Summer 2016

VARIATION IN ALKALOID DEFENSES OF THE DENDROBATID POISON FROG OOPHAGA PUMILIO LEAD TO DIFFERENCES IN AVAOIDANCE BY ARHTROPODS

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VARIATION IN ALKALOID DEFENSES OF THE DENDROBATID POISON FROG *OOPHAGA PUMILIO* LEAD TO DIFFERENCES IN AVOIDANCE BY ARTHROPODS

A Thesis Submitted to the
Office of Graduate Studies
College of Arts & Sciences of
John Carroll University
in Partial Fulfillment of the Requirements
for the Degree of
Master of Science

By
Sarah K. Bolton
2016

The thesis of Sarah K. Bolton is hereby accepted:

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Reader – Rebecca E. Drenovsky, Ph.D.	19 July 2016 Date
Advisor – Ralph A. Saporito, Ph.D.	07/19/2016 Date

I certify that this is the copy of the original document.

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ABSTRACT

Conspicuously colored dendrobatid frogs sequester alkaloid-based defenses from dietary arthropods, resulting in considerable alkaloid variation among populations. Although alkaloids act as a defense against predation, relatively little is known about how alkaloid variation is perceived and functions as a defense against predators. Throughout its geographic range, previous studies have found the dendrobatid frog *Oophaga pumilio* to have particularly variable alkaloids, and that differences in these alkaloids are associated with differences in toxicity to laboratory mice. Although toxicity is one measure of alkaloid variation, predator avoidance of dendrobatids might simply be due to the bitter or unpalatable nature of alkaloid defenses. Arthropods are natural predators that use chemoreception to detect prey, including frogs, and may therefore perceive variation in alkaloid profiles as differences in palatability. The goal of the present study is to gain an understanding of how arthropods respond to variable alkaloid defenses in *O. pumilio*. Frog alkaloids were sampled from individual *O. pumilio* from ten geographic locations throughout the Bocas del Toro region of Panama and the Caribbean coast of Costa Rica. Alkaloids were used in feeding bioassays with the fruit fly *Drosophila melanogaster* and the ant *Ectatomma ruidum* to investigate how arthropods respond to different suites of dendrobatid alkaloids. *Drosophila melanogaster* and *E. ruidum* feed less on frog alkaloid solutions when compared to controls, and variation in alkaloid profiles among *O. pumilio* populations result in differences in palatability. Differences in palatability were observed among populations, as well as between sexes and life stages of a single population. In particular, alkaloid quantity, diversity, and type appear to play an important and complex role in arthropod avoidance of alkaloid profiles. The findings of the present study

represent the first direct evidence of a palatability spectrum in a vertebrate that sequesters its chemical defenses from dietary sources. The presence of a palatability spectrum suggests that variable alkaloid defenses in *O. pumilio* (and likely other dendrobatids) are ecologically relevant and play an important role in natural predator-prey interactions, in particular with respect to arthropod predators.

INTRODUCTION

Chemical defenses are present in a variety of organisms and represent unique protective adaptations aimed at deterring microbial pathogens, parasites, and natural predators (Savitzky et al., 2012; Speed et al., 2012). Chemically defended organisms have a widespread distribution across many taxa, giving rise to several different classes of defensive chemicals such as terpenoids, phenolics, steroids, biogenic amines, peptides, proteins, and alkaloids (Hancock & Scott, 2000; Mithöfer & Wilhelm Boland, 2012). Most chemically defended organisms can synthesize their own defensive compounds, but others sequester defenses and are entirely dependent on external sources such as diet or symbionts for their chemical protection (Termonia et al., 2001; Hartmann et al., 2001; Williams, 2010).

Plants are well known for synthesizing a variety of chemical defenses that are used to deter pathogens and herbivores; however, certain phytophagous arthropods have evolved specialized adaptations to circumvent plant defenses and sequester them for use in their own defense (Wittstock & Gershenzon, 2002; Opitz & Müller, 2009; Savitzky et al., 2012). For example, certain brightly colored caterpillars and butterflies (e.g., buckeyes (*Junonia coenia*) and monarchs (*Danaus plexippus*)) feed on toxic plants for nutrition but sequester their host plant defenses as their own (e.g., monarchs sequestering cardiotonic steroids from milkweed plants; Nishida, 2002; Bowers, 2003). Leaf beetles in the genus *Platyphora* feed on toxic plants in Apocynaceae and sequester plant-based alkaloid chemical defenses (Hartmann et al., 2001). In addition to numerous invertebrates, certain vertebrates are also adapted to sequester chemical defenses from their diet (Savitzky et al., 2012; Saporito et al., 2012). Insectivorous birds in two genera

(*Pitohui* and *Ifrita*) from Papua New Guinea sequester alkaloid-based defenses from beetles (Dumbacher et al., 1992, 2000). North American snakes in the genus *Thamnophis* (Colubridae: Natricinae) feed on toxic newts (*Taricha*: Salamandridae) that contain tetrodotoxin (TTX), and accumulate this neurotoxin in their liver, which is hypothesized to deter predation (Savitzky et al., 2012; Williams et al., 2012). Additionally, the Asian natricine snake, *Rhabdophis tigrinus*, sequesters steroid-based bufadienolide defenses from toads that it consumes (Hutchinson et al., 2007). Anurans, however, represent the most well studied group of vertebrates that sequester defenses from their diet. Specifically, members of five different families of poison frogs have evolved the ability to use alkaloids obtained from dietary arthropods as a mode of defense. These include the dendrobatids (Dendrobatidae) from Central and South America, bufonids (*Melanophryniscus*) from South America, mantellids (*Mantella*) from Madagascar, myobatrichids (*Pseudophryne*) from Australia, and certain eleutherodacylids (*Eleutherodactylus*) from Cuba (reviewed in Saporito et al., 2012).

Vertebrates dependent on dietary sources for their chemical defenses often exhibit tremendous variation in the type and quantity of their sequestered defenses (Saporito et al., 2009; Savitzky et al., 2012; Speed et al., 2012). Variation in defense can occur geographically and temporally, and is largely attributed to differences in food availability, but also include age, size, and sex (Saporito et al., 2007a, 2012; Speed et al., 2012; Stynoski et al., 2014; Jeckel et al., 2015). For example, although the snake *R. tigrinus* possess bufadienolide-based defenses obtained from eating toads, there is one isolated island population that completely lacks these defenses because its habitat does not contain any toads (Hutchinson et al., 2012). Variation in diet for vertebrates

dependent on food sources for their chemical defense are hypothesized to result in differences in the ability of individuals, populations, and/or species to protect themselves from predation (Brower et al., 1968; Bowers, 2003; Saporito et al., 2007a; Savitzky et al., 2012); however, relatively little empirical work has tested how this variation might function as a defense against natural predators (however, see Murray et al., 2016). Recently, theoretical studies have proposed that variation in prey defenses are only important if predators are able to detect and respond to this variation (Speed et al., 2012; Summers et al., 2015). Therefore, experimental studies are necessary to fully understand the ecological and evolutionary importance of variable chemical defenses in vertebrates.

Conspicuously colored dendrobatid frogs represent a group of vertebrates that acquire defenses from dietary sources (Saporito et al., 2012). These aposematically colored frogs range from Central to South America and are known to sequester alkaloid-based chemical defenses from a diet of mites, ants, beetles, and millipedes (Daly et al., 2002; Saporito et al., 2007a, 2007b, 2009). Due to differences in the distribution, abundance, and availability of these dietary arthropods, dendrobatids often exhibit tremendous alkaloid variability within and among populations, between sexes, throughout life stages, and over time (Daly & Myers, 1967; Daly et al., 1978, 1987, 2002; Saporito et al., 2006, 2007a, 2010b, 2012; Stynoski et al., 2014a). Given the large degree of variation in chemical defenses exhibited by dendrobatids, it is possible that predators perceive differences in alkaloids as a spectrum of palatability (Fritz et al., 1981; Szelistowski, 1985; Saporito et al., 2007a; Murray et al., 2016). For example, chemically defended arthropods with variable defenses are known to exhibit 'palatability spectra' that are perceived by predators (e.g., leaf beetles in the genus *Platyphora*; Hartmann et

al., 2001). Furthermore, Brower et al. (1967, 1968) examined differences in predation upon the monarch butterfly (*Danaus plexippus*) by avian predators and found that butterflies differed in their relative 'palatability', which was attributed to differences in diet as caterpillars. Preferential feeding by predators on more palatable arthropods suggests that prey will differ in their risk of predation, with some experiencing more or less predation than others (Brower et al., 1968; Bowers, 2003; Saporito et al., 2007a). Whether or not dendrobatid frogs, with their variable sequestered defenses, exhibit a similar 'palatability spectrum' is not well understood and is the main focus of the present study.

The dendrobatid poison frog *Oophaga pumilio* is characterized by particularly variable alkaloid defenses throughout its geographic range from southern Nicaragua through northwestern Panama, differing in defense among populations, between sexes, among life stages, and over time (Saporito et al., 2006, 2007a). More than 232 different alkaloids have been identified in *O. pumilio* (categorized into 24 structural classes), and individual frogs possess between 4-44 different alkaloids (Saporito et al., 2007a). Ants and spiders are important natural predators of this species (Santos & Cannatella, 2011; Hovey et al., 2016; Murray et al., 2016), both of which use chemoreception to detect prey and therefore may be particularly sensitive to differences in alkaloid defenses. Recently, Murray et al. (2016) investigated differences in predation by bullet ants (*Paraponera clavata*) and red-legged banana spiders (*Cupiennius coccineus*) on different life stages of *O. pumilio*, and provided evidence that ant predators are sensitive to differences in alkaloid defenses among life stages. However, it is not known how alkaloid variation among populations in *O. pumilio* translates to predator avoidance. The extensive

population-level variation in chemical defenses in *O. pumilio*, coupled with the fact that their natural arthropod predators use chemoreception, makes it an ideal species to study how alkaloid variation in dendrobatids is perceived by arthropods.

Investigations on variable alkaloid defenses in *O. pumilio* have been predominantly focused on measuring alkaloid toxicity to laboratory mice in minimum lethal dose, LD_{50}, or irritability assays. Overall, these studies have shown that variation in alkaloids is related to differences in toxicity (Daly & Myers, 1967; Darst et al., 2006; Maan & Cummings, 2012). Daly & Myers (1967) investigated alkaloid toxicity in terms of minimal lethal dose for several populations of *O. pumilio* from Bocas del Toro, Panama. These assays were performed by injecting mice subcutaneously with naturally occurring alkaloids dissolved in saline, which served as a proxy for quantifying toxicity. Daly and Myers (1967) found considerable variation in toxicity among populations, of which the Isla Bastimentos population of frogs was more than 25 times more toxic than the least toxic frog population of Isla Colon. Maan & Cummings (2012) further expanded the research of Daly & Myers (1967) by adapting a slightly different model for quantifying toxicity (i.e., irritability assays), in order to study the relationship between aposematic coloration and toxicity among populations in the same island chain of Panama. These irritability assays were performed by injecting alkaloids from individual frogs into mice that were bred to sleep (CD-1 outbred strain; Harlan Laboratories), and toxicity was measured as the time it took these mice to return to sleep after being injected with alkaloids (Darst & Cummings, 2006; Darst et al., 2006; Maan & Cummings, 2012). Maan & Cummings (2012) found similar results to Daly & Myers (1967) in that there was considerable variation in toxicity among populations, of which frogs from Isla

Bastimentos were among the more toxic populations. However, the two studies also found dissimilarities among their toxicity scores for the same populations. For example, Maan & Cummings (2012) found frogs from Isla Solarte to be five times more toxic than did Daly & Myers (1967). These studies provide evidence that variation in the alkaloid defenses of *O. pumilio* leads to differences in toxicity to mice; however, mice are not natural predators of frogs, and it still remains unclear how arthropods (a natural group of predators) might perceive variable alkaloid defenses, and most importantly, how this translates to predator avoidance.

Most arthropods use chemoreception as their primary mode of foraging and detecting prey, and appear particularly sensitive to differences in bitter substances such as alkaloids (Fritz et al., 1981; Levings & Franks, 1982; Szelistowski, 1985; Lachaud, 1990; Gray et al., 2010; McGlynn et al., 2010). Fruit flies (*Drosophila melanogaster)* are a commonly used arthropod model in studies of taste perception and used specifically in studies to understand how alkaloids are perceived by arthropods (Meunier et al., 2003; Sellier et al., 2010; Devambez et al., 2013; Lee et al., 2015). Therefore, fruit flies represent a good model 'taste tester' for determining how arthropods might perceive variation in alkaloid defenses. Furthermore, the omnivorous neotropical ant, *Ectatomma ruidum* (Formicidae), was used recently to quantify the relative palatability of two species of dendrobatid frogs, *O. pumilio* and *Dendrobates auratus*, both of which are known to vary significantly in their alkaloid defenses (see Daly et al., 1984; Blanchard et al., 2014). Although *E. ruidum* is not a recorded predator of *O. pumilio*, it may serve as a good model for ant predators, such as the bullet ant (*P. clavata*) (Levings & Franks, 1982; Lachaud, 1990; Santos & Cannatella, 2011; Murray et al., 2016). Blanchard et al.

(2014) found that alkaloid defenses in one population of *O. pumilio* and *D. auratus* are considered unpalatable to the ant *E. ruidum*, and that 'palatability' differs between these two species of dendrobatids (*D. auratus* is less palatable than *O. pumilio*). Fruit flies and the ant *E. ruidium* represent good model organisms by which to study how arthropods perceive and respond to variation in the alkaloid-based defenses of *O. pumilio*.

The objective of the present study was to understand the ecological importance of variable chemical defenses in *O. pumilio* by examining differences in alkaloid palatability to two model arthropod species *D. melanogaster* and *E. ruidum*. To empirically investigate this, I – (1) used the fruit fly *D. melanogaster* as a taste tester to establish a palatability index for naturally occurring frog alkaloids among populations of *O. pumilio* in Panama, (2) examined the relationship between this measure of *O. pumilio* alkaloid palatability to previously reported *O. pumilio* alkaloid toxicity measured in mice models (Daly & Myers, 1967), and (3) used the ant *E. ruidum* as a more biologically relevant arthropod to examine how alkaloid variation within and among populations of *O. pumilio* in Costa Rica is related to palatability (i.e., a 'palatability spectrum').

METHODS

Experiment 1

Does alkaloid variation in Panamanian populations of *Oophaga pumilio* relate to differences in palatability to a fruit fly model?

Frog collection. In order to examine how alkaloid variation relates to palatability and toxicity, samples of *O. pumilio* were used that had been collected previously from the Bocas del Toro Archipelago, Panama, in 2005-2006 by R.A. Saporito as part of a larger study on alkaloid variation in *O. pumilio* (e.g., Saporito et al., 2007a, 2010a). These samples were collected from Isla Popa (9°11'57.84"N, 82°07'47.28"W), Isla Solarte (9°19'56.87"N, 82°13'07.76"W), Isla Bastimentos (9°20'20.16"N, 82°10'44.926"W), Isla San Cristobal (9°16'45.17"N, 82°17'26.56"W), and Cerro Brujo (9°12'07.20"N, 82°12'11.09"W) (10 adult frogs were collected from each population) (see Fig. 1 for map). Daly & Myers (1967) included four of these same locations (Isla Bastimentos, Isla Solarte, Isla San Cristobal, and Cerro Brujo; note: Cerro Brujo is listed as Mainland near Isla Split Hill in Daly & Myers, 1967) in their mouse minimal lethal dose assays, and found extensive variation in toxicity among these frog populations. In particular, they found one population, Isla Bastimentos, to have the most toxic frog alkaloids. The palatability data collected in the present study was compared to the toxicity data obtained in Daly & Myers (1967). All collection of *O. pumilio* was approved by the Convention on International Trade and Exportation of Species (CITES) research and collection permit (SEX/A-129-06) issued by the Panamanian government.

Alkaloid isolation (fractionation) from frog skins. From each of the five populations of *O. pumilio*, three randomly selected individuals were used (from the 10 individuals that were originally collected by R.A. Saporito), whose entire skin was removed in the field and stored at 20°C in 4 ml glass vials with a Teflon-lined lid containing 100% methanol. For each individual frog skin, two separate alkaloid fractionations were performed (n =

30 fractionations) to extract the alkaloids from the skin, one of which was used in palatability assays and the other for alkaloid identification and quantification.

For each alkaloid fractionation, 1 ml of the methanol/frog skin solution was removed from each 4 ml vial and placed in individual 15 ml conical glass vials. For fractionations used to quantify alkaloids (see methods below), 100 µl of nicotine standard (10 µg nicotine/100 µl methanol) was added to the conical vial; however, fractionations that were performed for palatability assays did not contain the nicotine standard. To the same conical vial, 50 µl of 1 N HCl was added to acidify the solution, followed by concentrating the sample with nitrogen gas until 100 µl remained. Once concentrated, equal parts of deionized water (200 µl) were added to draw the alkaloids into the aqueous layer. This solution was then extracted four times with 300 µl portions of hexane to remove fatty acids, fatty acid methyl esters, and other lipid-soluble artifacts from the solution. The remaining aqueous layer in the conical vial was then basified drop-wise with saturated $NaHCO_3$ until the pH reached 8-9. The basic solution was then extracted three times with 300 µl portions of ethyl acetate, and the top organic layer (containing the alkaloids) was transferred to a separate test tube containing anhydrous Na_2SO_4. The dried organic layer was then evaporated down to dryness by nitrogen gas, followed by re-suspending the alkaloids into an ethanol solution or 100% methanol, depending on the fractionation type. For fractionations that were used in palatability assays, 100 µl of a blue 20% sucrose/50% ethanol solution (see *Fruit fly palatability assays* below, for further discussion on the ethanol solution) was used to resuspend the alkaloids. This final solution was used to run palatability assays with fruit flies that reflected naturally occurring alkaloid concentrations (Daly et al., 1978, 1987; Saporito et al., 2007a, 2010a).

For fractionations that were used for alkaloid identification and quantification, 100 µl of 100% methanol was added to resuspend the alkaloids (see *Alkaloid characterization* below, for further details).

Fruit fly palatability assays. To test the palatability of *O. pumilio* alkaloids to fruit flies, two-choice feeding trials were conducted in which fruit flies were allowed the option to feed on two different sucrose solutions – one that contained alkaloids and one that did not (modified from Dyer et al., 2003). Previous studies have used *D. melanogaster* in multiple choice feeding trials and have demonstrated that fruit flies show no preference for different colored solutions (Meunier et al., 2003; Sellier et al., 2010), and therefore color was added to each of the treatments (alkaloid vs. no alkaloid). Fruit fly abdomens are transparent, which allowed for determining which colored solution they fed on during each trial, or in some cases a mixture of both colored solutions. Although research has shown that fruit flies do not exhibit color preference, a pilot study was conducted to determine if the fruit flies to be used in the present experiment show bias for red or blue food coloring. On the basis of this experiment, fruit flies exhibited no preference between red and blue food coloring ($t_{58} = 0.945$, $p = 0.349$), which supported the use of these two colors for non-alkaloid (control) and alkaloid treatments.

In developing the bioassay, it was important to determine if fruit flies were able to detect differences in alkaloid quantity on a biologically relevant scale and whether or not they exhibited a dose-dependent response. Using data from Saporito et al. (2010a), Stynoski et al. (2014a), and Saporito et al. (unpublished data), the average quantity of alkaloids in *O. pumilio* was calculated to be approximately 400 µg/frog, which is

approximately equivalent to a 1 μg/μl solution following an alkaloid fractionation. Most frog alkaloids are not available commercially, and obtaining adequate quantities of natural frog alkaloids was not feasible for piloting a bioassay. Therefore, synthetic decahydroquinoline (DHQ), an alkaloid class that is commonly found in skin secretions of *O. pumilio* (Saporito et al., 2007a), was used to create a 1 μg/μl solution for testing the effectiveness of the bioassay on a biologically relevant scale. To test fruit flies' ability to detect differences in palatability of DHQ at different concentrations, a two-choice feeding trial was conducted where the fruit flies had access to two sucrose solutions, one that contained 1 μg/μl DHQ and one that did not contain DHQ. On average, fruit flies significantly avoided the solution that contained the 1 μg/μl DHQ ($p < 0.001$), and when the concentration of DHQ underwent serial dilutions, fruit flies exhibited less of a preference for solution type (data not shown). The ability of fruit flies to detect differences in alkaloid concentration (quantity) and in a dose-dependent manner suggests that their ability to taste is sensitive enough to detect differences in alkaloids among individuals of *O. pumilio* at a biologically relevant concentration.

Following methods similar to these pilot experiments, a palatability assay was conducted using the naturally occurring frog alkaloids that were extracted from skins of *O. pumilio* (see *Alkaloid isolation* above). In this assay, red and blue food coloring were added to the control (no alkaloid) and treatment (alkaloid) solutions, respectively, in order to distinguish between feeding preferences during trials. Two stock solutions were made for use in the palatability assays, one for the control solution (no alkaloids) and one for the treatment solution (alkaloids). Each stock solution contained 20 ml of 20% sucrose/50% ethanol. For the control solution, 100 μl of red food coloring (Market

Pantry®) was added to one 20 ml stock solution. For the alkaloid treatment solution, 50 µl of blue food coloring (Market Pantry®) was added to the 20 ml of 20% sucrose/50% ethanol solution. The blue stock solution was used in the final step of the alkaloid fractionation to create the 100 µl of alkaloid solution that contained the naturally occurring alkaloids of an individual frog (Saporito et al., unpublished data – see *Alkaloid fractionation from frog skins* above). This procedure was performed for all 15 frogs skins, so that each treatment solution reflected an individual frogs' naturally occurring alkaloid defenses for use in the palatability assays.

Each fruit fly palatability assay was conducted using 10 *D. melanogaster* (wingless, wild type, Carolina Science) that were starved for 24 hours, were 3–11 days old (average five days old), and were grown on standard fruit fly media (Formula 4-24® Plain, Carolina Science). These 10 starved fruit flies were placed in a 9 cm petri dish (Fisherbrand, 100 mm x 15 mm, sterile, Polystyrene), lined with filter paper dampened with deionized water (to provide moisture for the fruit flies), which contained 10 µl of the control and 10 µl of the alkaloid solution on plastic cover slips (22 mm Fisherbrand® 2R Plastic Cover Slips; see Fig. 2 for experimental arena). Following the methods of previous studies (Sellier et al., 2010; Devambez et al., 2013, Saporito et al., unpublished), the fruit flies were allowed to feed on the solutions for 2 hours in the dark, and then euthanized by freezing to quantify feeding preferences. After freezing, fruit flies were examined under a dissecting microscope for the presence of red, blue, or purple (mixed) colored solutions in their abdomens and counted (Fig. 3). From this count, a palatability index for each assay was calculated to determine the relative palatability of each alkaloid solution. The palatability index is a value that ranges from -1 to +1, where zero and

positive values represent a palatable alkaloid solution and negative values indicate an unpalatable alkaloid solution relative to the control (modified from Dyer et al., 2003; Blanchard et al., 2014). This index was calculated as followed:

$$\frac{(\#\ of\ blue - \#\ of\ red - 0.5 * \#\ of\ purple)}{(\#\ of\ blue\ +\ \#\ of\ red\ +\ \#\ of\ purple)}$$

In order to examine whether or not alkaloid palatability is perceived by fruit flies in a dose-dependent manner, three alkaloid concentrations were tested for each individual frog in two-choice feeding assays, which represented 2.5%, 1.25%, and 0.625% of the total quantity of the alkaloids present in each individual frog skin sample. Each alkaloid extract from an individual frog was included in four independent replicate assays, and at three different concentrations (n = 12 for each individual frog skin extract). Palatability indices were used to (1) determine if frog alkaloids were considered unpalatable to fruit flies and if this was a dose-dependent response, (2) examine differences in frog palatability among replicates and geographic locations, (3) determine how palatability is related to alkaloid diversity and quantity, and (4) determine how palatability is related to toxicity by comparing this data to the toxicity data of Daly & Myers (1967).

Statistical analyses. *Alkaloid palatability and dose response.* One-tailed independent samples t-tests were used to test if frog alkaloids were considered unpalatable to fruit flies at each of the three concentrations (2.5%, 1.25%, 0.625%). Palatability index scores of zero or greater are considered palatable, and therefore average fruit fly palatability indices for all frogs were compared to a hypothesized mean of zero (Dyer et al., 2003;

Blanchard et al., 2014). A linear regression was used to determine if there was a dose response in palatability among concentrations.

Differences in palatability. Differences in fruit fly alkaloid palatability among *O. pumilio* populations were examined using a nested one-way ANOVA (replicates of each individual frog were treated as subsamples nested within individuals) with Tukey's post-hoc pairwise comparisons. Linear regressions were used to investigate the relationships between alkaloid palatability and frog alkaloid diversity, quantity, and toxicity (using toxicity scores measured as minimum lethal dose from Daly & Myers (1967)).

Alkaloid variation. Differences in alkaloid composition among frogs with respect to the number, quantity, and type of alkaloids were graphically visualized using non-metric multidimensional scaling (nMDS), and statistical differences in alkaloids among populations were examined with a one-way analysis of similarity (ANOSIM) for all individuals (n = 15). nMDS and ANOSIM statistics were based on Bray–Curtis similarity matrices.

All raw data were checked for normality using the Shapiro-Wilk test. All parametric statistical analyses were performed using GraphPad Prism Software (version 6.0h) and SPSS (version 14.0), and multivariate statistics were performed with PRIMER-E (version 5).

Experiment 2

Is alkaloid variation among and within Costa Rican populations of *Oophaga pumilio* related to differences in palatability to the ant *Ectatomma ruidum*?

Frog collection. In order to examine how variation in naturally occurring alkaloid defenses are related to differences in palatability, 70 samples of *O. pumilio* were collected from five different geographic locations throughout the neotropical lowlands of Costa Rica where the frogs are abundant and are known to differ greatly in their alkaloids (e.g., Saporito et al., 2007a). Samples were collected from 23 May 2015 to 17 June 2015 from the following localities, listed North to South: Tortuguero (10°35'14"N, 83°31'34"W), La Selva Biological Research Station (10°26'N, 83°59'W, Huertos plots, STR 1500), Rio Palmas (10°10'16"N, 83°36'26"W), Hone Creek (9°39'23"N, 82°47'6"W), and Gandoca (9°35'03"N, 82°37'13"W) (see Fig. 1 for map). Following the methods of Saporito et al. (2006, 2007a, & 2010a), from each of the five locations 10 adult frogs were collected (snout-to-vent length > 19.0 mm), five males and five females (with the exception of Rio Palmas, where four males and six females were collected), from a single 45 m x 45 m plot. At one location, La Selva Biological Research Station, an additional five males and five females were collected (for a total of 10 individuals of each sex) along with 10 juveniles, in order to examine differences in alkaloid palatability between frog sex and life stages.

Alkaloid sampling and fractionation from TAS samples. In the field, alkaloids were collected from frog skins using a Transcutaneous Amphibian Skin Stimulator (TAS; Grant & Land, 2002). The TAS applies a consistent electrical current that stimulates the release of alkaloids from granular glands onto the frog's dorsum, allowing for the alkaloids to be collected (Hantak et al., 2013). The TAS was applied to each frog for three minutes (Amplitude: 9V, Frequency: 50 Hz, Pulse width: 2ms), moving the

electrode up and down the frogs' dorsum between the head and thigh, holding the electrode in contact with the skin for no more than 3 seconds at a time. This technique is non-destructive and did not result in harm or death to any frogs (also see Grant & Land, 2002; Hantak et al., 2013; Saporito et al. unpublished data). At the end of the three-minute period, the skin secretions were collected by wiping the frogs' dorsum with a 6 mm circle of bibulous paper (created by hole punch) held by forceps. The frogs were wiped from head to thigh and on the dorsal and ventral sides, using as many bibulous circles as needed (average: 4.6 bibulous circles) until the frog skin was dry and all visible alkaloid was collected. The circles of bibulous paper containing alkaloids were then placed into individually marked 1.8 ml glass vials with a Teflon-lined lid containing 1 ml of 100% ethanol. Frogs were collected in the field and housed in 1 gallon Ziploc® bags with wet leaf litter from the time of capture until their release. Alkaloids were extracted from each individual frog on the day of collection and frogs were allowed a 10-20 minute recovery period after the TAS treatment. All frogs were returned alive to their point of capture the following day. The TAS samples were used to characterize the type and quantity of naturally occurring alkaloids present in each individual frog (see below) and in a series of palatability assays. All methods were approved by the John Carroll University Institutional Animal Care and Use Committee (IACUC protocol #1101) and collection of *O. pumilio* was approved by CITES research and collection permit 2015-CR1420/SJ(#S1487) issued by the Costa Rican government.

Alkaloids from *O. pumilio* collected with the TAS were used for both alkaloid characterization and ant palatability assays – 500 µl were used for alkaloid analyses and 500 µl were used in the palatability assay. A single fractionation was performed for each

individual frog (n = 66) using one of the 500 µl alkaloid solutions. Four samples were lost due to leakage in transit from Costa Rica to John Carroll University (Gandoca–two males; Hone Creek–one female; Rio Palmas–one male).

The fractionation method used was similar to the alkaloid fractionation that was performed using the whole frog skins (see *Alkaloid isolation (fractionation) from frog skins* above), with minor adjustments. Due to small amount of ethanol evaporation, each sample did not contain exactly 500 µl of solution. To account for this loss of solvent, a 1 ml stereological pipet was used to withdraw and measure the exact volume of alkaloid solution in each 1.8 ml vial. The solution was then placed into a 15 ml conical glass vial where 25 µl of 1N HCl was added to acidify the solution, as well as 100 µl of nicotine standard (10 µg/100 µl), followed by concentrating the sample with nitrogen gas until 100 µl remained. The remaining steps were identical to that of the whole frog skin alkaloid fractionation described above. Following the last step of the fractionation, the alkaloid residue was re-suspended in 100 µl of 100% methanol for alkaloid characterization.

Ant collection. Using the TAS-collected frog alkaloid samples from five locations throughout Costa Rica, ant palatability trials were conducted at La Selva Biological Research Station, which housed the necessary facilities for these experiments. Naïve *Ectatomma ruidum* were collected for use in palatability assays from various locations throughout the La Selva trail system (STR 0-10), arboretum (SURA 500-650), and lab clearing. Ants were collected each day (ca. 250-350 ants) between the hours of 0730–1400 (most often between 0800–1000), and housed in 25–35 different containers for the

duration of the 48-hour starvation period. In order to ensure that ants had no prior contact with frogs used in the present study, the frog and ant collection occurred in separate locations at La Selva. Ant nests were baited for collection with Jolly Ranchers® (variety pack). The ants were individually collected with pressure sensitive forceps and placed in small plastic containers (4 oz.) with lids, each of which housed approximately 10 ants from the same bait location. All ants placed into a single container were collected within a 2 m radius and were presumed to be members of the same nest (Lachaud, 1990). Various nests from one site were sampled each day, different sites were sampled each day, and no site was revisited more than three times throughout the study.

Ant palatability assay. Ant palatability assays were modified from Blanchard et al. (2014) in which the omnivorous ant *Ectatomma ruidum* was used in palatability assays consisting of two-choice feeding trials, which are described briefly here. In each trial, ants were presented with a control sucrose solution and an alkaloid-containing sucrose solution (Molleman et al., 2010; Blanchard et al., 2014). The number of ants that fed on each solution was recorded at sequential time increments throughout a 5-minute trial in order to establish a palatability index that ranged from -1 to +1 (similar to the methods described above for fruit flies). Blanchard et al. (2014) demonstrated that ants found the alkaloid-containing solution less palatable than the sucrose control. On the basis of this study and Saporito et al., unpublished data, a similar assay was conducted in the present study using the same ant species. In the present study, the control solution consisted of 20% sucrose/50% ethanol and the alkaloid-containing solution (treatment solutions, n = 70) consisted of 20% sucrose/50% ethanol plus naturally occurring alkaloids collected

using the TAS from an individual *O. pumilio*. The unique treatment solution created for each individual frog was presented along with a control solution in 15 separate two-choice feeding trials. In each of the 15 individual trials, ant feeding preference was determined and used to calculate a palatability index for each individual frog.

Palatability assays took place in open-faced glass petri dishes (arenas) that were 5, 6, or 7 cm in diameter. In each arena, 10 µl of each of the two solutions was placed on separate, pre-cut 1/6 pieces of a plastic cover slip (22 mm Fisherbrand® 2R Plastic Cover Slips) set approximately 3 cm apart. A single ant was placed in an individual arena with access to both the control and treatment solutions, alternating the location of the solutions between different trials. Up to three individual trials were carried out at one time (Molleman et al., 2010; see Fig. 2 for example experimental set up) for up to 5 minutes (Schulte et al., 2016), in which each ant was recorded for the number of times it sampled both control and treatment solutions, as well as the length of time it took for the ant to feed or "choose" one of the two solutions presented. Each palatability assay was terminated when the ant "chose" one of the two solutions presented, or at the end of the 5-minute time period. If the ant did not choose a feeding solution within the 5-minute time frame, the trial was discarded and repeated with a new ant. "Sampling" was considered any direct contact the ant had with its mandibles or antenna with a solution that did not result in the ant feeding or "choosing" the solution. An ant was considered to "choose" a solution if the ant's mandibles remained in constant contact with the solution for more than 3 seconds or if the ant was observed carrying a droplet of the solution (Schulte et al., 2016). If an ant was carrying a solution, it was presumed that the ant would feed on that solution (Breed et al., 1990; Lachaud, 1990).

From each trial, the solution that each ant "chose" was recorded, and used to calculate a palatability index for each individual frog. A total of 15 individual trials (using the same alkaloid solution from the same frog) were used to calculate a single average palatability index for each frog's unique alkaloid mixture. Similar to the palatability index for the fruit fly assays (see above), the index was calculated as follows:

$$\frac{(\#\ of\ ants\ fed\ on\ alkaloid\ sol. -\ \#\ of\ ants\ fed\ on\ control\ sol.)}{total\ \#\ of\ ants}$$

In order to examine whether or not alkaloid palatability is perceived by *E. ruidum* in a dose-dependent manner, three TAS alkaloid concentrations were tested for each individual frog, which represented 0.5%, 0.25%, and 0.167% of the total alkaloid quantity collected with the TAS. Each alkaloid extract from an individual frog was included in 15 independent trials, and at three different concentrations (n = 45 for each individual TAS extract). All 15 trials per individual frog were conducted in the same sitting due to logistic constraints, with each trial using a single ant from a randomly chosen nest to decrease the chance of bias due to ant nest. The order at which each frog sample was used in a trial was randomly selected among individuals, geographic locations, and concentrations. Palatability indices were used to (1) determine if frog alkaloids were considered unpalatable to ants and if this was a dose-dependent response, (2) examine differences in frog palatability within and among geographic locations as well as between life-stages and sexes, and (3) determine how palatability is related to alkaloid diversity and quantity.

(1) Differences in alkaloids and palatability among Costa Rican populations

Statistical analyses. *Alkaloid palatability and dose response.* One-tailed independent samples t-tests were used to test if frog alkaloids were considered unpalatable to ants at each concentration (0.5%, 0.25%, 0.167%). Palatability index scores of zero or greater are considered palatable, and therefore average palatability indices for all frog extracts were compared to a hypothesized mean of zero (Dyer et al., 2003; Blanchard et al., 2014). A linear regression was used to determine if there was a dose response in palatability among concentrations.

Differences in palatability. Differences in frog alkaloid palatability among populations were examined using a one-way ANOVA with Tukey's post-hoc pairwise comparisons. Linear regressions were used to investigate the relationships between alkaloid palatability and frog alkaloid diversity and quantity.

Alkaloid variation. Differences in alkaloid profiles with respect to the number, quantity, and type of alkaloids, were graphically represented using non-metric multidimensional scaling (nMDS), and statistical differences in alkaloids among populations were examined with a one-way analysis of similarity (ANOSIM) for all individuals that had alkaloid data (n = 46). nMDS and ANOSIM statistics were based on Bray–Curtis similarity matrices.

All raw data were checked for normality using the Shapiro-Wilk test. All parametric statistical analyses were performed using GraphPad Prism Software (version 6.0h) and SPSS (version 14.0), and multivariate statistics were performed with PRIMER-E (version 5).

(2) Differences in alkaloids and palatability among sexes and life stages

Statistical analyses. *Alkaloid palatability and dose response.* One-tailed independent samples t-tests were used to test if frog alkaloids were considered unpalatable to ants at each concentration (0.5%, 0.25%, 0.167%) for both sexes and life stages. Palatability index scores of zero or greater are considered palatable, and therefore average palatability indices for all frogs were compared to a hypothesized mean of zero (Dyer et al., 2003; Blanchard et al., 2014). A linear regression was used to determine if there was a dose response in palatability among concentrations.

Differences in palatability. Differences in frog alkaloid palatability among sexes and life stages were examined using a one-way ANOVA with Tukey's post-hoc pairwise comparisons. Linear regressions were used to investigate the relationships between alkaloid palatability and frog alkaloid diversity and quantity.

Alkaloid variation. Differences in alkaloid profiles with respect to the number, quantity and type of alkaloids, were graphically represented using non-metric multidimensional scaling (nMDS), and statistical differences in alkaloids among sexes and life-stages were examined with a one-way analysis of similarity (ANOSIM) for all individuals (n = 30). nMDS and ANOSIM statistics were based on Bray–Curtis similarity matrices. A univariate ANOVA with Tukey's post-hoc pairwise comparisons was used to examine differences in alkaloid quantity and diversity among sexes and life stages.

Alkaloid characterization. All alkaloids from individual alkaloid fractionations (whole skin and TAS samples; see above for details) were identified and quantified using Gas Chromatography-Mass Spectrometry (GC-MS)(Saporito et al., 2010a). The samples were

analyzed on the GC-MS instrument using a temperature program from 100 to 280°C at the rate of 10°C per minute with helium as a carrier gas (1 ml/min). The GC-MS was a Varian 3900 GC coupled with a Varian Saturn 2100 T ion trap MS with a 30 m x 0.25 mm i.d. Varian Factor Four VF-5 ms fused silica column. All alkaloid samples were analyzed using both electron impact mass spectrometry (EI-MS) and chemical ionization mass spectrometry (CI-MS), with methanol as the ionizing reagent.

Alkaloids isolated from whole skin fractionations were manually injected into the instrument using 1 µl of the final 100 µl alkaloid extract, whereas alkaloids isolated from TAS samples were analyzed by injecting 2 µl of the 100 µl final alkaloid extract using the auto-sampler function on the GC-MS. Each individual frog extract (n = 15 for whole frog skin fractionations; n = 66 for TAS fractionations) was run in triplicate on EI-MS for identification and quantification purposes (n = 45 for whole skin fractionations; n = 198 for TAS fractionations) and once on CI-MS for alkaloid identification, resulting in a total of 324 individual runs on the GC-MS. Individual alkaloids for each run on the GC-MS were identified by comparing retention times and mass spectral data to known alkaloids found in dendrobatids (see Daly et al., 2005; Saporito et al., 2006; Hovey, 2016). Dendrobatid alkaloids have been assigned a coding system with boldface numbers and letters that distinguish different alkaloids by molecular weight.

Alkaloid quantities for each individual frog were calculated by comparing the peak area of each alkaloid to the peak area of the nicotine standard using a Varian MS Workstation V.6.9 SPI. Only alkaloids that were present in quantities $\geq$ 0.5 µg were included in the analyses of whole frog skins, whereas alkaloids that were present in quantities $\geq$ 0.01 µg were included in the analyses for TAS samples. In the few instances

in which an alkaloid was not present in all triplicate EI-MS analyses (due to its extremely low abundance in a frog skin), the individual alkaloid was removed from the analysis.

RESULTS

Experiment 1

Does alkaloid variation in Panamanian populations of *Oophaga pumilio* relate to differences in palatability to a fruit fly model?

Alkaloid palatability and dose response. Frog alkaloids were significantly unpalatable to fruit flies at all three concentrations 2.5% ($t = 16.16$, $df = 14$, $p \leq 0.001$), 1.25% ($t = 12.78$, $df = 14$, $p \leq 0.001$), and 0.625% ($t = 4.68$, $df = 14$, $p \leq 0.001$). Furthermore, there was a significant dose response in palatability among concentrations ($F_{1,43} = 11.51$, $p = 0.002$, $R^2 = 0.21$), with the highest concentration of alkaloids being the most unpalatable and the lowest concentration of alkaloids being the most palatable. Given the high level of unpalatability for the highest concentration of alkaloids, the intermediate concentration of 1.25% was used for all remaining fruit fly analyses.

Differences in palatability. There were significant differences in palatability among populations of *O. pumilio* ($F_{4,10} = 9.53$, $p = 0.002$), with no differences in palatability among individual subsamples ($F_{10,45} = 0.467$, $p = 0.902$). The average population palatability indices (PI) ranged from -1.00 to -0.53, where Cerro Brujo, Isla Cristobal, and Isla Solarte were the most unpalatable frog locations. Pairwise comparisons showed significant differences in palatability between Isla Popa ($p < 0.02$)

and Isla Bastimentos ($p < 0.01$) when compared to Cerro Brujo, Isla Cristobal, and Isla Solarte, respectively (Fig. 4).

Alkaloid variation. GC-MS analysis of *O. pumilio* skin extracts from five different populations (n = 15 frogs) in Bocas del Toro, Panama led to the detection of 157 unique alkaloids (including isomers) organized into 18 structural classes (Table 1). Alkaloid composition was significantly different among *O. pumilio* populations (Global $R = 0.99$, $p = 0.001$), and each of the five locations were significantly different from each other (Global $R \geq 0.92$, $p = 0.001$; Fig. 5). Frogs contained an average of 20-45 different alkaloids among populations, and the most common alkaloid (present in each individual from all populations) was the mite derived alkaloid 5,8-disubstituted indolizidine (5,8-I) **205A**. In general, the most widespread alkaloids which composed 48% of alkaloid quantity in Panamanian *O. pumilio* were 5,8-Is and 5,6,8-trisubstituted indolizidines (5,6,8-I), which have branched carbon skeletons and are of oribatid mite origin (Takada et al., 2005; Saporito et al., 2007b, 2015). Additionally, alkaloids with unbranched carbon skeletons are derived from myrmicine ants, such as decahydroquinolines (DHQ) and 3,5-disubstituted pyrrolizidines (3,5-P; Jones et al., 1999; Spande et al., 1999; Daly et al., 2002; Saporito et al., 2007a, 2007b, 2009, 2015) and composed 23% of Panamanian alkaloid quantity. The widespread abundance of mite-derived 5,8-Is and 5,6,8-Is and ant-derived DHQs and 3,5-Ps in *O. pumilio* throughout Panama is consistent with previous reports of alkaloid abundance (Daly et al., 1987; Saporito et al., 2006; 2007a) and the predominance of mites and ants in the diet of *O. pumilio* (Donnelly, 1991). Although 5,8-Is, 5,6,8-Is, DHQs, and 3,5-Ps were the most common and widespread alkaloids, a variety of other alkaloids such as pumiliotoxins (PTX), allopumiliotoxins (aPTX), Tricyclics

(Tri), and Unclassified alkaloids (Unclass), contributed to the extensive variation observed among Panamanian populations of *O. pumilio*. Previous studies have demonstrated similar levels of alkaloids variation among some of these same populations (e.g. Saporito et al., 2006, 2007a; Daly et al., 1987, 2000). The average quantity and number of alkaloids, as well as the five most abundant alkaloids and their alkaloid classes in frogs from each of the five locations are indicated in Table 2.

Relationship between palatability and alkaloid composition. There was no relationship between alkaloid palatability and alkaloid quantity ($F_{1,13} = 2.65$, $p = 0.128$, $R^2 = 0.17$; Fig. 6); however, there was a significant negative relationship between alkaloid palatability and alkaloid diversity ($F_{1,13} = 15.87$, $p = 0.002$, $R^2 = 0.55$; Fig. 7), suggesting that diversity is a better predictor of alkaloid palatability in these populations with a fruit fly model.

Relationship between alkaloid palatability and toxicity. Alkaloid palatability, as measured by fruit fly palatability assay, was compared to the minimum lethal dose (toxicity) values reported in Daly & Myers (1967) for frogs from four locations (Isla Bastimentos, Isla Solarte, Isla Cristobal, and Cerro Brujo (Mainland near Isla Split Hill in Daly & Myers, 1967)). There was no significant relationship between alkaloid palatability and alkaloid toxicity ($F_{1,2} = 1.77$, $p = 0.315$; Fig. 8), suggesting that alkaloid palatability is not related to toxicity.

Experiment 2

Is alkaloid variation among and within Costa Rican populations of *Oophaga pumilio* related to differences in palatability to the ant *Ectatomma ruidum*?

(1) Differences in alkaloids and palatability among Costa Rican populations

Alkaloid palatability and dose response. Frog alkaloids were significantly unpalatable to ants at all three concentrations 0.5% ($t = 10.14$, $df = 49$, $p \leq 0.001$), 0.25% ($t = 5.79$, $df = 49$, $p \leq 0.001$), and 0.167% ($t = 3.96$, $df = 49$, $p \leq 0.001$). Furthermore, there was a significant dose response in palatability among concentrations ($F_{1,148} = 23.78$, $p \leq 0.001$, $R^2 = 0.14$), with the highest concentration of alkaloids being the most unpalatable and the lowest concentration of alkaloids being the most palatable. The quantity of alkaloids obtained using the TAS was less than the total quantity present in an individual frog skin (ranging from 25-50% of the total quantity; Seiter, Bolton, & Saporito, unpublished data); therefore, the maximum concentration of 0.5% was used for the remainder of the analyses.

Differences in palatability. There were significant differences in palatability among populations of *O. pumilio* ($F_{4,45} = 2.77$, $p = 0.038$). The average population palatability indices (PI) ranged from - 0.49 to - 0.16, where La Selva was the most palatable population with an average PI of - 0.16. Pairwise comparisons showed significant differences in palatability between La Selva and each of the four other populations (Tortuguero, Rio Palmas, Hone Creek, and Gandoca $p < 0.03$; Fig. 9).

Alkaloid variation. GC-MS analysis of *O. pumilio* TAS extracts from five different populations ($n = 46$ frogs) in Costa Rica led to the detection of 336 unique alkaloids (including isomers) organized into 22 different structural classes (Table 1). Alkaloid composition was significantly different among frog locations in Costa Rica (Global $R = 0.94$, $p = 0.001$) and each of the five locations were significantly different from each other (Global $R \geq 0.85$, $p = 0.001$; Fig. 10). Frogs contained an average of 11-

60 different alkaloids among populations, and the most frequently detected alkaloids in Costa Rica were the mite derived alkaloid 5,6,8-I **223A** and ant derived 3,5-P **223H**. Costa Rican populations are dominated by 5,8-Is and 5,6,8-Is, which have branched carbon skeletons and are of oribatid mite origin (Takada et al., 2005; Saporito et al., 2007b, 2015). Additionally, alkaloids with unbranched carbon skeletons and are derived from myrmicine ants, such as DHQs and 3,5-Ps (Jones et al., 1999; Spande et al., 1999; Daly et al., 2002; Saporito et al., 2007a; 2007b; 2009; 2015), made up 21% of Costa Rican alkaloid quantity. The widespread abundance of mite-derived 5,8-Is and 5,6,8-Is and ant-derived DHQs and 3,5-Ps in *O. pumilio* throughout Costa Rica is consistent with previous reports of alkaloid abundance (Daly et al., 1987; Saporito et al., 2006; 2007a) and the predominance of mites and ants in the diet of *O. pumilio* (Donnelly, 1991). Previous studies have demonstrated similar levels of alkaloids variation among some of these same populations (e.g., Saporito et al., 2006, 2007a; Daly et al., 1987, 2000). The average quantity and number of alkaloids, as well as the five most abundant alkaloids and their alkaloid classes in frogs from each of the five locations are indicated in Table 3.

Relationship between palatability and alkaloid composition. There was a significant negative relationship between alkaloid palatability and alkaloid quantity ($F_{1,44} = 23.78$, $p < 0.001$, $R^2 = 0.35$; Fig. 11) and alkaloid diversity ($F_{1,44} = 9.55$, $p = 0.004$, $R^2 = 0.18$; Fig. 12), suggesting that both quantity and diversity are predictors of alkaloid palatability in these populations to *E. ruidum*.

(2) Differences in alkaloids and palatability among sexes and life stages

Alkaloid palatability and dose response. Frog alkaloids were significantly unpalatable to ants at the 0.5% ($t = 5.04$, $df = 29$, $p < 0.001$) and 0.25% ($t = 2.66$, $df = 29$, $p = 0.013$) concentrations, but not at the 0.167% concentration ($t = 1.61$, $df = 29$, $p = 0.117$). Furthermore, there was a significant dose response in palatability among concentrations, with the highest concentration of alkaloids being the most unpalatable and the lowest concentration of alkaloids being the most palatable ($F_{1,88} = 5.92$, $p = 0.017$; $R^2 = 0.06$). The 0.5% concentration was much more unpalatable when compared to the other concentrations, and therefore the intermediate concentration of 0.25% was used for all remaining analyses.

Differences in palatability. There were significant differences in palatability among sexes and life stages ($F_{2,27} = 6.02$, $p = 0.007$). The average palatability indices (PI) for females, males, and juveniles ranged from - 0.35, 0.0, and -0.06, respectively. Pairwise comparisons showed significant differences in palatability between females and males ($p = 0.009$) as well as between females and juveniles ($p = 0.031$) (Fig. 13).

Alkaloid variation. GC-MS analysis of ten male, ten female, and ten juvenile *O. pumilio* from La Selva ($n = 30$ frogs) in Costa Rica led to the detection of 98 unique alkaloids (including isomers) organized into 16 different structural classes. La Selva was in general dominated by mite derived 5,8-Is (**195I** and **207A)** as well as Unclass **247L**. Alkaloid composition was significantly different among frog sexes and life stages (Global $R = 0.33$, $p = 0.001$), with females, males, and juveniles being significantly different from each other (Global $R \geq 0.23$; $p \leq 0.009$ for all comparisons; Fig. 14).

There were significant differences in alkaloid quantity among females, males, and juvenile *O. pumilio* ($F_{2,27} = 9.82$, $p = 0.001$). The average alkaloid quantity for females, males, and juveniles was 36 μg, 7 μg, and 2 μg, respectively. Pairwise comparisons showed significant differences in alkaloid quantity between females and males ($p = 0.002$) as well as between females and juveniles ($p < 0.001$)(Fig. 15, Table 4). There were significant differences in alkaloid diversity among females, males, and juvenile *O. pumilio* ($F_{2,27} = 21.36$, $p < 0.001$). The average alkaloid diversity for females, males, and juveniles was 30, 17, and 8, respectively. Pairwise comparisons showed significant differences in alkaloid diversity among females, males, and juveniles ($p \leq 0.013$)(Fig. 16, Table 4).

Relationship between palatability and alkaloid composition. There was a significant negative relationship between alkaloid palatability and alkaloid quantity ($F_{1,28} = 6.38$, $p = 0.018$, $R^2 = 0.19$; Fig. 17) and alkaloid diversity ($F_{1,28} = 6.10$, $p = 0.020$, $R^2 = 0.18$; Fig. 18), suggesting that both quantity and diversity are predictors of alkaloid palatability within this population to *E. ruidum*.

Discussion

Organisms that sequester chemical defenses from dietary sources typically exhibit extensive variation in both their quantity and type of defensive chemicals; however, there is little understanding of how this variation, particularly in vertebrates, is perceived and acted upon by potential predators (Bowers, 1992; Speed et al., 2012; Saporito et al., 2012). Dendrobatid frogs sequester a diversity of alkaloids from their diet, and possess significant variation in the quantity and type of alkaloid defenses (e.g., Saporito et al.,

2007a; Stynoski et al., 2014a; Jeckel et al., 2015). Arthropods are known predators upon dendrobatids (see Supplemental Table in Santos & Cannatella, 2011), and the results of the present study suggest that fruit flies (*D. melanogaster*) and ants (*E. ruidum*) find alkaloids of the dendrobatid frog *O. pumilio* to be unpalatable. Furthermore, the extensive differences in alkaloid defenses within and among populations of *O. pumilio* are largely perceived by these same arthropods as differences in palatability (i.e., a palatability spectrum). Although *D. melanogaster* and *E. ruidum* are not natural predators of *O. pumilio*, these findings suggest that arthropods in general (and likely arthropod predators) can perceive and respond differentially to variable alkaloid defenses. Phytophagous arthropods that sequester their variable chemical defenses from host plants are well known to differ in their palatability to natural predators, which has been shown to result in differential predation by both vertebrate and invertebrate predators (Brower, 1967, 1968; Bowers, 2003; Hartmann et al., 2001). The findings of the present study represent the first direct evidence for the presence of a palatability spectrum in a vertebrate that sequesters its chemical defenses from dietary sources. The presence of a palatability spectrum suggests that variable alkaloid defenses in *O. pumilio* (and likely other dendrobatids) are ecologically relevant and play an important role in natural predator-prey interactions, in particular with respect to arthropod predators.

Alkaloid palatability to arthropods

Overall, the quantity, diversity, and type of alkaloid defenses in *O. pumilio* each contributed to the observed differences in palatability within and among frog populations from Panama and Costa Rica. A strong dose response was observed in which higher

concentrations of alkaloids were considered less palatable to fruit flies and ants, suggesting that alkaloid quantity in *O. pumilio* is directly related to palatability. Alkaloid quantity is highly correlated with alkaloid diversity (Saporito et al., 2007a, 2010a), and it appears that both variables are important in alkaloid avoidance. Alkaloid quantity was a significant predictor of palatability for *E. ruidum*, in which populations of *O. pumilio* with more alkaloids were avoided more strongly by ants. Although populations of frogs that contained the highest quantity of alkaloids were also strongly avoided by *D. melanogaster*, high alkaloid diversity was a better predictor of palatability when compared to quantity for fruit flies. For both arthropod species, however, frog populations with the fewest alkaloid defenses (quantity and diversity) were always the most palatable. For example, frogs from Isla Popa contained about half the quantity of alkaloids when compared to all other Panamanian populations, and frogs from La Selva contained on average five to ten-fold less alkaloids when compared to the other Costa Rican populations. In both cases, arthropods found frogs from these populations to be the most palatable. Frogs from all other Panamanian and Costa Rican populations contained larger quantities of alkaloids, and were avoided more strongly by the both arthropods. With respect to alkaloid diversity, frogs from Isla Bastimentos and Isla Popa had the lowest alkaloid diversity (26 and 19 alkaloids, respectively), and were the most palatable Panamanian populations. Furthermore, and demonstrating the complexities between alkaloid quantity and diversity, frogs from Isla Bastimentos and Cerro Brujo shared similar quantities of alkaloids, but frogs from Cerro Brujo were considered significantly more unpalatable. Although alkaloid quantity was equivalent between these two locations, frogs from Cerro Brujo contained nearly double the alkaloid diversity (43

alkaloids) when compared to Isla Bastimentos (26 alkaloids). A similar pattern was observed among Costa Rican frogs, where frogs from Rio Palmas contained an average of 35 alkaloids, and were equally unpalatable to other populations that did not contain the same diversity, but instead possessed 1.5-2 times the quantity of alkaloids. Similarly, the predatory orb-weaving spider *Nephila clavipes* avoids the phytophagous arctiine moth *Utethesia ornatrix* that sequesters pyrrolizidine alkaloids from host plants and avoidance is dependent on alkaloid quantity and type (Silva & Trigo, 2002; Martins et al., 2015). Spiders avoid pyrrolizidine alkaloids in a dose-dependent manner such that large quantities render moths completely protected; however, similar to the present study, there were also differences in predator avoidance (independent of quantity) that are related to alkaloid diversity and alkaloid type (Silva & Trigo, 2002).

Alkaloid defenses of *O. pumilio* are also known to vary within populations (Daly et al., 1994; Saporito et al., 2010a, 2012; Stynoski et al., 2014a, 2014b), and in the current study, differences in alkaloid defenses and palatability were observed between sexes and life stages for one frog population. Assays with the ant *E. ruidum* at La Selva, Costa Rica found differences in palatability that were largely attributed to differences in alkaloid quantity. Female *O. pumilio* were considered more unpalatable to ants when compared to males and juveniles, both of which contained more than five-fold lower quantities of alkaloids. Murray et al. (2016) recently demonstrated that bullet ants (*Paraponera clavata*) could detect differences in the quantity of alkaloid defenses between adult and juvenile *O. pumilio*, resulting in higher levels of bullet ant predation upon juveniles. In the present study, juveniles and males had lower quantities of alkaloids and were considered more palatable to *E. ruidum*. Alkaloid quantity appears to be an

important predictor for understanding how strongly *O. pumilio* will be avoided by ant predators, both among populations as well as between sexes and life stages.

Different alkaloids are known to vary in their toxicity to certain vertebrates and invertebrates (Daly & Spande, 1986; Daly et al., 1994; Sellier et al., 2010; Weldon et al., 2013; see Table 21.2 in Santos et al., 2016), and therefore alkaloid type is likely related to arthropod palatability. Dendrobatid frogs obtain their alkaloid defenses by consuming a diversity of alkaloid-containing mites, ants, beetles, and millipedes (Donnelly 1991, Saporito et al., 2007a, 2009, 2012, 2015), and the nature of these different alkaloid sources may contribute to differences in palatability. The two most palatable frog populations in Panama were Isla Bastimentos and Isla Popa, which were dominated by mite alkaloids including 5,8-disubstituted indolizidines (5,8-Is), 5,6,8-trisubstituted indolizidines (5,6,8-Is), and 1,4-disbustituted quinolizidines (1,4-Qs). The most palatable population in Costa Rica was La Selva, which was also dominated by mite-derived alkaloids such as 5,8-disubstituted indolizidines. Weldon et al. (2013) recently demonstrated that 5,8-disubstituted indolizidines had the lowest levels of contact toxicity to the fire ant *Solenopsis invicata*, which is also consistent with toxicity scores for these alkaloids using LD_{50} assays on laboratory mice (Daly & Spande, 1986; see Table 21.2 in Santos et al., 2016). Collectively, these data suggest that frog populations dominated by mite-derived alkaloids might be more palatable to certain arthropod predators. Conversely, some populations of *O. pumilio* were dominated by ant-derived alkaloids, and these populations tended to be more unpalatable to both fruit flies and ants. For example, frogs from Rio Palmas, Costa Rica, had a relatively low average alkaloid quantity of 50 μg per frog, however, were dominated by ant-derived alkaloids such as

3,5-disubstituted indolizidines (3,5-Is) and 3,5-disubstituted pyrrolidines (3,5-Ps). Additionally, Isla Cristobal, Panama was largely dominated by two ant-derived alkaloids, (3,5-P) *trans*-**223B** and decahydroquinoline (DHQ) *trans*-**223F**, representing 42% of the total alkaloid quantity in these frogs. Isla Cristobal was the only Panamanian population that was completely avoided, and lacked large amounts of mite-derived pumiliotoxins or allopumiliotoxins. These findings suggest that populations of *O. pumilio* that were dominated by ant alkaloids, in general, were considered more unpalatable to arthropods. Alkaloids are commonly used as a chemical defense between ant species (Blum, 1981; Berenbaum & Seigler, 1992; Jones et al., 1999), and therefore frogs containing ant alkaloids may serve as a more effective defense towards predatory ants such as the bullet ant *Paraponera clavata* (Murray et al., 2016) or army ant *Eciton hamatum* (Yaeger et al., 2013), both of which sample and reject dendrobatids.

Certain pumiliotoxin alkaloids are known to be particularly toxic based on contact toxicity assays with the fire ant *Solenopsis invicta*, and LD_{50} assays with laboratory mice (Daly & Spande, 1986; Daly et al., 1994, 1999; Sellier et al., 2010; Weldon et al., 2013; see Table 21.2 in Santos et al., 2016), and therefore the presence of pumiliotoxin (PTX) and/or allopumiliotoxin (aPTX) alkaloids, in particular, also appear to be important in explaining differences in palatability in the present study. Frogs from Cerro Brujo, Panama were less palatable to the fruit fly *D. melanogaster* and were dominated by a combination of both ant-derived and mite-derived alkaloids; however, 10% of the total quantity of alkaloids consists of one major mite-derived alkaloid, PTX **307A**. Furthermore, an equally unpalatable population was Isla Solarte, Panama, which contained the mite-derived PTXs **307A** and **323A**, and collectively make up 38% of the

frog skin alkaloids in this population. Pumiliotoxins appear to also contribute to the avoidance of frogs from Rio Palmas, Costa Rica, which contained large amounts of aPTX **267A**. Weldon et al. (2013) demonstrated that pumiliotoxins and allopumiliotoxins, specifically aPTX **267A**, PTX **307A**, and PTX **323A**, are most effective at reducing ambulation in the fire ant *Solenopsis invicta* upon contact, and in some cases cause convulsions (PTX **251D**) at relatively low concentrations (0.001 - 0.33 mM). Therefore, the presence of pumiliotoxin and allopumiliotoxin alkaloids may be largely responsible for the unpalatability of frogs from Isla Solarte, Cerro Brujo, and Rio Palmas.

Collectively, alkaloid quantity, diversity, and type in *O. pumilio* appear to play a complex role in avoidance responses of fruit flies and ants, and provide insight into our understanding of how arthropod predators might similarly respond to variation in alkaloid defenses. Frogs that contain larger quantities of alkaloids may be equally protected from predators as frogs with lower quantities of alkaloids, if they contain a broader diversity or specific alkaloid defenses. However, different predators may perceive the same alkaloid profiles differently, and therefore it will be important for future studies to consider the mode by which predators are coming into contact with alkaloid defenses as well as how different predators respond to naturally occurring variable alkaloid defenses.

Alkaloid variation as an adaptive trait

Due to the aposematic nature of dendrobatid frogs, most studies have focused on how vertebrate predators, more specifically color-visioned avian predators (domestic chickens), perceive and respond to alkaloid-based defenses (e.g., Darst & Cummings, 2006; Darst et al., 2006; Stuckert et al., 2014). Avian predators rely largely on visual cues

to identify prey, and in general, experimental evidence suggests that chickens can learn to associate conspicuous coloration in dendrobatids with the presence of alkaloids, and avoid preying upon certain frogs (Darst & Cummings, 2006; Darst et al., 2006; Stuckert et al., 2014). Although the mechanisms by which birds perceive alkaloids is not known, it is assumed that alkaloids are simply considered distasteful and bitter, largely based on observations of rejection (Darst & Cummings, 2006; Darst et al., 2006) and beak wiping by chickens following contact with alkaloid-containing frogs (Stuckert et al., 2014). Differences in alkaloid defenses (quantity, diversity, and type) may not be as important to bird predators, as long as there are sufficient amounts of alkaloids to elicit a bitter or distasteful response (Darst & Cummings, 2006; Darst et al., 2006; Stuckert et al., 2014).

Alternatively, arthropods primarily use contact chemoreception to assess prey (Fritz, 1981; Szelistowski, 1985; Isman, 1992; Weldon et al., 2013; Hantak et al., 2016; Hovey et al., 2016; Murray et al., 2016), and have a diversity of chemoreceptors that are located on structures such as antenniform, maxillae, labium, pedipalps, etc. (Isman, 1992). Previous experimental studies have demonstrated that ctenid spiders do not learn to avoid dendrobatids, but instead indiscriminately attack frogs, and in most cases, reject alkaloid-containing dendrobatids (Szelistowski 1985; Gray et al., 2010; Hantak et al., 2016; Murray et al., 2016). Interestingly, there are differences in how certain predators respond to the dendrobatid frog *O. pumilio* within a population with more similar alkaloid defenses. A recent study that took place at La Selva, Costa Rica (one of the same locations as in the present study) found that ctenid spiders avoided all *O. pumilio*, whereas bullet ants were sensitive to differences in frog alkaloid quantity and preyed more often upon juveniles that contained less alkaloids (Murray et al., 2016). The fact

that certain arthropods respond differently to similar alkaloid profiles, likely has important implications on the degree of predation pressure frogs from a specific location are experiencing. For example, in the present study, La Selva, Costa Rica frogs were considered relatively palatable to the ant *E. ruidum* when compared to other locations. La Selva frogs had the lowest quantity of alkaloids and were dominated by mite-derived alkaloids, which may be effective against spider predation, but less effective against ant predation. The relative palatability of adult *O. pumilio* to *E. ruidum* at La Selva, coupled with experimental evidence that ctenid spiders will avoid all *O. pumilio* from La Selva (Murray et al., 2016), may indicate that spiders are a more significant predator for frogs at this location, and that alkaloid profiles in these frogs are effective against this particular predator assemblage. Conversely, populations whose predator assemblage might be dominated by ant predators may require having higher quantities of specific alkaloids, such as ant-derived alkaloids for protection. Therefore, different geographic locations may have different predator assemblages that apply specific selective pressures upon frogs, which could result in specific alkaloid profiles for adequate predator defense (Summers et al., 2015). Variable alkaloid defenses in dendrobatids are largely believed to be due to the availability of dietary arthropods (Saporito et al., 2009, 2012); however, nothing is known about whether or not frogs "choose" which alkaloid-containing prey to consume beyond what is available to them. Further research is necessary to understand the role that predation pressures may play in driving frogs to find more or specific alkaloid-containing prey to be protected from predation.

Variation in chemical defenses is common among organisms that sequester defenses, including dendrobatids, and this variation may or may not represent an adaptive

trait (Speed et al., 2012). According to recent theoretical studies, if variability in chemical defenses represents a non-adaptive trait, it is expected that the presence of these defenses alone (independent of variation) would result in equal predator avoidance and protection from pathogens (Ruxton et al., 2004; Speed et al., 2012). Alternatively, if variable defenses were an adaptive trait, it is expected that predators and pathogens would be sensitive to this variation, resulting in differential selection upon chemically defended frogs (i.e., a palatability spectrum) (Brower et al., 1968; Bowers, 1992; Speed et al., 2012). On the basis of the findings in the present study, in which arthropods responded differentially to variable alkaloid defenses, it is possible that alkaloid defense in *O. pumilio* represents an adaptive trait that is under selection by predators (or pathogens; see Mina at al., 2015). However, in order for alkaloid variation in dendrobatids to be considered adaptive with respect to predators/pathogens, frogs would need to exhibit a dietary preference for specific alkaloid-containing arthropods (with respect to quantity, diversity, or type) that operated in response to selective pressures from predators/pathogens (i.e., diet choice is linked to fitness differences). The diet of certain dendrobatids have been shown to vary with geographic location (e.g., Gómez-Hoyos et al., 2014), and male *O. pumilio* from one population in southern Costa Rica (Hitoy Cererse) are more likely to defend territories with a higher abundance of ants (Staudt et al., 2010), suggesting that there may be some degree of selection (or preference) by frogs for certain dietary arthropods. It is also possible that the genetic component(s) of alkaloid sequestration (e.g., alkaloid specificity, uptake efficiency) could be subject to selective pressures by predators/pathogens. If any combination of behavioral dietary preference or differences in alkaloid uptake has any selective advantage against predators/pathogens,

then variable alkaloid defenses in *O. pumilio* (and possibly other dendrobatids) may be an adaptive trait.

More specifically, in order for alkaloid variation in dendrobatids to be considered adaptive with respect to predators/pathogens, it would be expected that frogs have (1) some dietary preference(s) for which alkaloid-containing arthropods are being consumed, which could be with respect to quantity, diversity, or type, and that this preference operates in response to specific predation/pathogen pressure and/or (2) some currently unknown genetic component of alkaloid sequestration, which presumably influences the quantity, diversity, and type of alkaloids, is under strong selective pressure by specific predators/pathogens. If this were the case, then variable alkaloid defenses in *O. pumilio* (and possibly other dendrobatids) could be an adaptive trait. The presence of a palatability spectrum that results in differential feeding on alkaloids in *O. pumilio* by arthropod models (fruit flies and ants) only provides the first step into understanding how arthropod predators might respond to variable alkaloid defenses in dendrobatid frogs, and further research will be necessary to determine the potential adaptive nature of chemical defense in dendrobatids.

Alkaloid palatability vs. alkaloid toxicity

Previous studies aimed at understanding how variable alkaloid defenses in dendrobatids are related to predator avoidance have primarily been conducted using 'toxicity assays' by way of subcutaneous alkaloid injections into mice (e.g., Daly & Myers, 1967; Darst & Cummings, 2006; Darst et al., 2006; Maan & Cummings, 2012). Lethality assays, such as LD_{50} experiments, have demonstrated that variable alkaloid profiles among species and

populations of dendrobatids translate into differences in toxicity (Daly & Myers, 1967; Daly & Spande, 1986; Daly et al., 1987). Irritability assays, such as the sleeping mouse assay, which measures the length of time it takes a mouse (CD-1 outbred strain) to return back to sleep after a subcutaneous alkaloid injection, have also reported differences in alkaloid toxicity among species and populations of dendrobatids (Darst & Cummings, 2006; Darst et al., 2006; Maan & Cummings, 2012). Collectively, these types of studies have suggested that using mice in toxicity assays are necessary, due to the lack of a more biologically relevant and quantifiable measure of alkaloid defenses in dendrobatids. Although these types of toxicity assays offer informative and meaningful measures of alkaloid variation, they may not be the most appropriate measure of predator avoidance. For one, mammals are not known to be natural predators of dendrobatid frogs (see Supplemental Table in Santos & Cannatella, 2011; Murray et al., 2016). Furthermore, natural predators of dendrobatids are not injected with alkaloids, but are instead coming into contact with alkaloid defenses by sampling frogs during predation (feeding) events. Therefore, understanding predator avoidance may be more accurately understood by way of measuring alkaloid defenses in a manner more consistent with the mode by which predators are coming into contact with alkaloid defenses, such as the palatability assays with arthropods used in the present study; however, it is equally important to consider how different methods of measuring predator avoidance relate to one another.

In the present study, alkaloid palatability to fruit flies was not correlated with previously reported alkaloid toxicity measures using laboratory mice for certain populations of *O. pumilio* in Panama. Using different toxicity assays, Daly & Myers (1967) [LD_{50} assays] and Maan & Cummings (2012) [sleeping mouse assays] both found

that *O. pumilio* from Isla Bastimentos were among the most toxic populations present in Bocas del Toro, Panama. Furthermore, frogs from Isla Solarte, Panama were considered more toxic in the sleeping mouse assay as compared to the LD_{50} assays of Daly & Myers, (1967)(Maan & Cummings, 2012). In the present study, frogs from Isla Bastimentos were found to be the most palatable to fruit flies, which is contrary to the findings that frogs from this location are the most toxic to laboratory mice. Furthermore, frogs from Isla Solarte were found to be completely unpalatable to fruit flies, which is consistent with the toxicity measures for frogs from this same location in Maan & Cummings (2012), but does not match the toxicity measures for these same locations in Daly & Myers (1967). Although limited in scope, these finding suggest that palatability and toxicity assays are not strongly related, and that toxicity measures may not be a reliable predictor of predator response to frog alkaloid defenses. Therefore, measuring predator avoidance in terms of 'toxicity' to laboratory mice might not translate directly to how arthropod predators perceive alkaloid defenses. It is possible that the lack of congruence among these different assays is due to temporal or small spatial differences in alkaloid defenses (Daly et al., 1987; Saporito et al., 2006, 2007a), but addressing this question will require further research in which toxicity assays and palatability assays are conducted at the same time and with the same individual frogs. Finally, toxicity to mice may not be the most meaningful measure of predator avoidance, especially with respect to arthropod predators. Arthropod predators come into direct contact with chemically-defended frogs using their antenniform, pedipalps, etc. and sample or taste the prey before making decisions to consume them (Isman, 1992; Gray et al., 2010; Weldon et al., 2013; Hantak et al., 2016; Hovey et al., 2016; Murray et al., 2016). Therefore, the palatability assays

utilized in the present study may represent a more biologically relevant measure of alkaloid defenses against arthropod predators that use chemoreception.

Conclusions

Palatability assays provide a powerful tool to study chemical defenses and predator avoidance in dendrobatid frogs. Alkaloid defenses in *O. pumilio* were perceived as unpalatable, however, the degree of unpalatability differed among populations as well as between sexes and life stages. Arthropod models were sensitive to differences in alkaloid profiles and responded accordingly as differences in avoidance, which provides some of the first evidence of a palatability spectrum for vertebrates that sequesters chemical defenses. Differences in alkaloid profiles predict differences in palatability where alkaloid quantity, diversity, and type all appear to play an important role in the frogs' defenses. Dendrobatid frogs represent one of the few groups of vertebrates that sequester their defenses solely from diet, and therefore environmental heterogeneity (e.g., variation in dietary arthropod availability) likely plays a significant role in their ability to defend themselves from predators. However, if different predator assemblages respond to alkaloid profiles differently, this may have major implications in understanding predator-prey dynamics and the ecological significance of variable chemical defenses. Therefore, future studies should aim to further understand how different predators, both invertebrates and vertebrates, respond to the same alkaloid profiles in order to understand how frogs are protected from various predation pressures. Additionally, examining whether or not vertebrate predators such as birds are sensitive to a palatability spectrum or if palatability changes over time, still remains to be tested. The present study

represents an important step in understanding how arthropods perceive dendrobatid frogs with variable chemical defenses and provides important insight into the ecology and evolution of sequestered defenses in vertebrates.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor Dr. Ralph A. Saporito for the invaluable opportunity, experiences, and guidance that have inspired my work and me in infinite ways. I owe a tremendous thanks to my field assistant Kelsie Dickerson for her help collecting an enormous amount of data in the field. I would like to acknowledge my thesis committee members, Dr. Rebecca E. Drenovsky and Dr. Carl D. Anthony, for their support and valuable perspective that continually improved my thesis. I would also like to thank the Organization for Tropical Studies La Selva Biological Research Station, Canadian Organization for Tropical Education and Rainforest Conservation Caño Palma Biological Station, Emily Khazan, Andres Vega, Samasati Retreat, Scott McKenzie, Bungalows Kiré, and Hotel Río Palmas for their support in carrying out this research. I would also like to thank the Costa Rican government for permitting this research. Furthermore, I would like to thank the entire Saporito Lab, including M. Gade, K. Hovey, A. Blanchette, N. Bezca, M. Boyk, M. Russell, E. Seiter, N. Spies, and N. Woodcraft for continuous support and editorial comments. In addition, I would like to give a special thanks to M. Viloria and M. Russell for their additional help in frog collection, as well as C. Baggett for support and feedback on various aspects of my thesis. Lastly, I would like to thank John Carroll University for financial support as well as the Exploration Fund Grant from The Explorers Club.

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Table 1. Tabulation of all alkaloids identified from 81 *Oophaga pumilio*, organized by structural class and identified by dietary arthropod source, from Bocas del Toro region of Panama and Caribbean coast of Costa Rica.

Mite									Ant								Millipede
1,4-Q	5,8-I	5,6,8-I	Dehydro-5,8-I	PTX	aPTX	Deoxy hPTX	Tri	Unclass	3,5-P	3,5-I	DHQ	Izidine	Lehm	Pip	Pyr	HTX	Spiro
217A	195B	195G	205L	225F	225E	193F	191F	195E	195F	195B	195A	155	275A	211I	183B	283A	236
231A	195I	205G	221J	237A	241H	281B	193C	195K	223B	223AB	211A	191E	275G	211J	197B	285A	222
233A	203A	207C	233E	265D	253A		205B	207F	223H	275C	221C	195H	277A	213A	211T	285C	252A
233I	205A	209C	243F	277B	267A		207GH	209G	223R		223F	195K		223K	225C	287A	252B
237T	207A	223A	269D	277G	293K		221M	217Q	249I		233F	209L		225B	225H	291A	
257D	209I	223C		307A	305A		223P	235P	251K		237U	211B		225I	235F		
257Q	209S	225K		307B	307C		235I	235S	265J		249E	211C		239I	253I		
279E	217B	231B		307E	309D		235M	239T			251A	221N		239L	253U		
	219F	231K		307F	323B		245J	247L			269AB	225A		241D	277D		
	219J	233C		307G	325A		247N	247P			269B	225J		241G	279G		
	219L	235E		309A	341B		249Z	251HH			271D	239A		253J			
	221A	237C		321A			253G	261G			275B	247J		267K			
	221I	237L		323A			253S	261L				251C		267X			
	223D	249C					275L	265K				253L					
	223J	251M						267Q									
	231C	251S						269E									
	233D	251T						269K									
	235B	251V						271E									
	237D	253H						273F									
	239G	259C						275M									
	243B	261B						279I									
	243C	263A						311A									
	245I	263C															
	247E	265L															
	249J	267J															
	249O	275E															
	251B	277E															
	251N	293C															
	253B	353B															
	257C																
	259B																
	261D																
	263F																
	271A																
	273B																
	275F																

Five additional alkaloids were detected, but not included in the table due to space limitations. They are listed here as follows, along with their presumed dietary source: Polyzonamine **151B** (millipede/mite); homopumiliotoxin **223G** (mite); dehydropumiliotoxin **207O** (mite); 4,6-disubstituted quinolizidine **237I** (mite); and cyclopentaquinolizidine **249B** (mite).

Abbreviations for alkaloid classes are as follows: 1,4-Q (1,4-disubstituted quinolizidine); 5,8-I (5,8-disubstituted indolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine); Dehydro-5,8-I (dehydro-5,8-disubstituted indolizidine); PTX (pumiliotoxin); aPTX (allopumiliotoxin); Deoxy-hPTX (deoxy-homopumiliotoxin); Tri (Tricyclic); Unclass (Unclassified); 3,5-P (3,5-disubstituted pyrrolizidine); 3,5-I (3,5-disubstituted indolizidine); DHQ (decahydroquinoline); Lehm (lehmizidine); Pip (piperidine); Pyr (pyrrolidine); HTX (histrionicotoxin); Spiro (spiropyrroliizidine).

Table 2. Mean alkaloid quantity (μg per frog), alkaloid diversity, palatability, and five major alkaloids (organized by dietary arthropod source) based on structural classes for the five populations of *Oophaga pumilio* from Bocas del Toro, Panama.

	Mean alkaloid quantity	Mean alkaloid diversity	Mean palatability	Mite					Ant	
				5,8-I	5,6,8-I	1,4-Q	aPTX	PTX	DHQ	3,5-P
Isla Bastimentos	1,040	26	-0.53	◆◆◆◆	◆					
Isla Solarte	1,666	38	-1.00	◆			◆	◆◆	■	
Isla Cristobal	1,400	36	-0.99	◆◆◆					■	■
Cerro Brujo	923	43	-1.00	◆◆		◆		◆		■
Isla Popa	681	19	-0.60	◆◆		◆◆				■

The five most abundant alkaloids within each alkaloid class are based on the quantity of alkaloids in μg per frog.
See Table 1 for structural class abbreviations.

Table 3. Mean alkaloid quantity (μg per TAS sample), alkaloid diversity, palatability, and five major alkaloids (organized by dietary arthropod source) based on structural classes for the five populations of *Oophaga pumilio* from Costa Rica.

	Mean alkaloid quantity	Mean alkaloid diversity	Mean palatabiltiy	Mite						Ant		
				5,8-I	5,6,8-I	Unclass	1,4-Q	Tri	aPTX	DHQ	3,5-I	3,5-P
Tortuguero	90.0	33	-0.48	◆◆	◆◆							■
La Selva	10.5	21	-0.16	◆◆		◆◆						■
Rio Palmas	51.2	35	-0.49		◆			◆	◆		■	■
Hone Creek	72.1	34	-0.45		◆		◆			■	■	■
Gandoca	102.7	48	-0.47	◆	◆◆			◆◆		■		

The five most abundant alkaloids within each alkaloid class are based on the quantity of alkaloids in μg per frog.
See Table 1 for structural class abbreviations.

Table 4. Mean alkaloid quantity (μg per TAS sample), alkaloid diversity, and palatability for female, male, and juvenile *Oophaga pumilio* from La Selva, Costa Rica.

	Mean alkaloid quantity	Mean alkaloid diversity	Mean palatability
Females	35.8	29	-0.351
Males	6.7	17	0.00
Juveniles	1.8	8	-0.06

Table 5. Tabulation of new identified alkaloid isomers in *Oophaga pumilio* from Costa Rica and Panama. Following the methods of Jeckel et al. (2015), the retention time (Rt) for each alkaloid is reported as 'Adjusted Rt', to account for differences in elution time (approximately 0.34 seconds slower) for alkaloids in the present study compared to the alkaloid library of Daly et al. (2005).

Structural class	Alkaloid	Adjusted Rt
Izidine	**191E**	8.58
Tri	**191F**	8.07
Tri	**193C**	5.92
5,6,8-I	**193G**	7.13
DHQ	**195A**	8.00
Unclass	**195E**	9.36
Izidine	**195H**	7.53
Pyr	**197B**	7.48
Pyr	**197B**	7.65
5,6,8-I	**205G**	6.09
Dehydro-5,8-I	**205L**	8.92
5,8-I	**207A**	6.73
5,8-I	**207A**	7.93
5,8-I	**207A**	8.06
5,8-I	**207A**	8.11
5,8-I	**207A**	8.34
5,6,8-I	**207C**	7.39
Tri	**207GH**	8.15
5,8-I	**209S**	7.99
Izidine	**211B**	9.53
Pip	**211I**	7.95
Pyr	**211T**	7.24
5,8-I	**219J**	11.45
5,8-I	**221A**	8.69
DHQ	**221C**	7.51
5,6,8-I	**223A**	8.99
5,6,8-I	**223A**	10.75
3,5-P	**223B**	9.18
3,5-P	**223B**	10.25
5,6,8-I	**223C**	11.25
3,5-P	**223H**	8.72

Structural class	Alkaloid	Adjusted Rt
3,5-P	**223H**	8.93
3,5-P	**223H**	9.15
3,5-P	**223H**	9.64
3,5-P	**223H**	9.82
5,8-I	**223J**	8.32
5,8-I	**223J**	8.71
Tri	**223P**	9.60
PTX	**225F**	10.91
PTX	**225F**	10.99
Pyr	**225H**	9.43
Pip	**225I**	9.66
5,6,8-I	**231B**	10.88
5,6,8-I	**233C**	8.90
5,8-I	**233D**	12.23
5,8-I	**233D**	12.65
DHQ	**233F**	9.44
5,8-I	**235B**	10.62
5,8-I	**235B**	8.80
Tri	**235M**	9.18
Tri	**235M**	9.27
Unclass	**235P**	9.46
5,6,8-I	**237C**	9.35
5,8-I	**237D**	10.62
4,6-Q	**237I**	10.39
5,6,8-I	**237L**	9.31
5,6,8-I	**237L**	9.52
DHQ	**237U**	9.86
Pip	**239I**	11.50
Pip	**239L**	12.43
Pip	**241D**	12.37
Pip	**241D**	12.47

Structural class	Alkaloid	Adjusted Rt
aPTX	**241H**	12.49
5,8-I	**245I**	11.90
5,8-I	**245I**	12.29
Tri	**245J**	11.89
Tri	**247N**	12.60
Tri	**247N**	12.64
5,6,8-I	**249C**	9.82
5,6,8-I	**249C**	9.93
5,6,8-I	**249C**	11.55
DHQ	**249E**	11.38
5,8-I	**249O**	11.38
DHQ	**251A**	11.87
5,8-I	**251B**	11.10
5,6,8-I	**251T**	9.75
5,6,8-I	**251T**	11.36
5,6,8-I	**251V**	13.29
aPTX	**253A**	12.08
5,6,8-I	**253H**	11.98
Pip	**253J**	12.54
Tri	**253S**	12.30
Tri	**253S**	12.56
Tri	**253S**	12.69
1,4-Q	**257D**	12.89
5,6,8-I	**259C**	11.21
5,6,8-I	**259C**	11.52
5,6,8-I	**259C**	11.98
5,8-I	**261D**	12.92
5,6,8-I	**263A**	12.78
5,8-I	**263F**	12.78
Unclass	**265K**	11.20

Structural class	Alkaloid	Adjusted Rt
Pip	**267K**	14.45
Pip	**267K**	14.09
DHQ	**269B**	14.84
DHQ	**271D**	15.02
Unclass	**271E**	14.36
5,8-I	**273B**	13.80
5,8-I	**273B**	13.88
Lehm	**275A**	13.42
Lehm	**275A**	13.66
Lehm	**275A**	14.27
DHQ	**275B**	13.64
DHQ	**275B**	14.19
Lehm	**277A**	13.88
PTX	**277B**	14.07
PTX	**277B**	14.66
Pyr	**277D**	13.91
5,6,8-I	**277E**	12.01
1,4-Q	**279E**	12.80
Pyr	**279G**	13.97
HTX	**285A**	15.47
HTX	**285C**	15.82
HTX	**285C**	16.01
HTX	**287A**	16.14
HTX	**291A**	15.40
aPTX	**293K**	16.10
PTX	**323A**	16.69
PTX	**323A**	18.13
aPTX	**323B**	16.39
aPTX	**323B**	16.69
aPTX	**323B**	17.05

See Table 1 for structural class abbreviations.

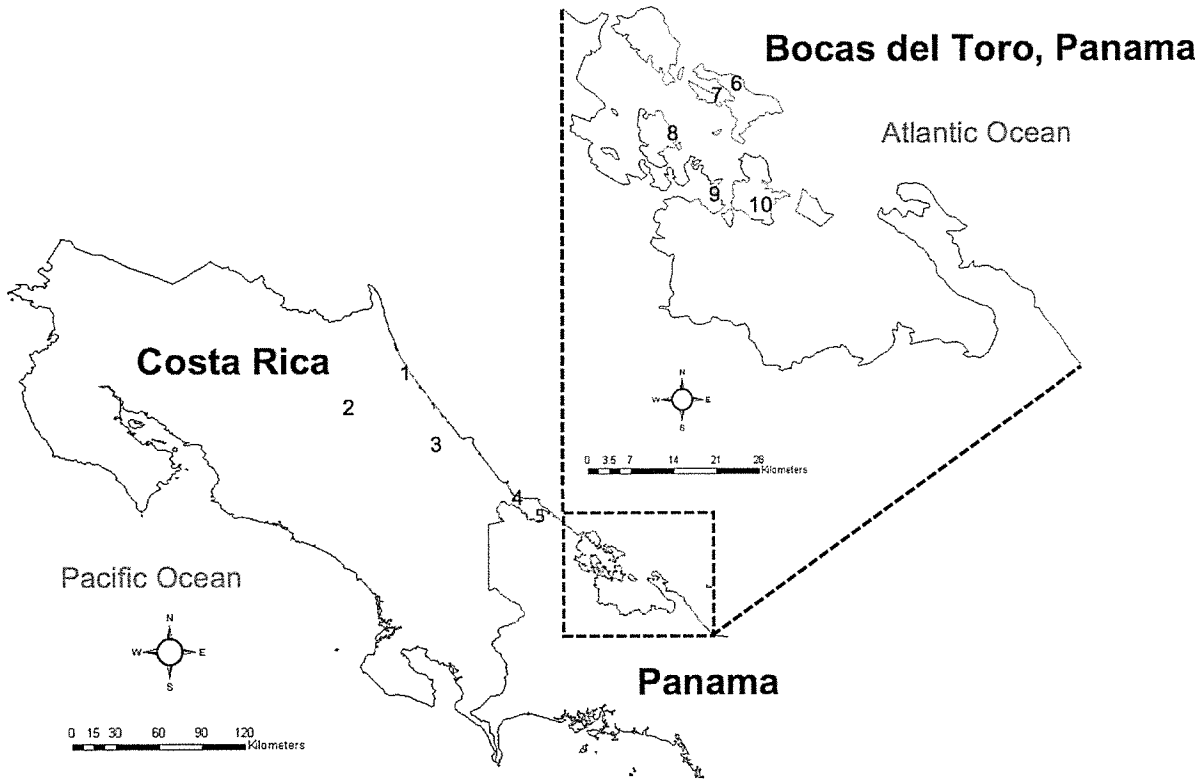


Figure 1. Map of collection sites for *Oophaga pumilio* in Costa Rica and Panama: (1) Tortuguero; (2) La Selva; (3) Rio Palmas; (4) Hone Creek; (5) Gandoca; (6) Isla Bastimentos; (7) Isla Solarte; (8) Isla Cristobal; (9) Cerro Brujo; and (10) Isla Popa.

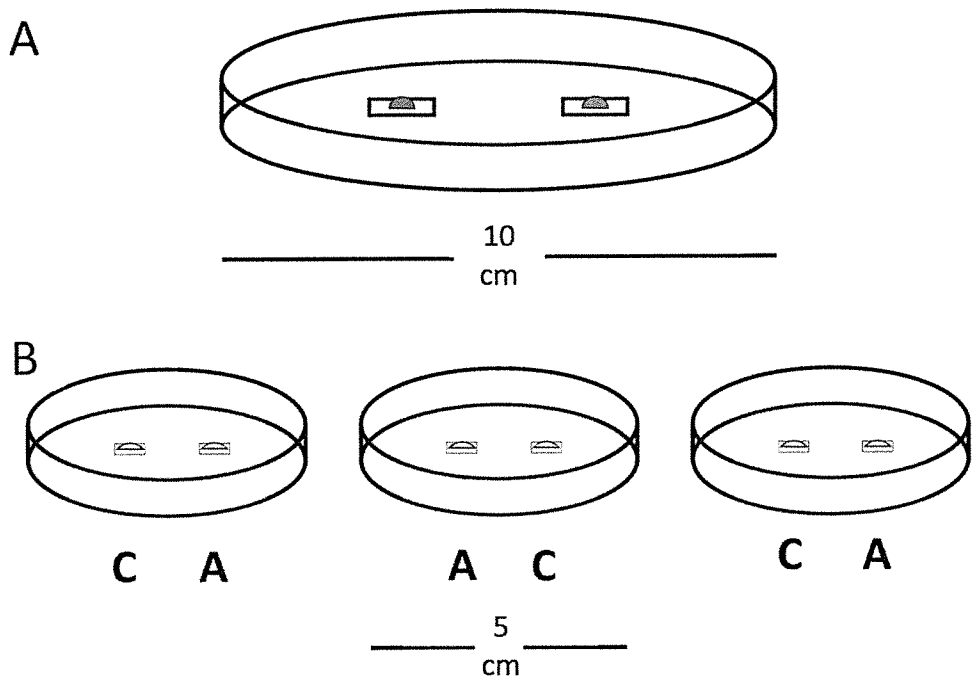


Figure 2. Experimental arenas for (A) *Drosophila melanogaster* (fruit fly) and (B) *Ectatomma ruidum* (ant) palatability assays. A total of 10 fruit flies were added to arena (A) for assays with fruit flies, whereas only one ant was added to each of the three different arenas (B) at the same time. Fruit flies and ants were allowed to choose between a control and alkaloid solution in each assay (colored solutions in fruit fly assays), and the location of control and alkaloid treatment was randomized between trials.

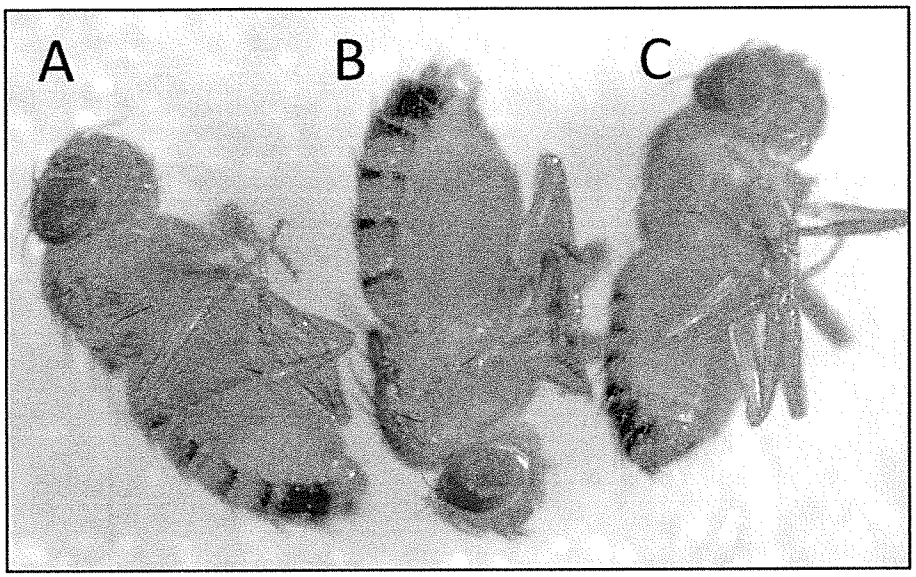


Figure 3. An example of *Drosophila melanogaster* (fruit flies) that have fed on the (A) control solution (red color), (B) both control and alkaloid solution (purple color) or (C) alkaloid solution (blue color), in the palatability assays for Panamanian *Oophaga pumilio*.

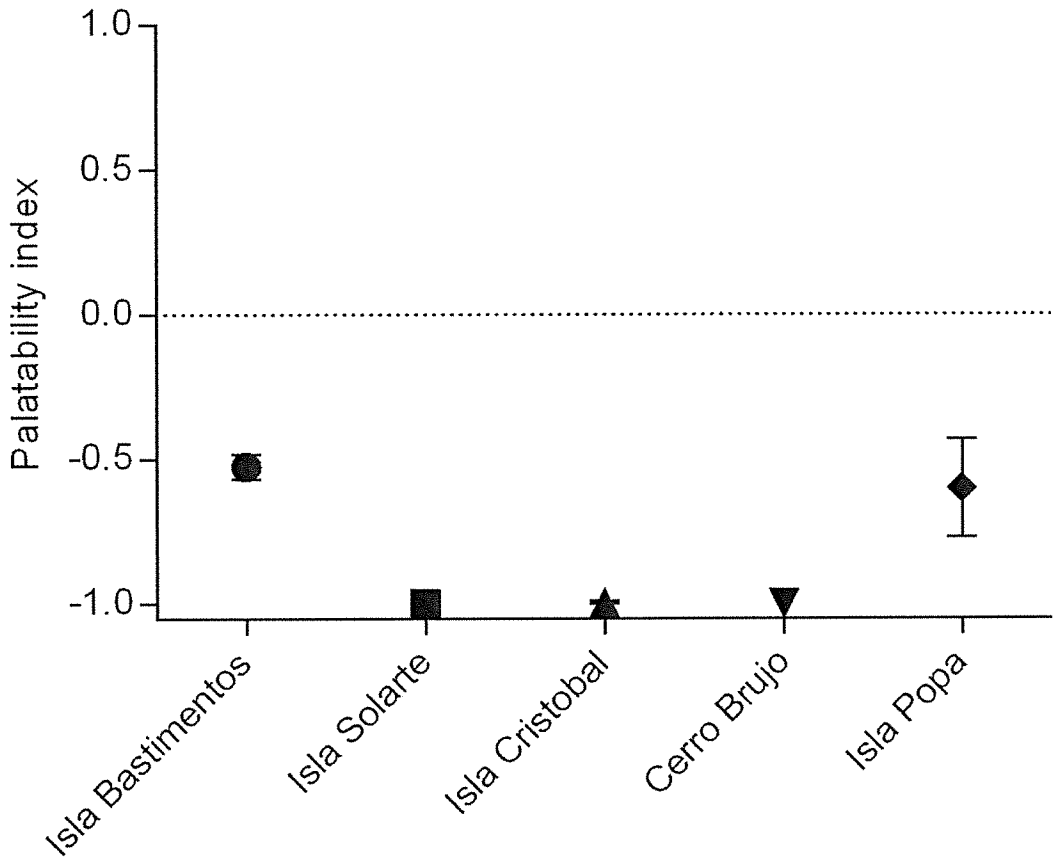


Figure 4. Mean palatability scores (±1 S.E.) for *Drosophila melanogaster* (fruit fly) palatability assays at 1.25% alkaloid concentration for each of the five populations of *Oophaga pumilio* from Bocas del Toro, Panama. The dotted line represents the point at which the solution of alkaloids is considered palatable.

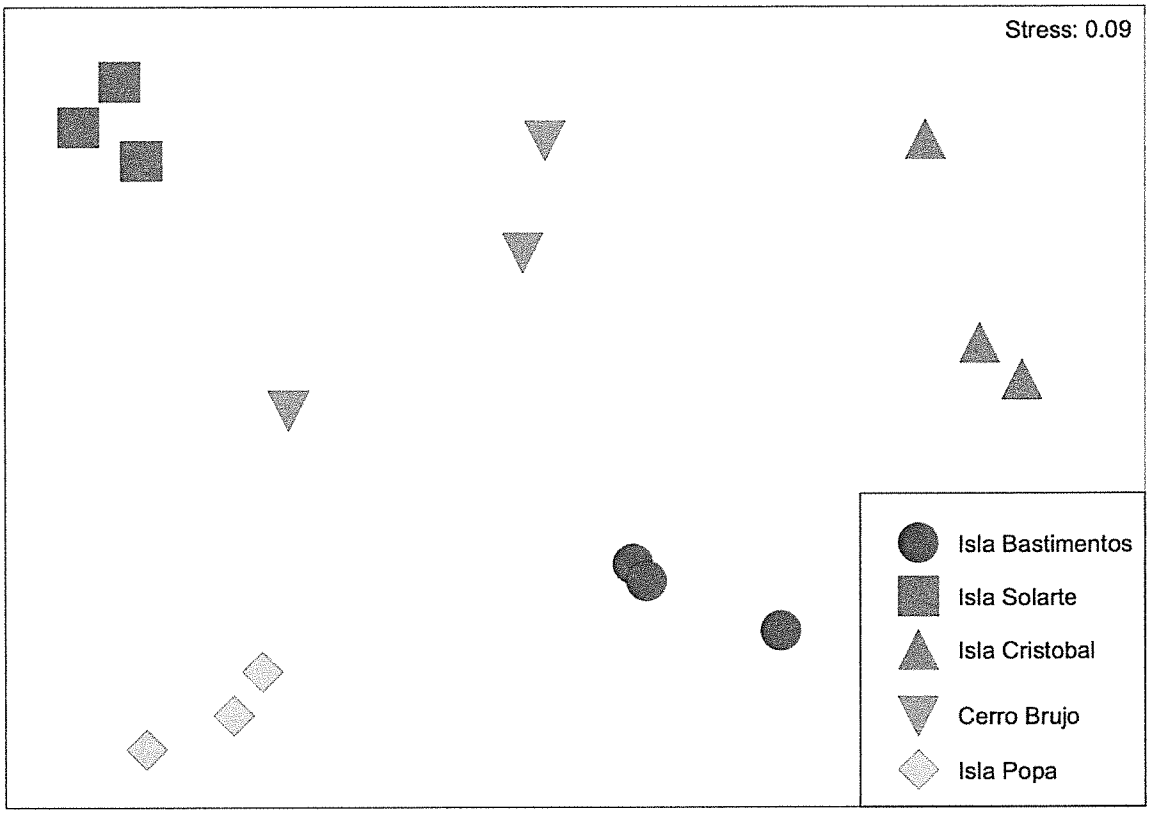


Figure 5. nMDS plot of variation in alkaloid composition of *Oophaga pumilio* among the five locations examined in Bocas del Toro, Panama. Each symbol represents an individual frog from a specific location. The distance between any two symbols (frogs) represents the proportional difference in alkaloid composition between those two individual frogs.

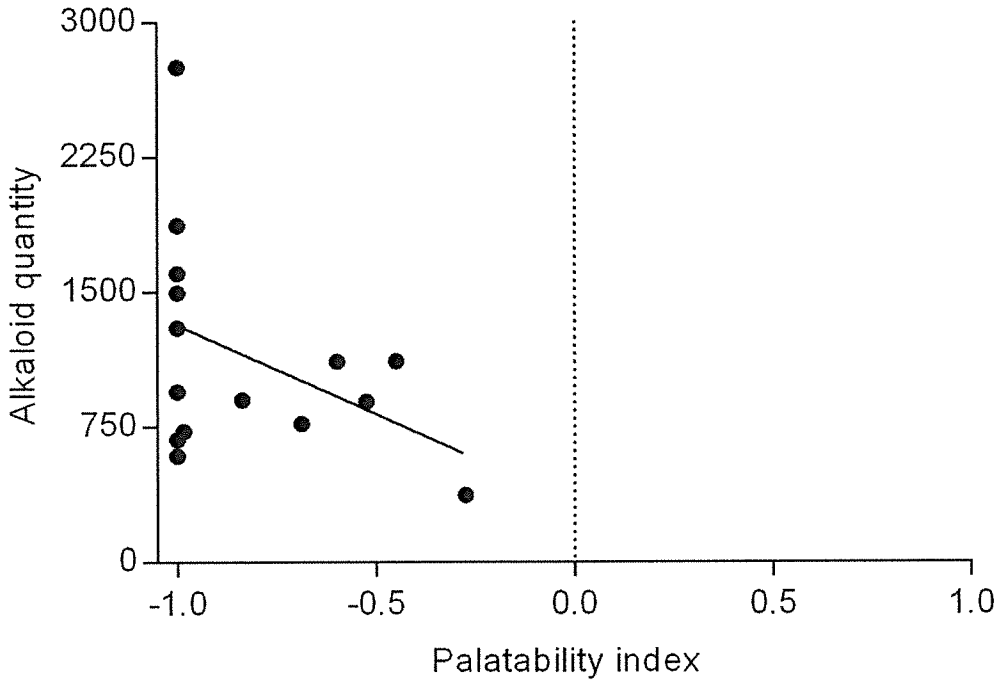


Figure 6. The relationship between alkaloid palatability and alkaloid quantity for *Oophaga pumilio* from Bocas del Toro, Panama to fruit flies (*D. melanogaster*) at 1.25% alkaloid concentration (μg per skin). The dotted line represents the point at which the solution of alkaloids is considered palatable.

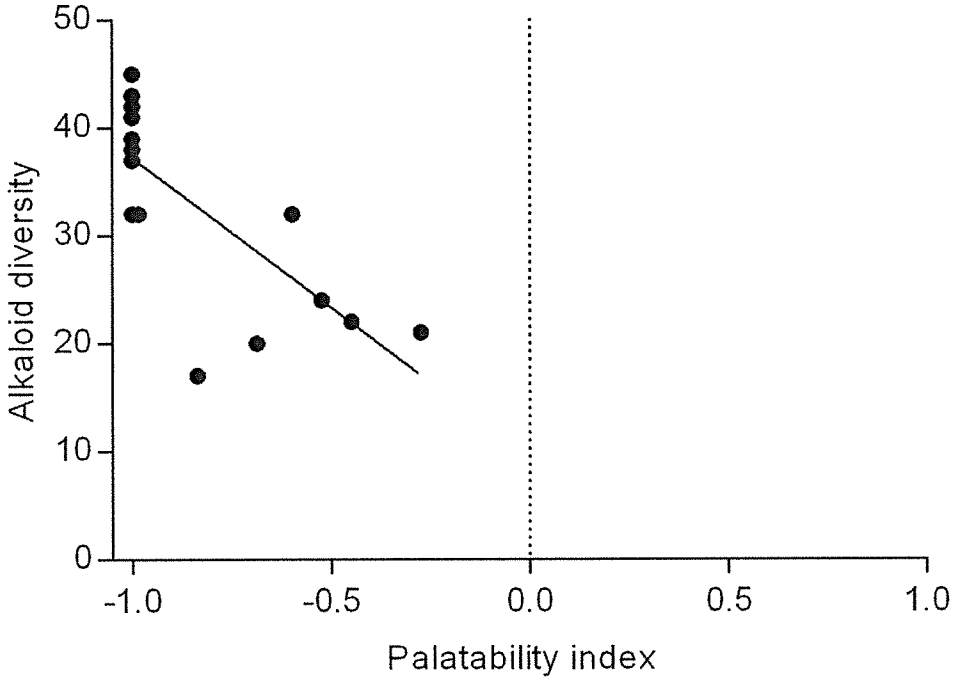


Figure 7. The relationship between alkaloid palatability and alkaloid diversity for *Oophaga pumilio* from Bocas del Toro, Panama to fruit flies (*D. melanogaster*) at 1.25% alkaloid concentration (μg per skin). The dotted line represents the point at which the solution of alkaloids is considered palatable.

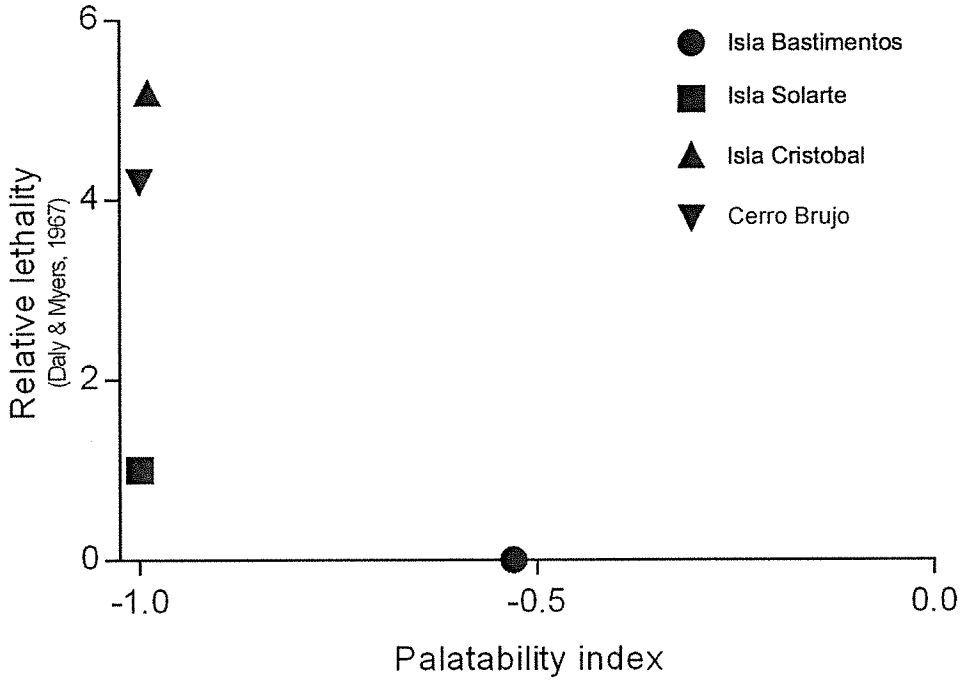


Figure 8. The relationship between alkaloid palatability and relative lethality reported in Daly & Myers (1967) for *Oophaga pumilio* in Bocas del Toro, Panama to *Drosophila melanogaster* (fruit flies) at 1.25% alkaloid concentration.

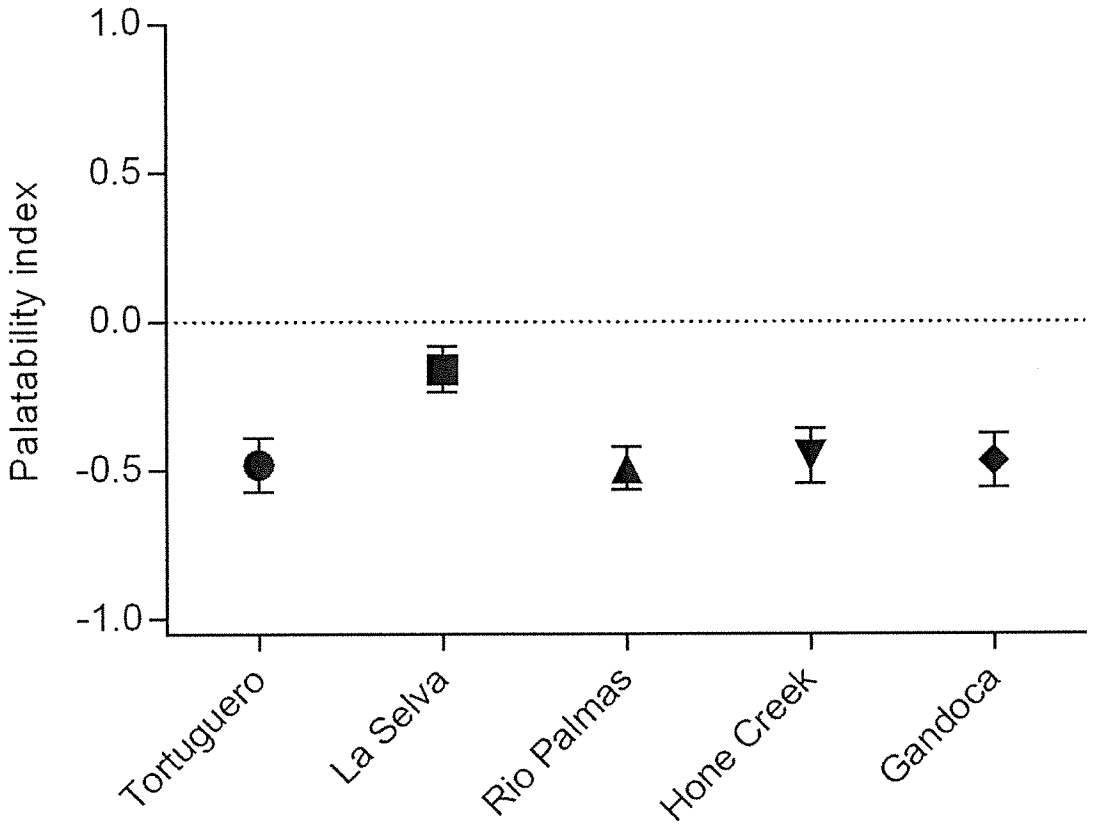


Figure 9. Mean palatability scores (±1 S.E.) for *Ectatomma ruidum* (ant) palatability assays at 0.5% TAS alkaloid concentration for each of the five populations of *Oophaga pumilio* from Costa Rica. The dotted line represents the point at which the solution of alkaloids is considered palatable.

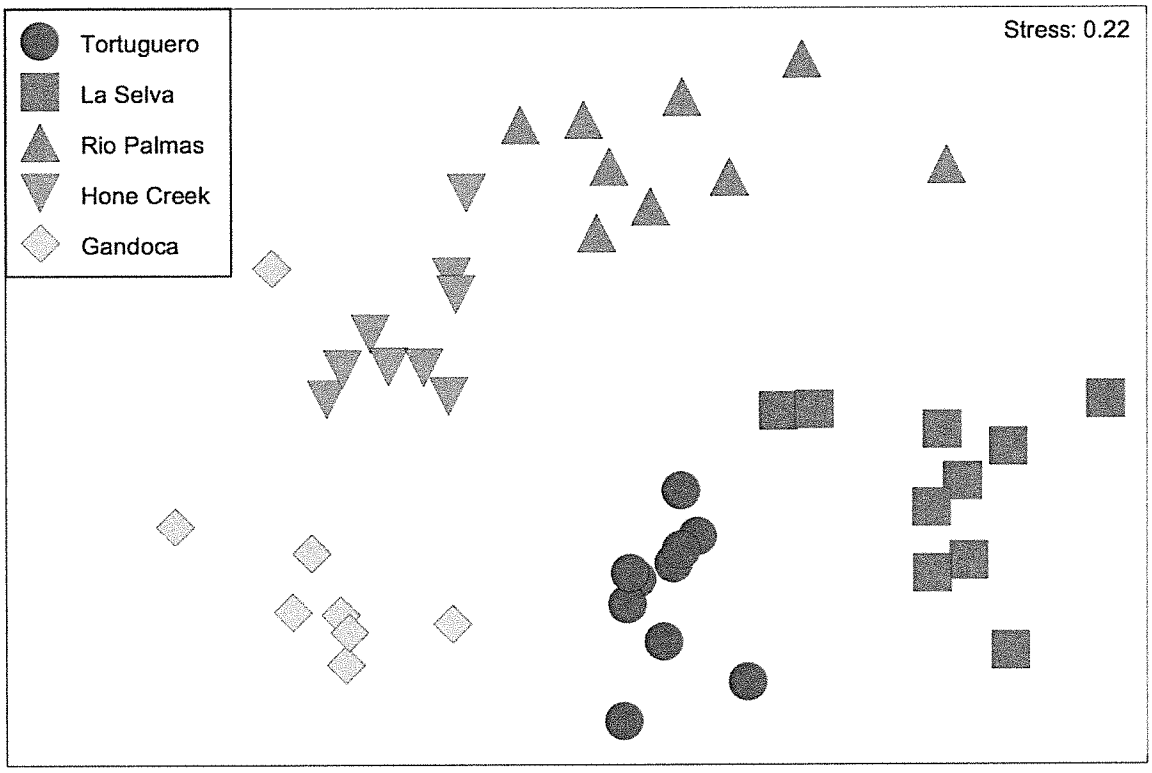


Figure 10. nMDS plot of variation in alkaloid composition of *Oophaga pumilio* among the five locations examined in Costa Rica. Each symbol represents an individual frog from a specific location. The distance between any two symbols (frogs) represents the proportional difference in alkaloid composition between those two individual frogs.

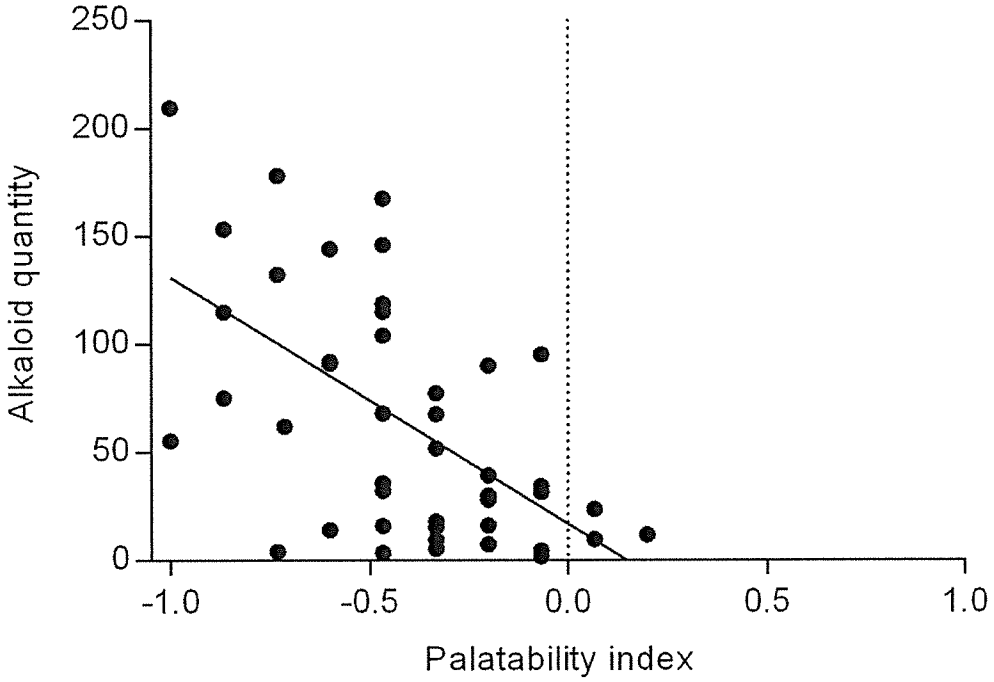


Figure 11. The relationship between alkaloid palatability and alkaloid quantity for *Oophaga pumilio* from Costa Rica to the ant *Ectatomma ruidum* at 0.5% TAS alkaloid concentration (μg per TAS sample). The dotted line represents the point at which the solution of alkaloids is considered palatable.

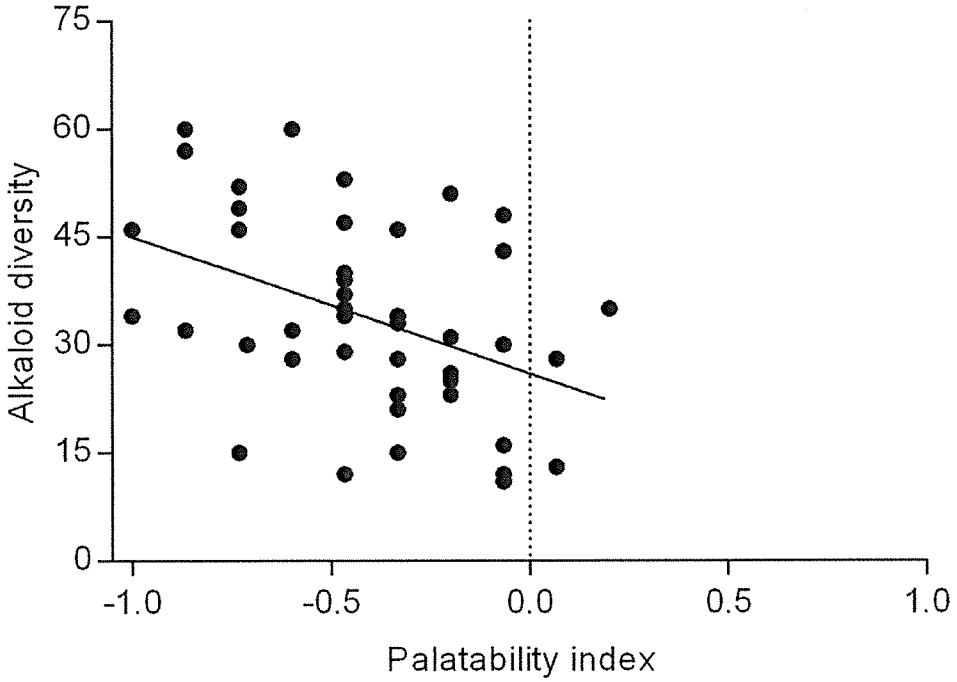


Figure 12. The relationship between alkaloid palatability and alkaloid diversity for *Oophaga pumilio* from Costa Rica to the ant *Ectatomma ruidum* at 0.5% TAS alkaloid concentration (μg per TAS sample). The dotted line represents the point at which the solution of alkaloids is considered palatable.

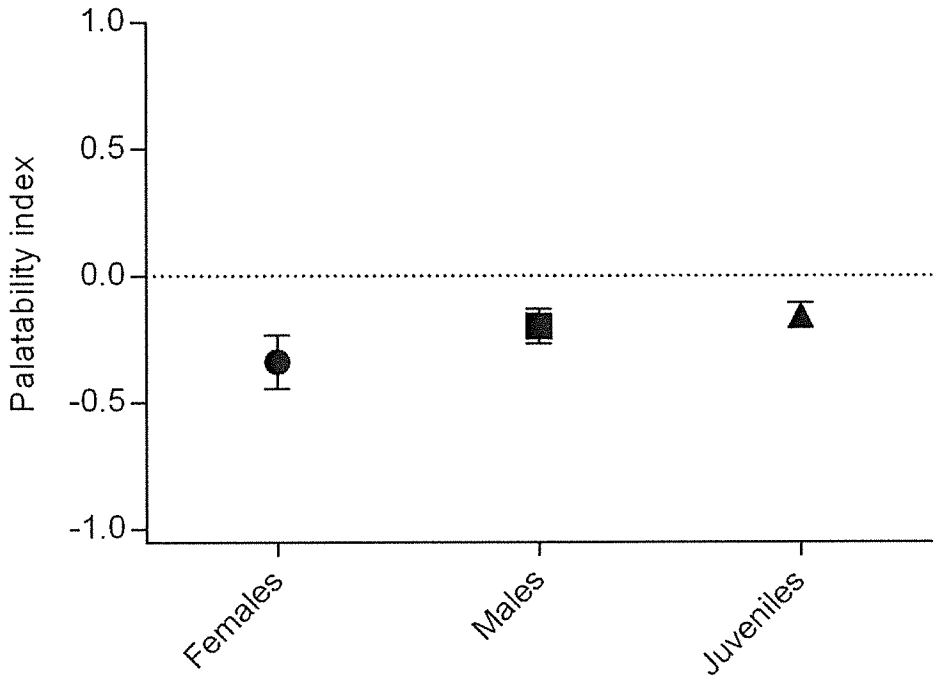


Figure 13. Mean palatability scores (±1 S.E.) for *Ectatomma ruidum* (ant) palatability assays at 0.25% TAS alkaloid concentration for each of the female, male, and juvenile of *Oophaga pumilio* from La Selva, Costa Rica. The dotted line represents the point at which the solution of alkaloids is considered palatable.

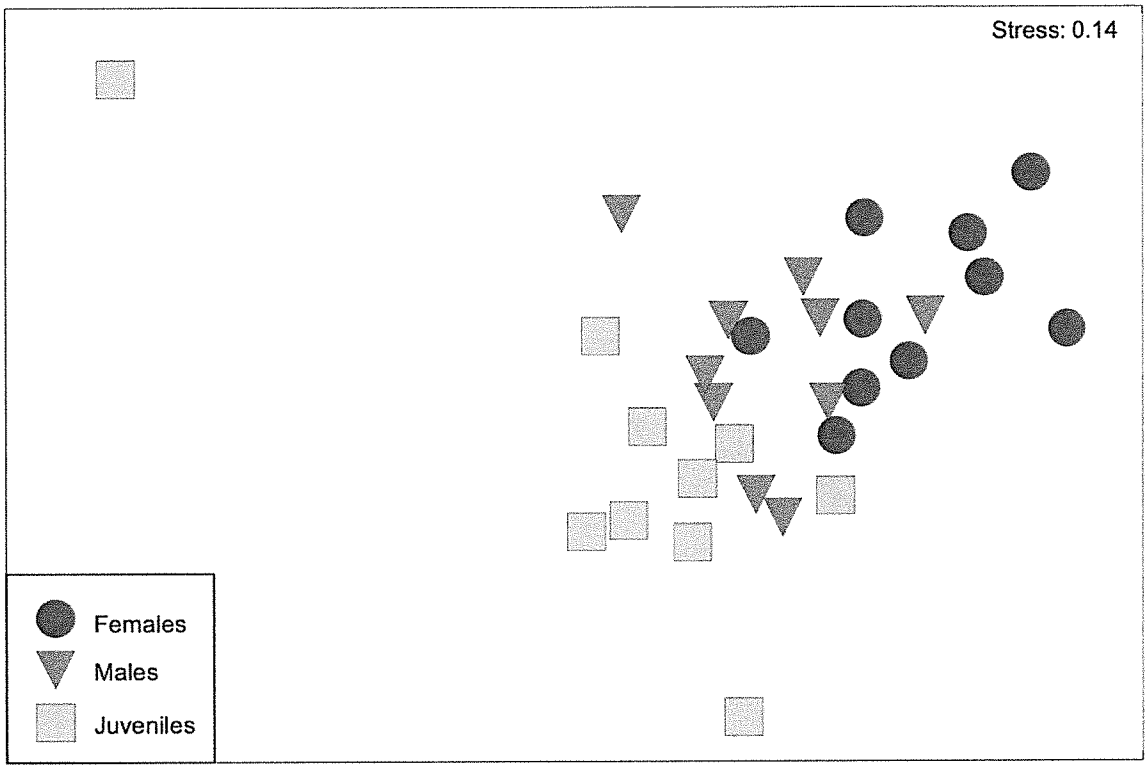


Figure 14. nMDS plot of variation in alkaloid composition of female, male, and juvenile *Oophaga pumilio* at La Selva, Costa Rica. Each symbol represents an individual frog from a specific sex/life stage. The distance between any two symbols (frogs) represents the proportional difference in alkaloid composition between those two individual frogs.

Note: One juvenile frog contained very small quantities of alkaloid (top left), which gave it a very different alkaloid composition to all other frogs in the analysis.

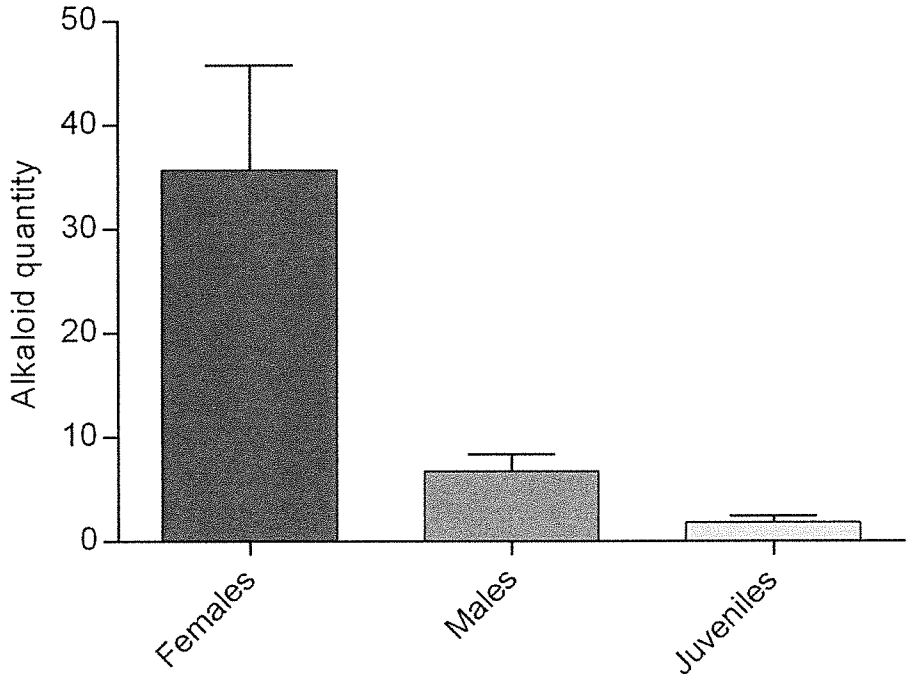


Figure 15. Mean alkaloid quantity (μg per TAS sample) (± 1 S.E.) for female, male, and juvenile *Oophaga pumilio* from La Selva, Costa Rica.

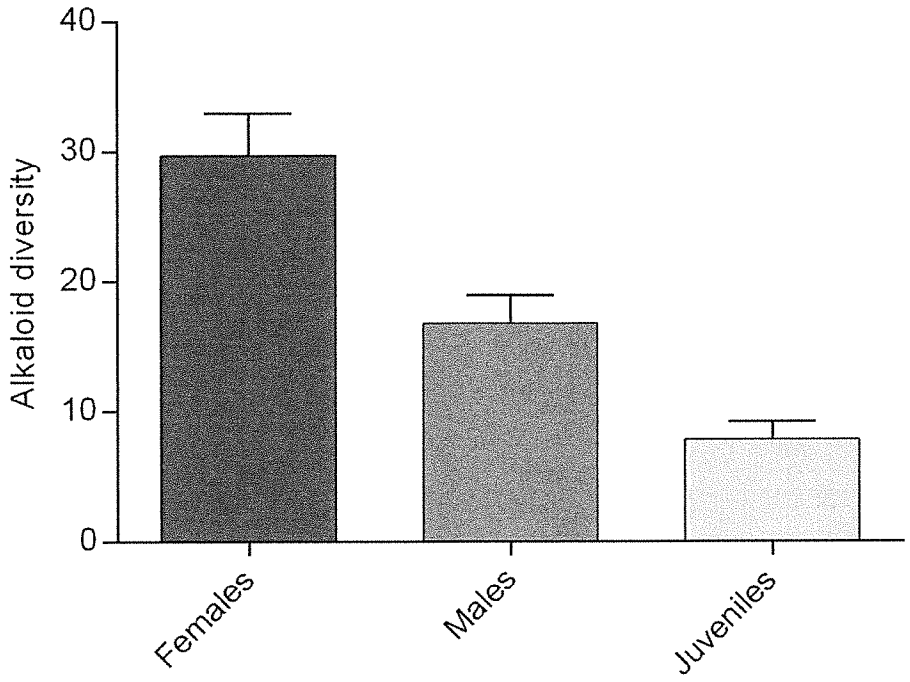


Figure 16. Mean alkaloid diversity (μg per TAS sample) (± 1 S.E.) for female, male, and juvenile *Oophaga pumilio* from La Selva, Costa Rica.

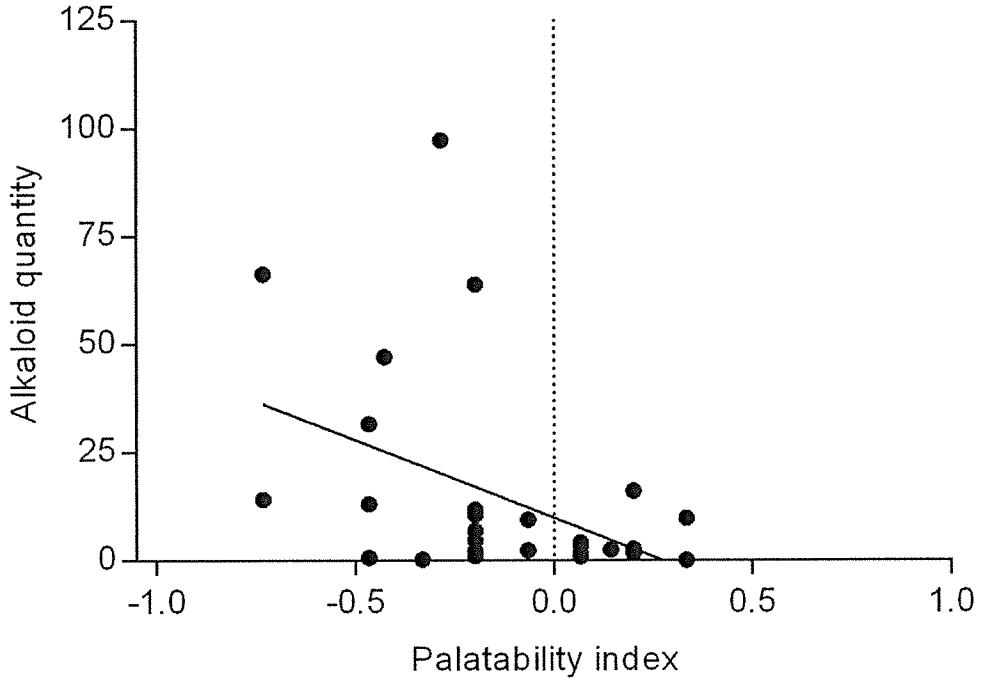


Figure 17. The relationship between alkaloid palatability and alkaloid quantity for female, male, and juvenile *Oophaga pumilio* at La Selva, Costa Rica to the ant *Ectatomma ruidum* at 0.25% TAS alkaloid concentration (µg per TAS sample). The dotted line represents the point at which the solution of alkaloids is considered palatable.

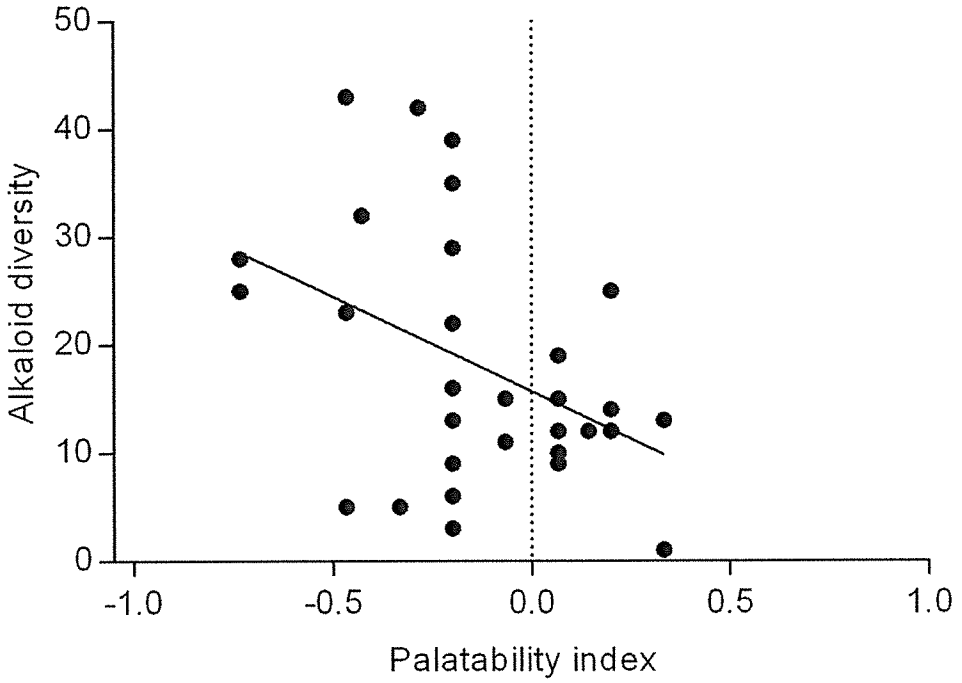
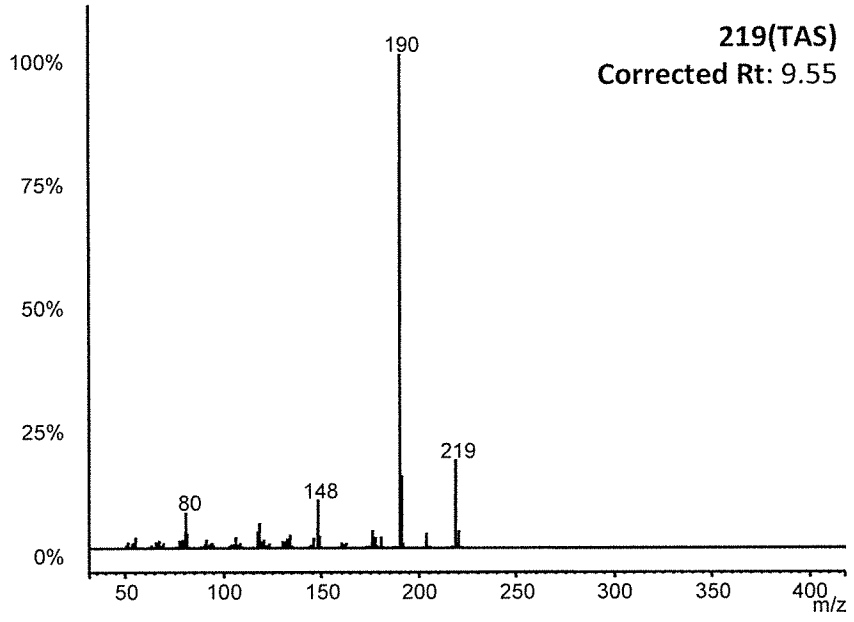
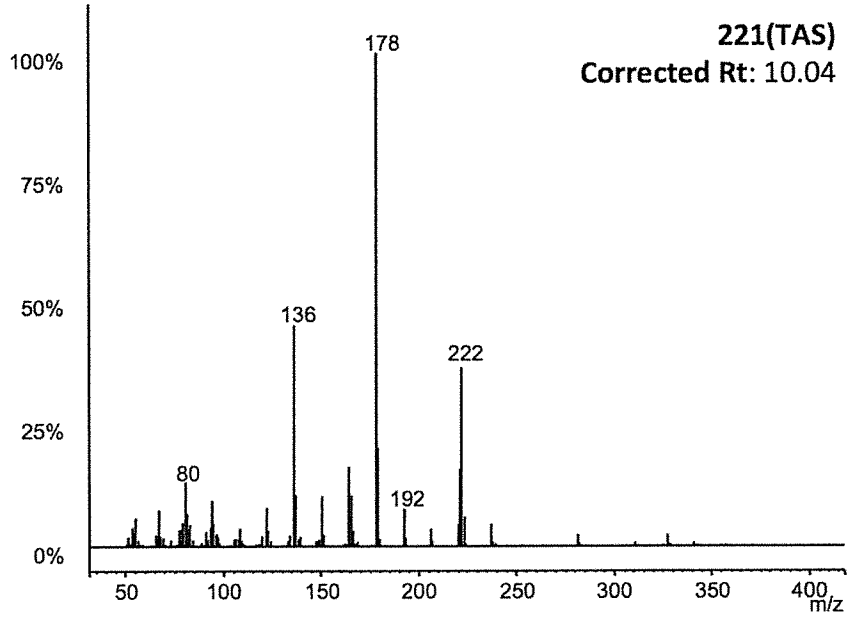


Figure 18. The relationship between alkaloid palatability and alkaloid diversity for female, male, and juvenile *Oophaga pumilio* at La Selva, Costa Rica to the ant *Ectatomma ruidum* at 0.25% TAS alkaloid concentration (µg per TAS sample). The dotted line represents the point at which the solution of alkaloids is considered palatable.

Appendix 1. Mass spectral data for the eight tentatively new alkaloids detected in TAS samples of *Oophaga pumilio* from Hone Creek, Costa Rica. Following the methods of Jeckel et al. (2015), the retention time reported (Rt) for each alkaloid is the 'Corrected Rt', to account for differences in elution time (approximately 0.34 second slower) for alkaloids in the present study compared to the alkaloid library of Daly et al. (2005). The alkaloids reported here were given code names that correspond to their molecular mass, but also include "**TAS**" to indicate that they were identified using a Transcutaneous Skin Stimulator (TAS) and not from whole skins. **Note**: All tentatively new alkaloids were present in three or more frogs, with at least one of the three frogs containing at least >0.5 μg of alkaloid per TAS sample.



Unclass



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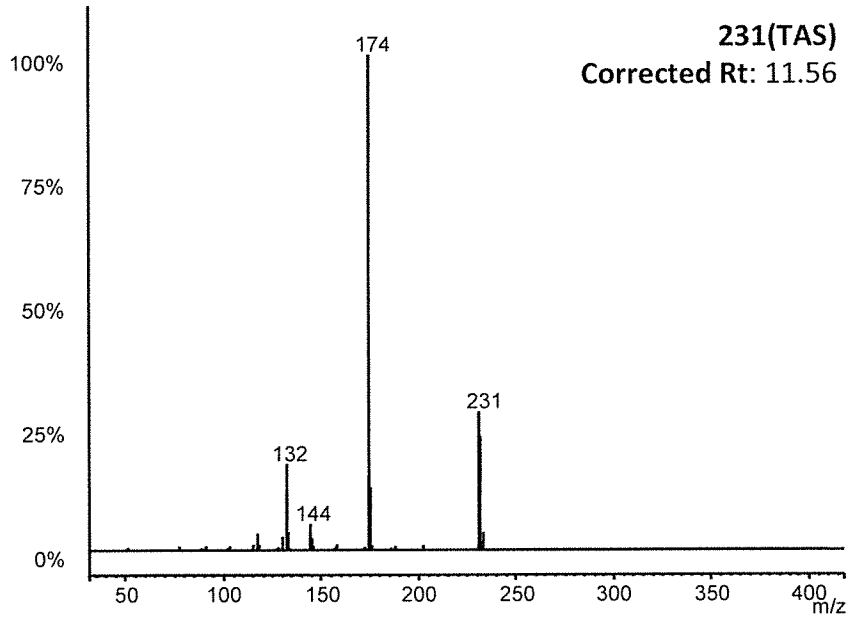

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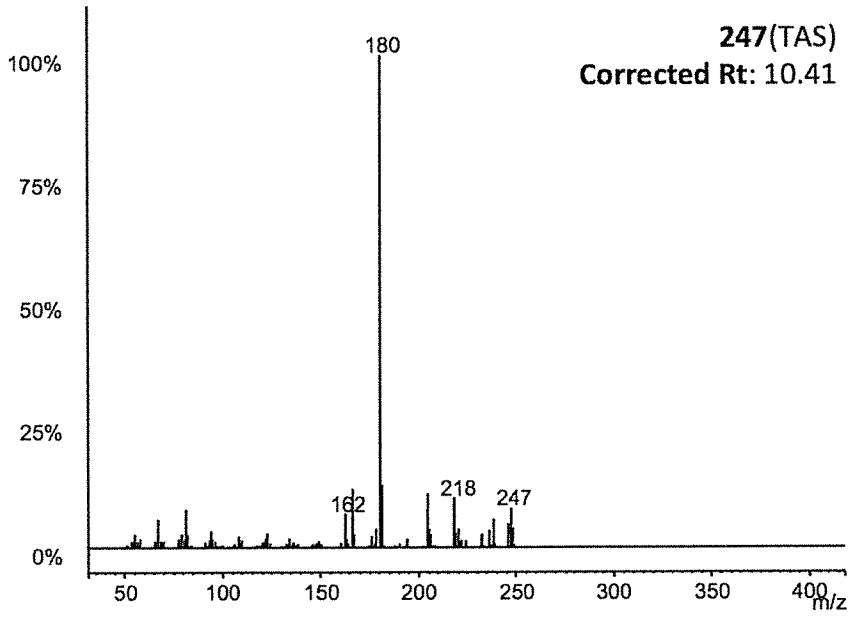
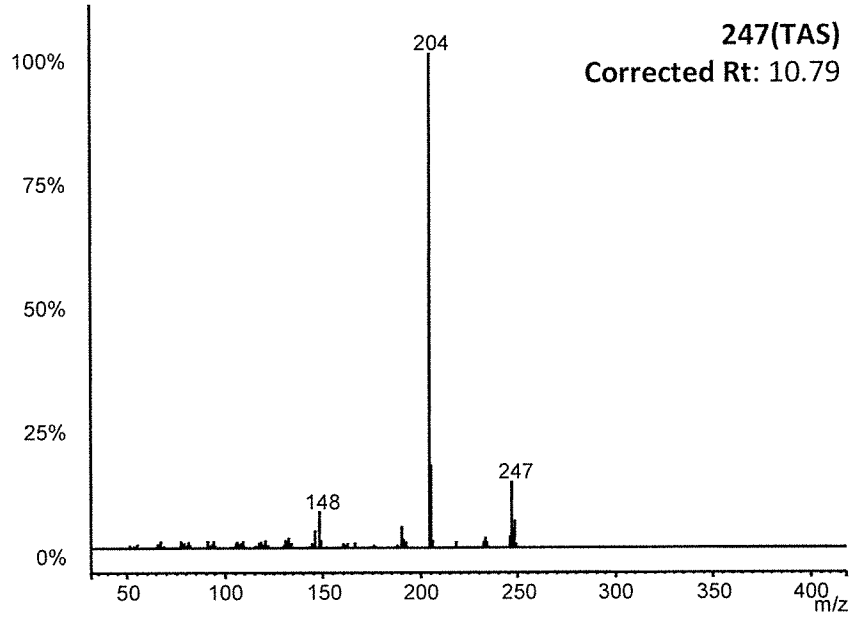
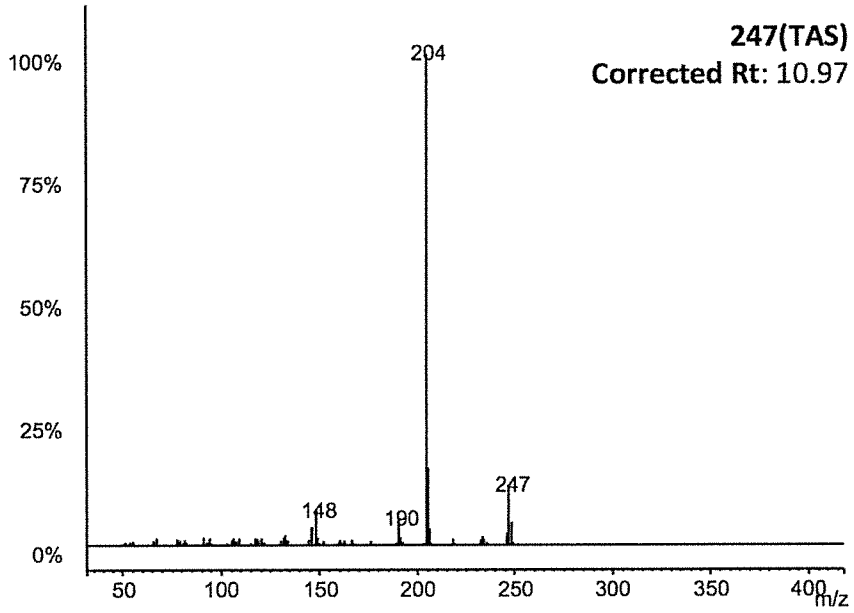

247(TAS)
Corrected Rt: 10.41
180
100%
75%
50%
25%
0%
162
218
247
50
100
150
200
250
300
350
400
m/z

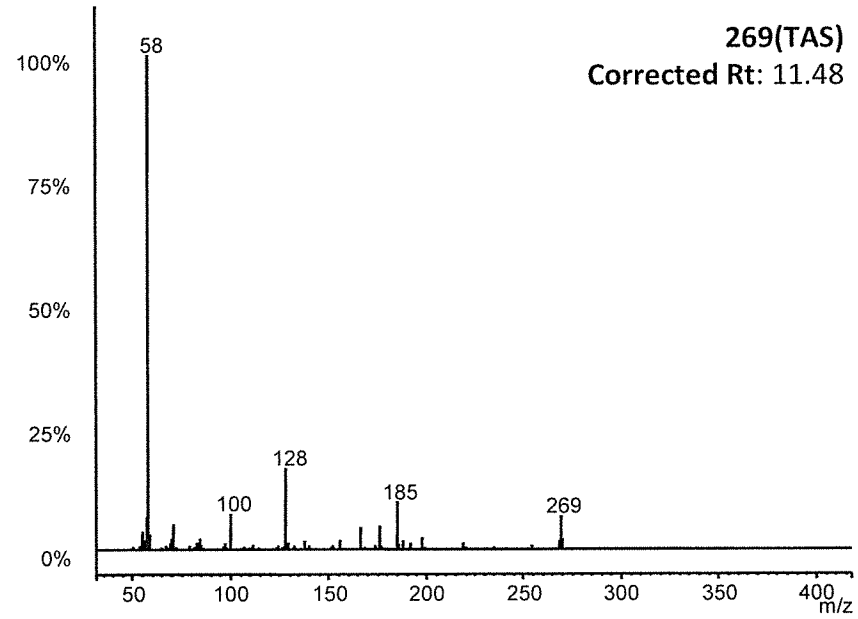
Unclass


247(TAS)
Corrected Rt: 10.79
204
100%
75%
50%
25%
0%
247
148
50
100
150
200
250
300
350
400
m/z

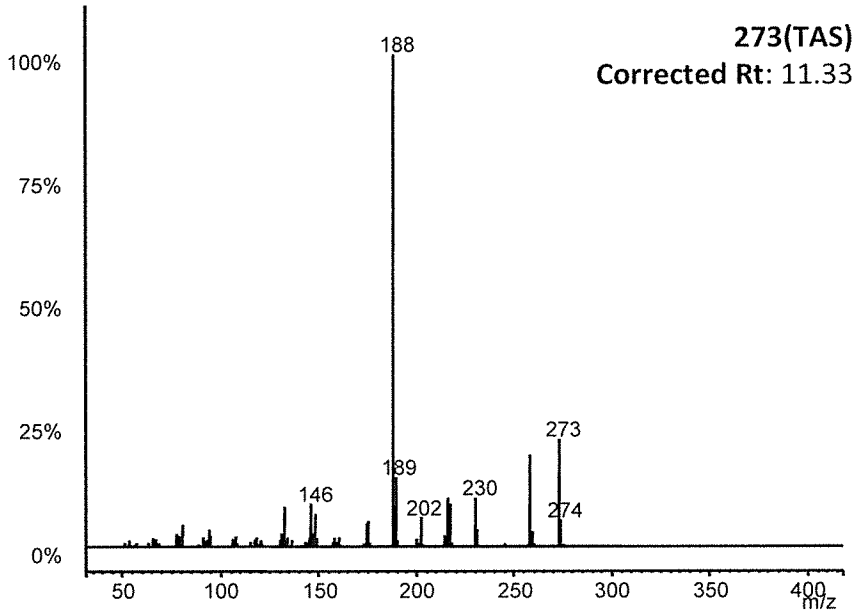
DHQ


247(TAS)
Corrected Rt: 10.97
100%
75%
50%
25%
0%
204
247
148
190
50
100
150
200
250
300
350
400
m/z

DHQ


269(TAS)
Corrected Rt: 11.48
100%
75%
50%
25%
0%
58
128
100
185
269
50
100
150
200
250
300
350
400
m/z

Unclass


273(TAS)
Corrected Rt: 11.33
100%
75%
50%
25%
0%
188
189
146
202
230
273
274
50
100
150
200
250
300
350
400
m/z

Unclass