in both OD and CFU assays for each microbe. A two-tailed, unpaired, independent samples *t*-test was used to determine the presence of a dose-response relationship between 2.4 and 4.8 μL doses in both OD and CFU assays for each microbe. The effects of alkaloids on microbial growth were assessed using one-way ANOVA; final optical densities (growth inhibition) and CFU/mL after treatment (remaining viable cells) of frog alkaloid and methanol treatments in the 4.8 μL treatment were compared among populations and for each pathogen. Multiple comparisons (Tukey's HSD) were used to identify differences in microbial growth inhibition of frog alkaloids from each population compared to methanol treatments. To determine the effects of frog population and sex on growth inhibition, two-way ANOVA was conducted for each assay with each microbe. Linear regression analyses were performed to assess relationships between alkaloid composition (diversity and quantity) and growth inhibition (OD and CFU assays) for each microbe, and to test the relationship between OD and CFU assays for each microbe. All parametric statistical analyses were performed using SPSS version 14.0 and GraphPad Prism version 6.05.

Results

Alkaloid Analysis GC-MS analysis of 70 *Oophaga pumilio* from five Costa Rican populations resulted in the identification of 353 unique alkaloids (including isomers) represented by 19 different structural classes (see **Online Resource Table 1** for the most common alkaloids identified in each population and **Online Resource Table 2** for all identified alkaloids). The majority of these alkaloids are derived from mites and ants. Alkaloid composition of *O. pumilio* differed significantly among the five populations (Global $R = 0.91$; $P = 0.001$; **Online Resource Fig. 1a**), but not between sexes (Global $R = 0.03$; $P = 0.966$). Frogs from Finca los Nacientes contained the largest number and greatest quantity of alkaloids, while frogs from Gandoca contained the lowest number of alkaloids and contained similarly low quantities of alkaloids as frogs from La Selva (see **Table 1** for details on alkaloid composition by population and sex). In general, frogs from Tortuguero and Finca los Nacientes contained about three times as much alkaloid (in terms of quantity, μg per frog skin) as frogs from La Selva, Río Palmas, and Gandoca (**Online Resource Fig. 1b**).

Table 1 Diversity (alkaloids per frog skin) and quantity (μg per frog skin) of alkaloid defenses from each population of *Oophaga pumilio*

	Average diversity	Average ♀ diversity	Average ♂ diversity	Total diversity	Total ♀ diversity	Total ♂ diversity	Average quantity	Average ♀ quantity	Average ♂ quantity	Total quantity	Total ♀ quantity	Total ♂ quantity
Tortuguero	37	39	36	521	272	249	986	1224	748	13,807	8567	5239
La Selva	33	36	30	458	250	208	344	462	225	4811	3233	1578
Finca los Nacientes	54	55	53	758	441	317	1181	1219	1130	16,528	9750	6778
Río Palmas	39	42	36	548	334	214	264	295	223	3697	2357	1340
Gandoca	26	21	31	364	147	217	344	280	524	4812	1148	3665
All populations	38	39	37	2649	1444	1205	624	677	564	43,656	25,056	18,600

Total diversity represents the sum number of alkaloids identified in all 14 frogs from each location. Total quantity represents the sum quantity of alkaloid present in all 14 frogs from each location

Alkaloid quantity and diversity are also strongly correlated ($F_{1,68} = 49.47$, $P < 0.001$; $R^2 = 0.421$; **Online Resource Fig. 2**), although the relationship appears to be exponential rather than linear. In general, frogs containing higher quantities of alkaloids also possessed more diverse alkaloid cocktails, but fewer new types of alkaloids were represented with increasing alkaloid quantities.

Dose-Response Relationship between 2.4 μL and 4.8 μL Treatments Exposure of both *A. hydrophila* and *K. pneumoniae* to increased doses of alkaloid treatments indicates that there is a clear dose-response relationship between treatments and microbial growth inhibition. With both *A. hydrophila* and *K. pneumoniae*, 4.8 μL treatments resulted in consistently lower final optical density readings ($t_{138} = 13.19$, $P < 0.001$; $t_{138} = 10.15$, $P < 0.001$) and reduced viable cell counts ($t_{138} = 8.47$, $P < 0.001$; $t_{138} = 6.65$, $P < 0.001$), respectively, in comparison to 2.4 μL treatments. In accordance

with the dose-response relationship, differences in microbial growth inhibition between control and alkaloid treatments were more evident in 4.8 μL treatments; therefore, the results for all following assays and comparisons are reported for 4.8 μL treatments only (Figs. 1, 2, 3, 4 and 5).

***Aeromonas hydrophila* Optical Density Assays** Mean optical density of *A. hydrophila* treated with alkaloid cocktails from the five locations of *O. pumilio* differed significantly among the five populations of *O. pumilio* and methanol controls ($F_{5,78} = 13.04$; $P < 0.001$; Fig. 1a); however, mean optical density of *A. hydrophila* treated with alkaloids from individual frogs did not differ within samples ($F_{18,312} = 1.09$; $P = 0.360$). The mean optical density of *A. hydrophila* treated with alkaloids from Tortuguero, La Selva, and Finca los Nacientes frogs was significantly less than the methanol control, while the mean optical density of *A. hydrophila* treated with alkaloids from Río Palmas and Gandoca frogs showed no change.

Fig. 1 Mean final optical densities (OD_{620}) (a) and viable cell counts (CFU/mL) (b) of *Aeromonas hydrophila* after treatment with alkaloid cocktails extracted from *Oophaga pumilio*. The dotted line represents the mean OD_{620} and CFU/mL for the methanol control, error bars represent ± 1 SEM, and treatment means that are significantly different from each other are indicated by different letters (Tukey's HSD, $P < 0.05$)

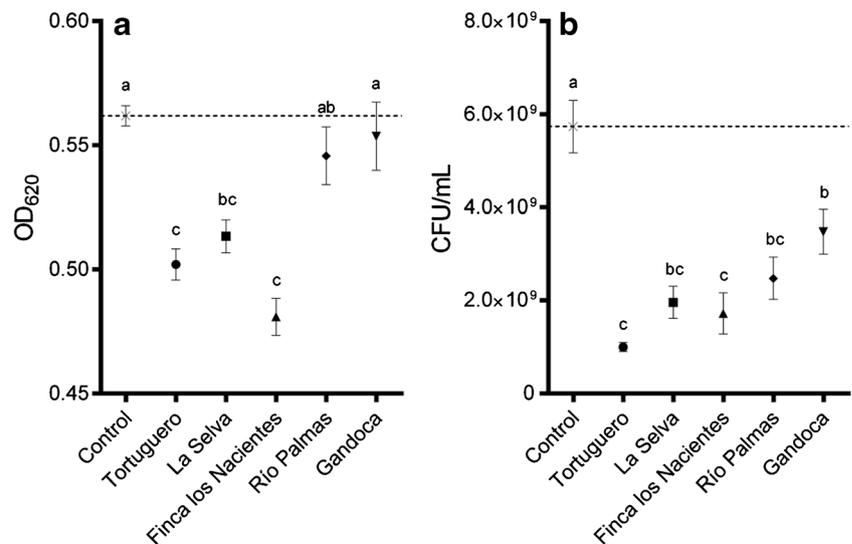
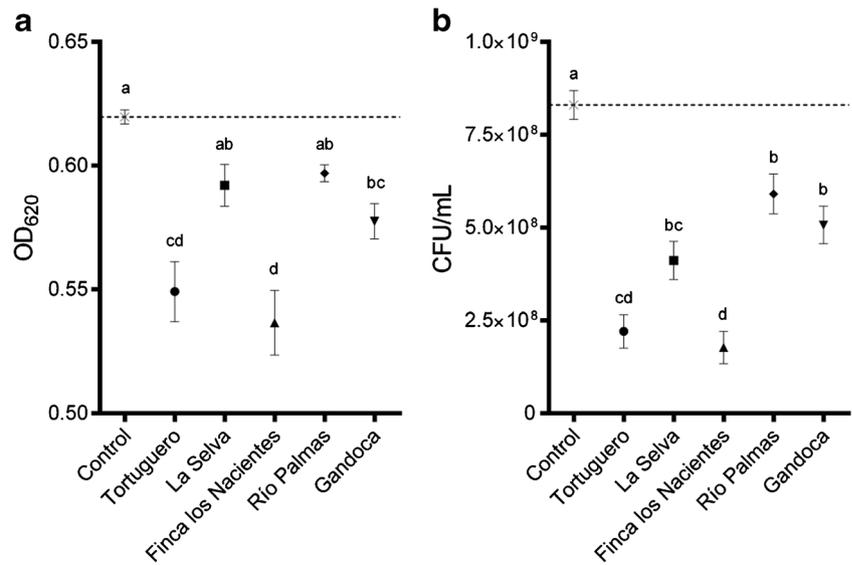


Fig. 2 Mean final optical densities (OD₆₂₀) (a) and viable cell counts (CFU/mL) (b) of *Klebsiella pneumoniae* after treatment with alkaloid cocktails extracted from *Oophaga pumilio*. The dotted line represents the mean OD₆₂₀ and CFU/mL for the methanol control, error bars represent ± 1 SEM, and treatment means that are significantly different from each other are indicated by different letters (Tukey's HSD, $P < 0.05$)



There was no interaction between the effects of frog population and sex for optical density assays with *A. hydrophila* ($F_{4,60} = 0.92$, $P = 0.459$).

***Aeromonas hydrophila* Colony-Forming Unit Assays** The number of viable cells of *A. hydrophila* treated with alkaloid cocktails differed significantly among the five populations of *O. pumilio* and methanol controls ($F_{5,78} = 15.89$; $P < 0.001$; Fig. 1b); however, mean viable cell counts of *A. hydrophila* treated with alkaloids from individual frogs did not differ within samples ($F_{12,234} = 0.90$; $P = 0.545$). The CFU's recovered from *A. hydrophila* treated with alkaloids from all populations were significantly less than the methanol control. There was a statistically significant interaction between frog population and sex in CFU assays with *A. hydrophila* ($F_{4,60} = 3.79$, $P = 0.008$), although there was no difference in original cell density between sexes after treatment with alkaloid cocktails from frogs ($F_{1,60} = 0.99$, $P = 0.324$); in other words, the more inhibitory sex varied among populations.

***Klebsiella pneumoniae* Optical Density Assays** Mean optical density of *K. pneumoniae* treated with alkaloid cocktails from the five locations of *O. pumilio* differed significantly among the five populations of *O. pumilio* and methanol controls ($F_{5,78} = 12.67$; $P < 0.001$; Fig. 2a); however, mean optical density of *K. pneumoniae* treated with alkaloids from individual frogs differed within samples ($F_{18,312} = 2.93$; $P < 0.001$). The mean optical density of *K. pneumoniae* treated with alkaloids from Tortuguero, Finca los Nacientes, and Gandoca frogs was significantly less than the methanol control, while the mean optical density of *K. pneumoniae* treated with alkaloids from La Selva and Río Palmas frogs showed no change. There was no interaction between the effects of frog population and sex for optical density assays with *K. pneumoniae* ($F_{4,60} = 1.99$, $P = 0.107$).

***Klebsiella pneumoniae* Colony-Forming Unit Assays** The number of viable cells of *K. pneumoniae* treated with alkaloid cocktails differed significantly among the five populations of

Fig. 3 Relationship between optical density and colony-forming unit assays for (a) *Aeromonas hydrophila* and (b) *Klebsiella pneumoniae* treated with alkaloids extracted from *Oophaga pumilio*

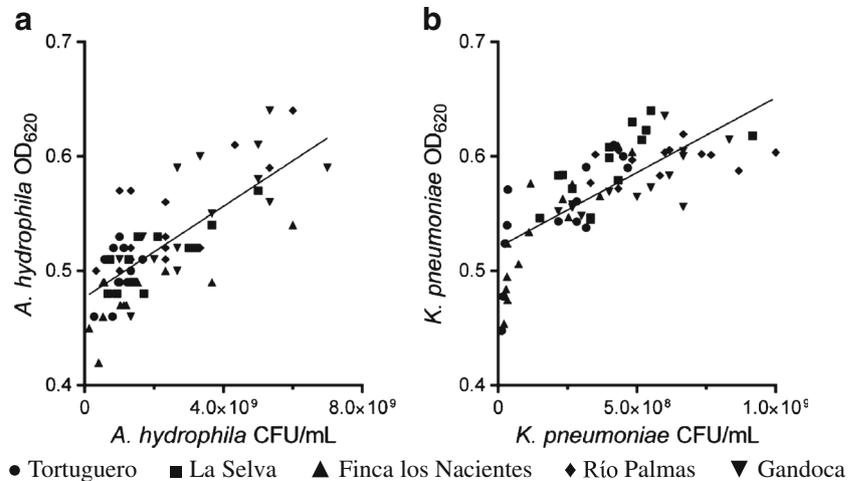
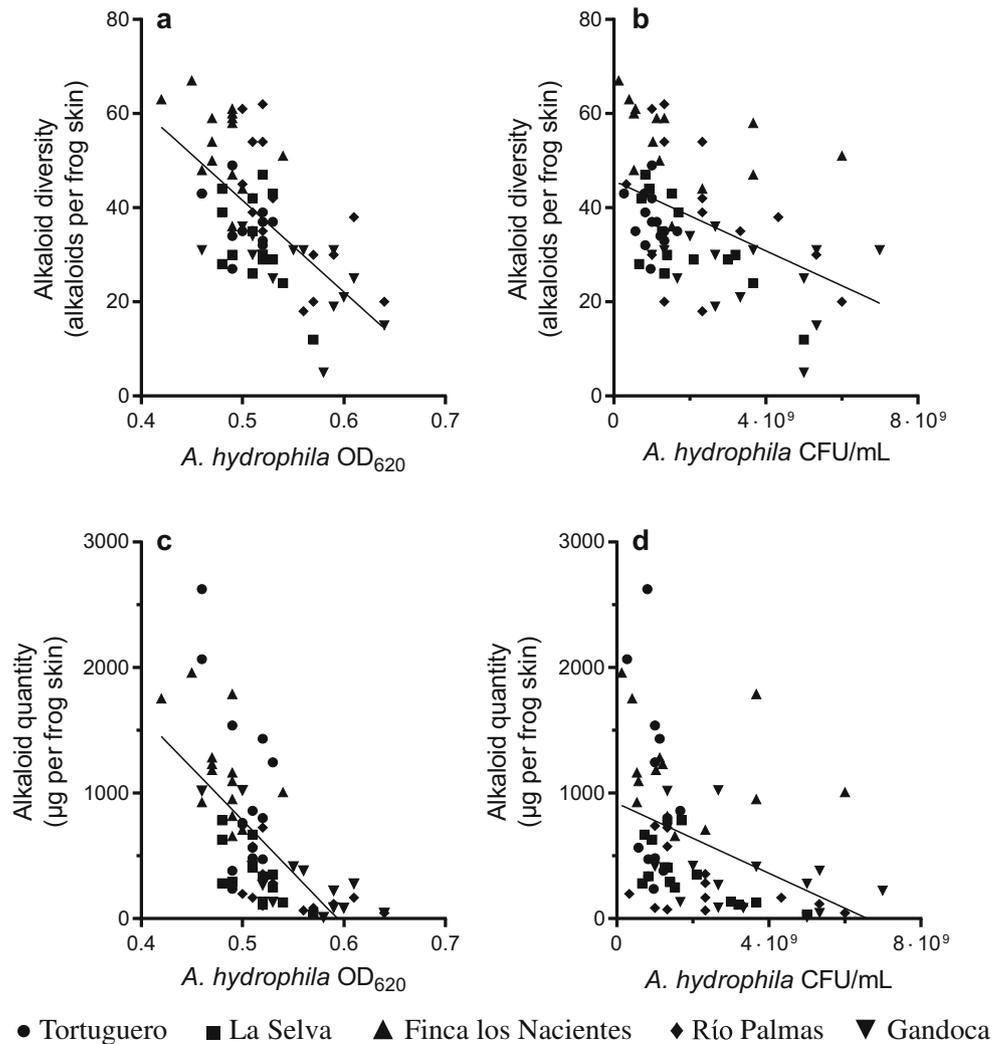


Fig. 4 Relationship between alkaloid profiles of individual *Oophaga pumilio* and corresponding growth inhibition of *Aeromonas hydrophila* [(a) alkaloid diversity (alkaloids per frog skin) and optical density assay; (b) alkaloid diversity (alkaloids per frog skin) and colony-forming unit assay; (c) alkaloid quantity (μg per frog skin) and optical density assay; (d) alkaloid quantity (μg per frog skin) and colony-forming unit assay]



O. pumilio and methanol controls ($F_{5,78} = 26.41$; $P < 0.001$; Fig. 2b); however, mean viable cell counts of *K. pneumoniae* treated with alkaloids from individual frogs did not differ within samples ($F_{12,234} = 0.52$; $P = 0.899$). The CFU's recovered from *K. pneumoniae* treated with alkaloids from all populations was significantly less than the methanol control. There was a statistically significant interaction between frog population and sex in colony-forming unit assays with *K. pneumoniae* ($F_{4,60} = 2.81$, $P = 0.033$), although there was no difference in original cell density between sexes after treatment with alkaloid cocktails from frogs ($F_{1,60} = 0.14$, $P = 0.706$); similarly to colony-forming unit assays with *A. hydrophila*, the more inhibitory sex varied among populations.

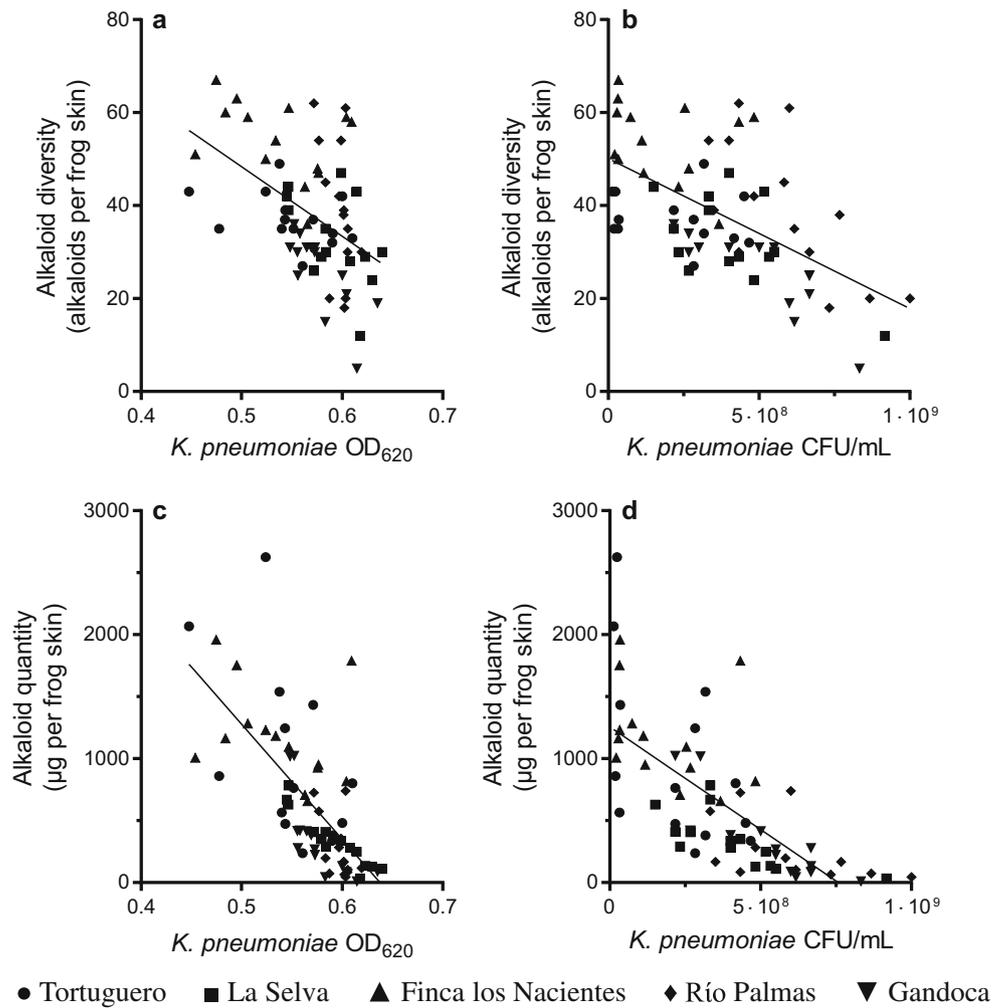
Relationship between Optical Density and Colony-Forming Unit Assays Optical density assays and colony-forming unit assays were strongly correlated for both *A. hydrophila* ($F_{1,68} = 80.88$, $P < 0.001$, $R^2 = 0.543$; Fig. 3a) and *K. pneumoniae* ($F_{1,68} = 85.52$, $P < 0.001$, $R^2 = 0.557$; Fig.

3b). The patterns in growth inhibition among *O. pumilio* populations were identical between the two assays for both microbes (Figs. 1 and 2).

Relationships between Alkaloid Composition and Growth Inhibition Alkaloid-based growth inhibition of *A. hydrophila* was correlated with both alkaloid diversity and composition of *O. pumilio* (Fig. 4). Alkaloid diversity was a moderate predictor of growth inhibition by proxy of both OD ($F_{1,68} = 48.60$, $P < 0.001$, $R^2 = 0.417$; Fig. 4a) and CFU assays ($F_{1,68} = 17.92$, $P < 0.001$, $R^2 = 0.209$; Fig. 4b). Alkaloid quantity was also a moderate predictor of growth inhibition by proxy of both OD ($F_{1,68} = 53.46$, $P < 0.001$, $R^2 = 0.440$; Fig. 4c) and CFU assays ($F_{1,68} = 13.79$, $P < 0.001$, $R^2 = 0.169$; Fig. 4d).

Alkaloid quantity, but not diversity, was a better predictor of growth inhibition of *K. pneumoniae* compared to *A. hydrophila* (Figs. 4 and 5). Alkaloid diversity was a moderately weak predictor of both OD ($F_{1,68} = 19.05$, $P < 0.001$, $R^2 = 0.219$; Fig. 5a) and CFU assays ($F_{1,68} = 34.22$, $P < 0.001$, $R^2 = 0.335$; Fig. 5b). However, alkaloid quantity was a

Fig. 5 Relationship between alkaloid profiles of individual *Oophaga pumilio* and corresponding growth inhibition of *Klebsiella pneumoniae* [(a) alkaloid diversity (alkaloids per frog skin) and optical density assay; (b) alkaloid diversity (alkaloids per frog skin) and colony-forming unit assay; (c) alkaloid quantity (μg per frog skin) and optical density assay; (d) alkaloid quantity (μg per frog skin) and colony-forming unit assay]



comparatively stronger predictor of both OD ($F_{1,68} = 63.35$, $P < 0.001$, $R^2 = 0.482$; Fig. 5c) and CFU assays ($F_{1,68} = 68.73$, $P < 0.001$, $R^2 = 0.503$; Fig. 5d) for *K. pneumoniae*.

Discussion

Alkaloid-based chemical defenses in poison frogs are unpalatable and/or toxic to a variety of invertebrates and vertebrates, and function accordingly as a defense against predators (Brodie and Tumbarello 1978; Fritz et al. 1981; Szelistowski 1985; Weldon et al. 2013; Stynoski et al. 2014; Hantak et al. 2016; Hovey et al. 2016; Murray et al. 2016). Individual alkaloids and natural alkaloid cocktails from the dendrobatid poison frog *O. pumilio* have also recently been shown to inhibit the growth of select microbes (Macfoy et al. 2005; Mina et al. 2015), suggesting that alkaloids may provide a dual defense against both predators and infection. The present study supports this hypothesis and demonstrates that sequestered alkaloid defenses from various populations of *O. pumilio* inhibit the growth of microbes that infect anurans and

contribute to substantial mortality in captivity and in the wild (i.e., pathogenic microbes); however, populations of *O. pumilio* also appear to vary in their ability to inhibit these pathogens.

Poison frogs accumulate unique suites of alkaloids gradually over the course of their lifetime as they consume alkaloid-containing arthropods (Saporito et al. 2009). In the present study, the composition of alkaloid defenses (i.e., quantity and diversity) differed significantly among populations, reflecting disparities in the type and availability of dietary arthropods at each location (Saporito et al. 2007a, 2009, 2012). Similarly, the extent of growth inhibition of both *A. hydrophila* and *K. pneumoniae* differed significantly among populations, most likely as a direct result of each population's distinct alkaloid composition.

Alkaloid quantity and alkaloid diversity are strongly correlated in the studied populations of *O. pumilio*. As the quantity of alkaloids in individual frogs increased, so did the number of unique alkaloids acquired. Likewise, alkaloid cocktails in the present study inhibited the growth of *A. hydrophila* and *K. pneumoniae* more effectively as alkaloid quantity and

alkaloid diversity increased. Accordingly, alkaloid cocktails from Finca los Nacientes and Tortuguero frogs inhibited growth of *A. hydrophila* and *K. pneumoniae* most effectively of all populations in both assays. Finca los Nacientes frogs were protected by alkaloid defenses that were, on average, nearly threefold greater in quantity and 40–100% more diverse than those La Selva, Río Palmas, and Gandoca frogs. Alkaloid cocktails from Tortuguero frogs were comparable in quantity to alkaloids from Finca los Nacientes frogs, but were instead similar in diversity to alkaloids from La Selva and Río Palmas frogs and higher in diversity than Gandoca. Additionally, the predominant alkaloids present in Finca los Nacientes frog alkaloid cocktails (comprising about half of the total quantity) included 5,8-disubstituted indolizidines, decahydroquinolines, and histrionicotoxins, whereas the most abundant alkaloids in Tortuguero frogs (over 60% of the total quantity) were 5,8-disubstituted indolizidines and 5,6,8-trisubstituted indolizidines. On the basis of these findings, larger quantities of more diverse alkaloids provided enhanced antimicrobial protection, but 5,8-disubstituted indolizidines, 5,6,8-trisubstituted indolizidines, decahydroquinolines, and histrionicotoxins may also be particularly inhibitory alkaloid classes.

All populations of *O. pumilio* in the present study contained disproportionately high quantities and numbers of mite-derived, branched-chain alkaloids, such as 5,8-disubstituted indolizidines and 5,6,8-trisubstituted indolizidines, which is typical of Costa Rican *O. pumilio* (Saporito et al. 2007b, 2009, 2011, 2015; Bolton et al. 2017). However, the alkaloid cocktails from the same populations also varied widely in inhibitory efficacy despite sharing large proportions of these common mite-derived alkaloids. The large quantities of these common alkaloids present in the more inhibitory Finca los Nacientes and Tortuguero frogs suggest that these alkaloids may become particularly inhibitory above a certain threshold, which could explain the comparatively reduced inhibition by other populations that share these same alkaloids, but in lower quantities. Alternatively, Finca los Nacientes frogs also contained distinctly high quantities of ant-derived decahydroquinoline and histrionicotoxin alkaloids when compared to other populations, which may also play an important role in Finca los Nacientes frogs' markedly effective inhibition. Although these findings from both mite- and ant-derived alkaloids suggest that alkaloid quantity contributes more to microbial growth inhibition than alkaloid diversity, the acquisition of such extreme quantities of alkaloid defenses presumably takes years to accrue in individual frogs. Interestingly, inhibition by La Selva frogs indicates that tremendous quantities of alkaloids, such as those present in frogs from Finca los Nacientes and Tortuguero, are not necessary for comparable protection.

In the present study, alkaloid diversity varied considerably, from an average of 26 unique alkaloids in Gandoca frogs to 54

unique alkaloids in Finca los Nacientes frogs, although Tortuguero, La Selva, and Río Palmas frogs contained the average 38 unique alkaloids per frog. The alkaloid defenses of frogs from La Selva and Gandoca were virtually identical in terms of average quantity, but Gandoca alkaloids were about 25% less diverse than those from La Selva, representing a unique opportunity to compare microbial growth inhibition and alkaloid diversity with quantity held constant. In both OD and CFU assays with *A. hydrophila*, Gandoca frog alkaloids were the least inhibitory of all populations, in accord with their low diversity. In contrast, La Selva frog alkaloid cocktails inhibited *A. hydrophila* virtually as well as those from Finca los Nacientes and Tortuguero, despite having 300% lower quantities of less diverse alkaloids. These findings may indicate that microbial inhibition increases at a progressively reduced rate after a certain threshold in the quantity and/or diversity of alkaloids (e.g., average quantity and diversity of La Selva frogs), and also clearly demonstrates that increased alkaloid diversity has the potential to considerably enhance microbial growth inhibition.

In certain cases, however, a high diversity of alkaloids may not be enough to compensate for low quantities of alkaloids. For instance, the alkaloid cocktails of Río Palmas frogs were marginally more diverse than those from Tortuguero frogs, yet inhibited growth of *K. pneumoniae* the least effectively of all populations. Such substantially reduced growth inhibition of *K. pneumoniae* by Río Palmas frog alkaloids was most likely a result of having the lowest quantities of alkaloids of all populations, despite their high diversity. In this case, the average quantity of alkaloids in Río Palmas frogs may have been comparatively far below the hypothetical threshold quantity after which gains in microbial inhibition diminish. Moreover, if such a threshold quantity exists, then particular alkaloid classes are likely required rather than any arbitrary suite of alkaloids. Coincidentally, the already low quantities of alkaloids in Río Palmas frogs were also evenly distributed among the identified alkaloid structural classes, which was not the case in other populations. For example, the five most abundant alkaloids among Río Palmas frogs were each of a different structural class and comprised just 28% of the population's total ~3700 µg of alkaloids, whereas the five most abundant alkaloids in the more inhibitory Tortuguero frog alkaloid cocktails comprised just the two most common structural classes (5,8-disubstituted and 5,6,8-trisubstituted indolizidines) and 62% of Tortuguero's total ~14,000 µg of alkaloids. Accordingly, optimal growth inhibition likely requires certain quantities of specific types of alkaloids, although even comparatively low quantities (e.g., Río Palmas) and diversities (e.g., Gandoca) of alkaloids can inhibit the growth of these pathogens to an extent.

Alkaloid defenses from all five populations of *O. pumilio* effectively inhibited growth of the opportunistic, Gram-negative bacteria *A. hydrophila* and *K. pneumoniae*, although

OD and CFU assays suggest slightly different interpretations of the results. For example, growth inhibition approximated by OD assays indicated that certain populations of *O. pumilio*, such as Río Palmas and Gandoca, did not effectively inhibit the growth of these pathogens compared to control, which may indicate that they are susceptible to infection. However, as evident from CFU assays, treatment with alkaloid cocktails from these same locations significantly reduced the amount of viable bacterial cells persisting after 24.5 hr in *A. hydrophila* and *K. pneumoniae* cultures compared to control, which suggests that alkaloid cocktails from Río Palmas and Gandoca are effective antimicrobial agents. These observations of effective growth inhibition in CFU assays but not OD assays demonstrate the value of CFU assays, and may in fact provide a more ecologically valid assessment of antimicrobial efficacy. The discrepancies in effective growth inhibition between OD and CFU assays for Río Palmas and Gandoca frogs are likely explained by each assay's measured growth variable; OD assays measured the density of both live and dead cells suspended in solution, whereas CFU assays evaluated the quantity of viable (i.e., able to multiply via binary fission) bacterial cells remaining after treatment with *O. pumilio* alkaloid cocktails. Moreover, OD assays and CFU assays were strongly correlated, and the relative patterns of growth inhibition among populations are nearly identical between OD assays and CFU assays, wherein the most (Finca los Nacientes and/or Tortuguero) and least inhibitory (Río Palmas and/or Gandoca) populations were consistent between assays for each pathogen.

On the basis of the relationships among *O. pumilio* alkaloid quantity, diversity and microbial growth inhibition in the present study, the efficacy of alkaloid-based microbial growth inhibition appears to increase most notably when alkaloid defenses comprise sufficient (e.g., >400 µg per frog skin) quantities of diverse suites of alkaloids (e.g., >30 individual alkaloids), and when there are high quantities of certain types of alkaloids. In other words, although the general quantity and diversity of alkaloids in poison frog defenses undoubtedly drive variation among populations, the presence of specific types of alkaloids also likely plays an important role in microbial growth inhibition. Overall, these findings support the hypothesis that alkaloid defenses from distinct populations of *O. pumilio* inhibit pathogens to different degrees, which may influence how those populations respond to emerging infectious pathogens.

Individual alkaloids inhibit the growth of the bacteria *B. subtilis* and *E. coli* to varying degrees or not at all (Macfoy et al. 2005), which indicates that alkaloid types differ in their inhibitory activity against specific types of microbes (e.g., Gram-negative or Gram-positive bacteria, or various fungi, viruses, etc.). For instance, Macfoy et al. (2005) report

that nearly all individual assayed alkaloids inhibited the growth of *B. subtilis*, whereas only a piperidine alkaloid inhibited *E. coli*. Similarly, certain individual pyrrolidine, piperidine, decahydroquinoline, and pumiliotoxin alkaloids effectively inhibited growth of the fungus *C. albicans* (Macfoy et al. 2005). In contrast, Mina et al. (2015) reported that *C. albicans* was inhibited only by Isla Solarte (Panama) frog alkaloid cocktails containing primarily decahydroquinolines and pumiliotoxin alkaloids, although alkaloid cocktails from Puerto Viejo (Costa Rica) frogs, consisting of large amounts of decahydroquinolines and histrionicotoxins, also inhibited *C. albicans* somewhat effectively. These findings are consistent with results from the present study in concluding that certain alkaloid classes such as decahydroquinolines and histrionicotoxins, which are unbranched-chain alkaloids derived from ants (Spande et al. 1999; Daly et al. 2005; Jones et al. 2012), may be particularly inhibitory. However, microbial inhibition by alkaloid defenses appears to be influenced by more than just the presence of specific alkaloid types.

Important complementary and/or synergistic relationships among sequestered alkaloids may enhance protection from pathogens in poison frogs. For example, while the aforementioned individual alkaloids differ in terms of which types of bacteria they can inhibit (Macfoy et al. 2005), natural alkaloid cocktails from *O. pumilio* that contain a diversity of alkaloids consistently inhibit the same bacteria (Mina et al. 2015). In synthesized amphibian chemical defenses, such as antimicrobial peptides, a combination of various peptides is more effective than the individual peptides at inhibiting pathogenic microbes, including *A. hydrophila* (Rollins-Smith et al. 2002a, b; Rollins-Smith 2005; Rosenfeld et al. 2006; Conlon 2011a). Additionally, more diverse suites of peptides have been proposed to provide protection from a wider range of pathogens by enhancing other peptides' inhibitory activity (i.e., synergy), or by different peptides or classes targeting different classes of microbes (i.e., complementary) (Nicolas and Mor 1995; Simmaco et al. 1998b; Zasloff 2002; Tennessen et al. 2009). Based on previous alkaloid-based microbial inhibition research and relationships between inhibition and alkaloid composition from the present study, similar synergistic and/or complementary effects may occur in sequestered alkaloid defenses, where comparatively diverse alkaloid cocktails provide enhanced protection from a wider range of pathogens.

Much like antimicrobial peptides, alkaloid defenses in poison frogs may provide dual predator and pathogen protection. For example, prospective predators that sample and ultimately reject poison frogs as a prey item (Brodie and Tumbarello 1978; Fritz et al. 1981; Szelistowski 1985; Gray et al. 2010; Stynoski et al. 2014; Hantak et al. 2016; Hovey et al. 2016; Murray et al. 2016) stimulate the secretion of defensive alkaloids onto the skin, and may inflict wounds that could be

potentially fatal if infected. In this case, poison frog alkaloid secretions could potentially reduce wound infection and inhibit the growth of non-residential microbes including pathogens. While most amphibian antimicrobial peptides exert activity through pore formation in cellular membranes (Yang et al. 2001; Brogden 2005; Diamond et al. 2009; Chen et al. 2012), more research is needed on the antimicrobial capacity and mechanism of action of poison frog alkaloids. However, it should be noted that a resident microbiota community almost certainly persists in the presence of alkaloid defenses (Culp et al. 2007; Lauer et al. 2007; Walke et al. 2015). Additionally, it is feasible that incipient infections in poison frogs may elicit a stress response that stimulates smooth muscle around granular glands, thereby inducing the secretion of additional alkaloids onto the skin to reduce the viable infection load. For instance, *Rana esculenta* and *Bombina orientalis* induce and/or upregulate synthesized antimicrobial peptide defenses after exposure to microbes, including *A. hydrophila* (Miele et al. 1998; Simmaco et al. 1998a; Mangoni et al. 2001). Sequestration of arthropod-derived alkaloids by poison frogs may therefore be analogous in some ways to synthesized peptide defenses (Zaslhoff 1987; Pask et al. 2013) and play an important role in protection from infectious pathogens, especially when the innate immune system is compromised or the frog is wounded.

Amphibians are inhabited by communities of microbes (e.g., gut-associated microbes and skin-associated microbes), some of which are symbiotic and complement their innate immune system by defending the host from infectious pathogens (Culp et al. 2007; Lauer et al. 2007, 2008; Woodhams et al. 2007a, b, c; Myers et al. 2012; Park et al. 2014; Becker et al. 2015). Conversely, certain Gram-negative gut- or skin-resident microbes that are frequently isolated from wild and captive frogs, such as *A. hydrophila* and *K. pneumoniae* (Bradford 1991; Barra et al. 1998), can cause fatal systemic infections in immunocompromised amphibians (Carr et al. 1976; Hubbard 1981). Of these microbes, *A. hydrophila* in particular has been linked to wild and captive population amphibian declines (Nyman 1986; Pearson et al. 2000; Miller et al. 2008; Hill et al. 2010). The resistance and resulting virulence of *A. hydrophila* appears to be due to its production of extracellular proteases that break down amphibian antimicrobial peptides, rendering these synthesized chemical defenses largely ineffective (Rollins-Smith et al. 2002a; Schadich and Cole 2009). Metabolites of *Phyllomedusa distincta* skin microbiota also effectively inhibited growth of *E. coli* and other microbes in an optical density-based growth inhibition assay, whereas *A. hydrophila* was most resistant to growth inhibition. Additionally, *K. pneumoniae* was not inhibited by the same metabolites, although in a separate streak assay (Brito de Assis et al. 2016). In the present study,

alkaloid cocktails from all populations of *O. pumilio* clearly inhibited the resistant pathogen *A. hydrophila* and the relatively resistant, more pathogenic, *K. pneumoniae*. The conclusion that poison frog alkaloids can reliably inhibit *A. hydrophila* may indicate that the extracellular proteases produced by the pathogen are inactive against sequestered alkaloids, especially given that those proteolytic compounds confer *A. hydrophila* resistance to antimicrobial peptides (Rollins-Smith et al. 2002a; Tennessen et al. 2009; Brito de Assis et al. 2016). This is consistent with the hypothesis that *A. hydrophila* proteases coevolved specifically with widespread amphibian chemical defenses, such as synthesized peptides or microsymbiont-derived metabolites, as an adaptation to survive on amphibian skin (Schadich and Cole 2009). Alternatively, the mechanism of action of poison frog alkaloids may simply be more effective against *A. hydrophila* and *K. pneumoniae* than that of the antimicrobial peptides and metabolites studied so far. However, the same antimicrobial peptides that do not inhibit *A. hydrophila* can effectively inhibit the pathogen most closely associated with global amphibian population declines and biodiversity loss, *Batrachochytrium dendrobatidis* (Berger et al. 1998; Rollins-Smith et al. 2002b). The inhibitory activity of sequestered alkaloid defenses against *Bd* is not yet known. Thus, although findings from the present study demonstrate that poison frog alkaloids can function as an effective defense against virulent pathogens, the inhibitory activity of alkaloid defenses against other highly-relevant pathogens remains to be determined.

Our results demonstrate that alkaloid-based chemical defenses in the dendrobatid poison frog *O. pumilio* inhibit the growth of known anuran pathogens, and may function as a defense against both predators and pathogenic microorganisms. Much like variation in alkaloid defenses is hypothesized to play a role in defense against predators (Saporito et al. 2006, 2007a; Murray et al. 2016), these findings suggest that the natural variation in poison frog alkaloid defenses influences protection from pathogenic microbes. Although the antimicrobial activity of alkaloid cocktails from other poison frog lineages have not been studied, similar variation may be observed given the comparable variation in dietary arthropods (Clark et al. 2006; Bonansea and Vaira 2007; Daly et al. 2008a; Quiroga et al. 2011; Andriamaharavo et al. 2015). However, the pathogens used in the present study do not pose a particularly significant threat to amphibian biodiversity. Future studies should aim to assess the effectiveness of sequestered alkaloid defenses from multiple poison frog lineages against pathogens associated with emerging infectious diseases and widespread amphibian population declines, such as ranaviruses and *Bd* (Pessier 2002; Whitfield et al. 2013).

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