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Geographic variation in alkaloid-based microbial defenses of the strawberry poison frog, Oophaga pumilio

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Abstract

Frogs of the family Dendrobatidae use alkaloids as a chemical defense against predators. Alkaloids are not manufactured by frogs, but instead sequestered from a diet of arthropods. Alkaloid defenses in dendrobatids differ with geographic location, mainly because arthropod availability varies with location. It has been postulated that alkaloids also function as a defense against microbes and different frog alkaloids might be more/less effective against microbes. We assessed this hypothesis by testing the effectiveness of alkaloids in the dendrobatid frog *Oophaga pumilio* from five geographic locations in Costa Rica and Panama against different microbes. Alkaloids were tested against the bacteria *Escherichia coli* and *Bacillus subtilis*, and the fungus *Candida albicans*. Optical density assays indicated alkaloids from each location inhibited microbial growth, suggesting alkaloids function as a defense against microbes. Furthermore, there are differences in the degree of microbial inhibition among frog locations, suggesting different frog alkaloids vary in effectiveness against microbes.

Introduction

Defense against predation is fundamental to the survival of all organisms. As a result, many defensive strategies are present in organisms to provide protection against predators (Saporito et al., 2010). One mechanism by which plants, animals, and other organisms defend themselves is through the use of noxious, poisonous, or toxic chemicals (Daly, 1994a; Saporito et al., 2010). These toxins are generally acquired in one of two ways: (1) the organism manufactures them *de novo* or (2) sequesters them from external dietary sources (Daly, 1994a; Saporito et al., 2010). Amphibians are well known for using toxins as a chemical defense (Daly, 1994a) and contain many biologically active compounds such as peptides, proteins, steroids, and alkaloids (Daly et al., 1992; Macfoy et al., 2005).

Alkaloids have been studied extensively in the poison frog family Dendrobatidae (Saporito et al., 2004). Over 800 lipophilic alkaloids from more than 20 different structural classes have been found in the skin of five different families of poison frogs (Saporito et al., 2011). It was initially believed that these alkaloids were synthesized *de novo* by the frogs (Daly et al., 2002). However, many studies have confirmed that the alkaloids present in poison frogs are sequestered (acquired) from a diet of arthropods such as mites, beetles,

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ants, and millipedes (Saporito et al., 2011). It has been experimentally demonstrated that captive-raised frogs sequester alkaloids into the poison glands in their skin when fed a diet of fruit flies dusted with alkaloid powder (Daly, et al., 1994b). Conversely, alkaloids are not found in the skin of captive frogs fed a diet of fruit flies (Daly et al., 1996; Daly et al., 2002; Daly et al., 2003; for review, see Saporito et al., 2009).

The types and quantities of alkaloids found in poison frogs (hereafter referred to as alkaloid composition) are related to geographic distance (Saporito et al., 2005). Poison frogs that are found close together are more similar in alkaloid composition when compared to frogs that are more geographically distant (Clark et al., 2006). The dissimilarities in alkaloid composition among frogs is largely attributed to differences in arthropod assemblages and their availability among localities (Clark et al., 2006; Andriamaharavo et al., 2010). Arthropod diversity varies from location to location and different arthropod species contain different alkaloids available to frogs (Saporito et al., 2009, 2011). For example, the most common alkaloids found in mites are 5,8-disubstituted indolizidines and 5,6,8-trisubstituted indolizidines, whereas, ants are the most common source of 3,5-disubstituted indolizidines and 3,5-pyrrolizidines, and millipedes have been found to be a common source of spiropyrrolizidine alkaloids (Saporito et al., 2011). These arthropods are very common in the diet of poison frogs, yet their distributions differ, which leads to differences in frog alkaloids (Clark et al., 2006; Saporito et al., 2011).

Most of the studies conducted regarding alkaloid function have examined their use as a defense against predation. However, alkaloids have recently been hypothesized to have antimicrobial function (Macfoy et al., 2005). Amphibians such as salamanders and frogs can live in a wide variety of habitats, ranging from deep freshwater lakes to dry deserts due to the function of their skin (Clarke, 1996). Amphibian skin is moist and plays an important part in respiration, water and temperature homeostasis, and defense (Clarke, 1996). The moisture in amphibian skin provides a perfect medium for the growth of aerobic microbes like bacteria and fungi (Clarke, 1996). This can be a disadvantage for amphibians because their skin must be kept moist to a certain degree in order for them to function properly. Despite the fact that the majority of amphibians inhabit environments well-suited for pathogen growth, they rarely acquire microbial or fungal infections. Thus, a microbial and fungal defense system must be present in order to keep the organism alive (Clarke, 1996).

Initially, it was believed only peptides (present in many frogs and salamanders) had antimicrobial function to serve as a defense against infections. However, a recent study conducted has shown that certain alkaloids from the skin of dendrobatid poison frogs are effective against the gram-positive bacterium *Bacillus subtilis*, the gram-negative bacterium *Escherichia coli*, and the fungus *Candida albicans* (Macfoy, et al., 2005). The results demonstrated that certain alkaloids from the pyrrolidine, decahydroquinoline, indolizidine, and pumiliotoxin structural classes had significant resistance against *E. coli*. In addition, decahydroquinolines and indolizidines were shown to have significant antifungal activity. Thus, certain alkaloids may better function against different classes of microbes than others (Macfoy et al., 2005).

The study conducted by Macfoy et al. (2005) demonstrated that certain individual alkaloids can be effective against specific microbes; however, my study will utilize the natural composition of alkaloids present in frogs (i.e., alkaloid composition) to gain a further understanding of how alkaloid variation affects microbial growth. Poison frog skin possesses many different alkaloids from different structural classes (Daly et al., 2002). The dendrobatid poison frog *Oophaga pumilio* contains approximately 232 alkaloids throughout its natural range in southern Central America. Different populations of *O. pumilio* vary significantly in the type, number, and quantity of alkaloids (Saporito et al., 2007), which suggests that different populations may differ in their antimicrobial defense. Herein, I studied how antimicrobial function differs among populations of *O. pumilio*. This study tested microbial resistance among different geographically isolated populations of *O. pumilio* and subsequently determined the efficacy of these alkaloids against a gram-positive bacterium, a gram-negative bacterium, and a fungus.

Methods

Frog Collections. The frog skins used in this study belong to the dendrobatid species *Oophaga pumilio*, which ranges from southern Nicaragua through the Caribbean Slope of Costa Rica and into the northwest region of Panama (Savage, 2002). Five geographically isolated populations were chosen for study, which spanned a portion of the species range from northeast Costa Rica to northwest Panama. From each of the 5 populations, 3 individual frogs were chosen for a total of fifteen frogs used in the present study. The frogs

were collected from the following locations: (1) Tortuguero, Costa Rica; (2) La Selva Biological Station, Costa Rica; (3) Puerto Viejo de Talamanca, Costa Rica; (4) Isla Solarte, Panama; and (5) Isla San Cristobal, Panama (Figure 1). All of the frog specimens were collected by R.A. Saporito and M.A. Donnelly as part of a larger, ongoing study aimed at characterizing differences in alkaloids among populations of *O. pumilio* throughout its entire natural range (R.A. Saporito, unpublished data).

Alkaloid Fractionation. In order to isolate the alkaloids from frog skins to use in microbial inhibition assays, individual alkaloid fractionations were conducted for each individual frog skin. After collection, whole frog skins were individually stored in glass vials with 4 mL of 100% methanol and sealed with Teflon lined caps (referred to as a methanol extract). Alkaloids were extracted from frog skins using the procedure outlined in Saporito et al. (2010). One mL of each methanol extract was transferred into a 10mL conical vial and 50 µL of 1N hydrochloric acid was added. Each sample was then mixed and evaporated with nitrogen gas to a volume of 100 µL. Subsequently, each sample was diluted with 200 µL of deionized water. The samples were then extracted with four 300 µL portions of hexane. The resulting hexane (organic) layer was disposed of and the remaining aqueous layer was basified with saturated sodium bicarbonate. Basicity was tested with pH paper. Once basic, each sample was extracted with three 300 µL portions of ethyl acetate, and anhydrous sodium sulfate was added to the newly extracted mixture to remove any excess water. The remaining samples were carefully evaporated to dryness with nitrogen gas. After evaporation, 100 µL of methanol was added to resuspend the concentrated and pure alkaloids. The completed alkaloid fractions were mixed and transferred into a small sample vial and stored at -15°C until its use in each optical density assay (see below).

Microbial Growth and Maintenance. To quantify the efficacy of skin alkaloids against microbial growth, the optical density of each microbe was measured every six hours over a set period of time based on the appropriate growth pattern of each microbe (Gronemeyer, 2013). All three microbes used in this study were purchased from Fischer Scientific and included: (1) *Bacillus subtilis* (a gram-positive bacterium); (2) *Escherichia coli* (a gram-negative bacterium); and (3) *Candida albicans* (a fungus). Upon receiving each microbe, a loopful was transferred (via inoculating loop) into 1 mL of nutrient broth and placed in a

shaking incubator (New Brunswick Scientific 12400 Incubator Shaker). *Escherichia coli* was maintained in Luria-Bertani (LB) nutrient broth at 37.3°C and 200 rpm. All *E. coli* assays were conducted in LB nutrient broth and grown on LB nutrient agar. *Bacillus subtilis* was maintained and grown in Difco nutrient broth and Difco nutrient agar at 27°C and 200 rpm. *Candida albicans* was grown at 37.3°C while maintained and grown in Sabourad dextrose (SD) broth and on SD agar. All subcultures, or new cultures made by transfer of the previous culture into fresh growth media, were left to incubate for 24 hours before 0.5 mL of each sample were transferred to a 250 mL Erlenmeyer flask containing 100 mL of the nutrient broth in which each microbe was initially grown. Each subculture was allowed to grow an additional 24 hours before the assay was performed.

Optical Density Assay. All optical density assays were carried out in a HEPA filtered biological hood. At the completion of the growth period, each subculture was gram stained (*C. albicans* was stained with methylene blue) to ensure purity. After no contamination was found, all cultures were diluted with nutrient broth relative to the McFarland standard (No. 0.5, Hardy Diagnostics). When the appropriate turbidity was reached (1.5 x 108), the solution was mixed and poured into a plastic trough (Diversified Biotech). Using a multichannel pipet (20-200 μL, Labnet), 200 μL of the solution were pipetted into 36 wells of a 96 well plate. Each horizontal row on the plate represented an individual frog (the bottom row represented a methanol control) and each vertical row was assigned a specific alkaloid concentration of 1.6 μL, 3.2 μL, or 4.8 μL of the original 100 μL extracted from each frog (Figure 2). This process was repeated four more times for a total of five plates per microbe (one plate per population). Following the completion of each plate, the optical density was measured at the 620 nm wavelength (Fisher Scientific Multiskan FC). Optical density values were measured every six hours for a set period of time: (1) *E. coli* was measured over 36 hours for a total of seven measurements; (2) *B. subtilis* was measured over a period of 48 hours for a total of nine measurements; and (3) *C. albicans* was measured over a period of 36 hours for a total of seven measurements. Due to high variation in the methanol control during the first *C. albicans* trial, this assay was repeated a second time at the 4.8 μL concentration using the aforementioned method, and optical density was measured every six hours over a period of 24 hours (five measurements total).

All 96 well plates were incubated in the shaking incubator at the appropriate temperature (see above) to ensure log phase growth was occurring.

Statistical Analysis. In order to evaluate the effect of alkaloids on microbial growth, independent sample t-tests were performed to compare the final optical densities between *O. pumilio* and the methanol controls for each of the three microbes examined in this study. T-tests were performed for each frog population, using only the highest alkaloid concentration (4.8 μL). To determine the final optical density value to be used in each comparison, a growth curve was constructed for each microbe relative to the methanol control (Figures 3-5). The point at which growth lag began was chosen as the point of comparison for each independent samples t-test. In order to evaluate the influence of geographic alkaloid variation on microbial growth, a one-way analysis of variance (ANOVA) comparing the final optical densities among all all five populations was performed. In addition, Tukey post-hoc tests were performed to examine pair-wise comparisons in antimicrobial inhibition between the frog populations.

Results

Optical density measurements among *Oophaga pumilio* populations

Escherichia coli. Mean optical densities differed significantly among the five populations of *O. pumilio* ($F_{4,40}$=1.840; p=0.0005) at the 4.8 μL alkaloid volume and 30-hour growth measurement (Figure 6A). Additionally, mean optical densities of the La Selva population were significantly different from the San Cristobal and Puerto Viejo populations (p=0.037; p=0.020, respectively). In addition, mean optical densities of the Tortuguero and Puerto Viejo populations (p=0.003) and the Tortuguero and San Cristobal populations (p =0.006) were significantly different. Mean optical densities were not significant among populations at 1.6 or 3.2 μL alkaloid volumes.

Bacillus subtilis. Mean optical densities differed significantly among the five populations of *O. pumilio* ($F_{4,40}$=1.716; p< 0.0001) at the 4.8 μL alkaloid volume and 36-hour growth measurement (Figure 6B). Additionally, mean optical densities of the Puerto Viejo population were significantly different from the La Selva (p<0.0001), Isla Solarte

(p<0.0001), San Cristobal (p<0.0001), and Tortuguero (p<0.0001) populations. Mean optical densities were not significant among populations at 1.6 or 3.2 µL alkaloid volumes.

Candida albicans. Mean optical densities differed significantly among the five populations of *O. pumilio* ($F_{4,40}$=3.370, p=0.0328) at the 4.8 µL alkaloid volume and 24 hour growth measurement (Figure 6C). Additionally, mean optical densities of the La Selva population were significantly different from the Isla Solarte population (alpha = 0.10; p=0.097). Mean optical densities were not significant among populations at 1.6 or 3.2 µL volumes.

Optical density measurements within populations of *Oophaga pumilio*

Escherichia coli at La Selva. Mean optical densities differed significantly among individual frogs from La Selva ($F_{3,8}$=1.040; p=0.0413); however, each individual demonstrated a varying response in comparison to the methanol control (Figure 7A). Mean optical density of individual 1 was not statistically significant from the methanol control (p=0.9547), whereas mean optical density of individual 2 (p =0.0319) and individual 3 (p=0.0007) was significantly different from the methanol controls.

Escherichia coli at Isla Solarte. Mean optical densities did not differ significantly among frogs from the Isla Solarte population ($F_{3,8}$=0.4947; p=0.3297) (Figure 8A). Additionally, mean optical densities were not statistically significant from methanol controls for any individuals in the population (p=0.3163, p=0.0761, p=0.2930, respectively).

Escherichia coli at San Cristobal. Mean optical densities did not differ significantly among individual frogs at San Cristobal ($F_{3,8}$=0.6900; p=0.1298) (Figure 9A). Additionally, mean optical densities were not statistically significant from methanol controls for any individuals in the population (p=0.1295, p=0.1822, p=0.1690, respectively).

Escherichia coli at Tortuguero. Mean optical densities did not differ significantly among individual frogs at Tortuguero ($F_{3,8}$=0.5988; p=0.2361) (Figure 10A). Additionally, mean optical densities were not statistically significant from methanol controls for any individuals in the population (p=0.1683, p=0.6956, p=0.3571, respectively).

Escherichia coli at Puerto Viejo. Mean optical densities differed significantly among individual frogs at Puerto Viejo ($F_{3,8}$=0.3395; p=0.0030) (Figure 11A). Additionally, mean optical densities were statistically significant from methanol controls for all individuals in the population (p=0.0086, p=0.0036, p=0.0104, respectively).

Bacillus subtilis at La Selva. Mean optical densities differed significantly among individual frogs at La Selva ($F_{3,8}$=0.4752; p<0.0001) (Figure 7B). Additionally, mean optical densities were statistically significant from methanol controls for all individuals in the population (p<0.0001, p=0.0003, p=0.0045, respectively).

Bacillus subtilis at Isla Solarte. Mean optical densities differed significantly among individual frogs at Isla Solarte ($F_{3,8}$=0.6083; p<0.0001) (Figure 8B). Additionally, mean optical densities were statistically significant from methanol controls for all individuals in the population (p<0.0001, p=0.0004, p=0.0001, respectively).

Bacillus subtilis at San Cristobal. Mean optical densities differed significantly among individual frogs at San Cristobal ($F_{3,8}$=0.4775; p <0.0001) (Figure 9B). Additionally, mean optical densities were statistically significant from methanol controls for all individuals in the population (p=0.0007, p=0.0001, p=0.0023, respectively).

Bacillus subtilis at Tortuguero. Mean optical densities differed significantly among individual frogs at Tortuguero ($F_{3,8}$=0.07125; p=0.0042) (Figure 10B). Additionally, mean optical densities were statistically significant from methanol controls for all individuals in the population (p=0.0184, p=0.0034, p=0.0330, respectively).

Bacillus subtilis at Puerto Viejo. Mean optical densities differed significantly among individual frogs at Puerto Viejo ($F_{3,8}$=1.858; p<0.0001) (Figure 11B). Additionally, mean optical densities were statistically significant from methanol controls for all individuals in the population (p=0.0002, p=0.0021, p=0.0029, respectively).

Candida albicans at La Selva. Mean optical densities did not differ significantly among individual frogs at La Selva ($F_{3,8}$=0.4636; p=0.1950) (Figure 7C). Additionally, mean optical densities were not statistically significant from methanol controls for all individuals in the population (p=0.7920, p=0.0626, p=0.6074, respectively).

Candida albicans at Isla Solarte. Mean optical densities did not differ significantly among individual frogs at Isla Solarte ($F_{3,8}$=0.1651; p=0.3759) (Figure 8C). Additionally, mean optical densities were not statistically significant from methanol controls for all individuals in the population (p=0.0887, p=0.8424, p=0.6100, respectively).

Candida albicans at San Cristobal. Mean optical densities differed significantly among individual frogs at San Cristobal ($F_{3,8}$=0.4182; p=0.0451) (Figure 9C); however, individuals demonstrated varying responses to methanol controls. Mean optical density of individual 1 was statistically significant from methanol controls (p=0.0155), whereas mean optical density of individual 2 (p=0.4507) and individual 3 (p=0.4249) were significantly different from methanol controls.

Candida albicans at Tortuguero. Mean optical densities did not differ significantly among individual frogs at Tortuguero ($F_{3,8}$=0.3681; p=0.9659) (Figure 10C). Additionally, mean optical densities were not statistically significant from methanol controls for all individuals in the population (p=0.9346, p=0.7283, p=0.8754, respectively).

Candida albicans at Puerto Viejo. Mean optical densities did not differ significantly among frogs at Puerto Viejo ($F_{3,8}$=0.8742; p=0.4524) (Figure 11C). Additionally, mean optical densities were not statistically significant from methanol controls (p=0.2799, p=0.4661, p=0.7203, respectively.

Discussion
My study found that alkaloids from the poison frog *Oophaga pumilio* significantly inhibit the growth of microbes. Previously, alkaloids were thought to largely function in predator defense, with only one study demonstrating that certain individual skin alkaloids may also

function as a protection against skin infections by inhibiting microbial growth (MacFoy et al., 2005). My results complement Macfoy et al. (2005) by establishing that the complex mixture of alkaloids present naturally in frog's skins are also an effective defense against certain microbes.

Alkaloid inhibition depends on microbe type. This study assayed alkaloids against a gram-positive (*B. subtilis*) and gram-negative (*E. coli*) bacterial species as well as a fungus (*C. albicans*). Overall, alkaloids from *O. pumilio* demonstrated greater inhibition towards bacteria than fungi (Figure 3-5), with *B. subtilis* being the most significantly inhibited bacterial species (Figure 6B). These findings vary slightly from Macfoy et al. (2005) in which inhibition among certain classes of alkaloids was greatest against both *B. subtilis* and *C. albicans.* Both MacFoy et al. (2005) and the present study did not find substantial inhibition against the gram-negative bacteria *E. coli*. In my study, only alkaloids from individuals from La Selva and Puerto Viejo showed statistically significant inhibition against *E. coli* (Figure 7A, Figure 11A, respectively). Overall, the alkaloids in my study did not demonstrate substantial inhibition against *C. albicans* (Figure 5), and only one individual in the San Cristobal population showed significant inhibition against *C. albicans*. These findings suggest that most alkaloids from the strawberry poison frog are ineffective at inhibiting growth of *C. albicans*. MacFoy et al. (2005) found that one class of decahydroquinolines was particularly inhibitory against *C. albicans*, and it is possible that this class of alkaloid is not present in the *O. pumilio* specimens examined in my study. Although *O. pumilio* is able to inhibit microbial growth in certain species of bacteria, it is least effective in fungal inhibition, possible leaving poison frogs more susceptible to fungal infection. Furthermore, the occurrence of *E. coli* inhibition only in select populations suggests that inhibition varies with alkaloid composition and location. Further analyses must be conducted to determine the alkaloid composition of frogs used in my study, which will aid in determining the specific structural classes (and alkaloids) that are more or less inhibitory towards certain microbes.

Alkaloid-based microbial inhibition varies with geographic location. The composition of alkaloids present in poison frogs is the result of dietary accumulation of certain types of arthropods (Saporito et al., 2006). The alkaloids sequestered by poison frogs vary among localities, primarily due to differences in arthropod type and availability between

geographic locations (Clark et al., 2006; Saporito et al., 2011). Previous studies have indicated that poison frogs living in closer proximities to one another have similar alkaloid profiles, suggesting that alkaloid composition is related to geographic location (Saporito et al., 2006; Clark et al., 2006). The results of my study demonstrate significant differences in microbial inhibition among alkaloids from all five populations at the highest concentration (Figure 6A-C), with alkaloids from Puerto Viejo frogs being the most inhibitory against *E. coli* (Figure 3E) and *B. subtilis* (Figure 4E). These results suggest that frogs from some locations are more potent than others, which could result in differential inhibition against microbial infection. The differences in alkaloid potency are likely the result of differences in alkaloids among localities, which can be explained by variation in arthropod availability between locations. Geographic variation in frog alkaloids is thought to play an important role in defense against predators (Saporito et al., 2006), and the findings of my study suggest that geographic location is also an important determinant in a frog's defense against microbes.

Alkaloid-based microbial defenses also vary within individuals among localities. In certain populations, some frogs significantly inhibited microbial growth whereas others did not show significant inhibition. For example, among individuals within the La Selva location, only alkaloids from frogs 2 and 3 significantly inhibited *E. coli* growth (Figure 3A), whereas all 3 frogs inhibited *B. subtilis* growth (Figure 4A), and none were particularly effective against *C. albicans* (Figure 5A). Conversely, alkaloids from individual frogs from San Cristobal did not demonstrate significant inhibition against *E. coli* (Figure 3C), but inhibition was observed in all individuals against *B. subtilis* (Figure 4C), and in frog 1 against *C. albicans* (Figure 5C). Additionally, alkaloid composition has been shown to vary among individuals within a geographic location (Saporito et al., 2006). The results of my study suggest that there are differences in alkaloids among individual frogs within a geographic location, and these differences appear to result in differences in microbial growth inhibition. The differences in alkaloids among individuals from the same location are likely attributed to small differences in arthropod availability within each collection site (Saporito et al., 2006, 2007). Interestingly, although differences in frog alkaloids are small within a location, these differences are still large enough to result in differential microbial inhibition among individual frogs.

In conclusion, my study demonstrated that the complex mixture of alkaloids present in the poison frog *Opumilio* inhibit microbial growth. These findings suggest that alkaloids in frog skin may serve as both a defense against predators as well as a defense against microbes. Furthermore, microbial inhibition is highly dependent upon microbe type, with *O. pumilio* alkaloids being most effective against bacteria and only moderately effective against a fungus. The degree of microbial inhibition varies significantly among geographic locations and individuals within a location, which is consistent with differences in frog alkaloids on similar scales. These findings illustrate that differences in arthropod derived alkaloids in *O. pumilio* may play a significant role in a poison frogs ability to defend itself from certain microbial infections. This study has proven valuable in demonstrating the relationship between alkaloid inhibition, microbial type, and geographic location, yet further research remains necessary. The alkaloids responsible for microbial inhibition were not characterized in the present study, preventing a more complete understanding of how differences in specific alkaloids function in the defense against certain microbes. Furthermore, the microbes used in the present study represent common, relatively non-pathogenic strains of microbes that are not likely to infect frogs. Future studies should aim to examine biologically relevant microbes, such as the chytrid fungus *Batrachochytrium dendrobatidis*, which has been shown to be the cause of major amphibian declines worldwide (Lips et al., 2006).

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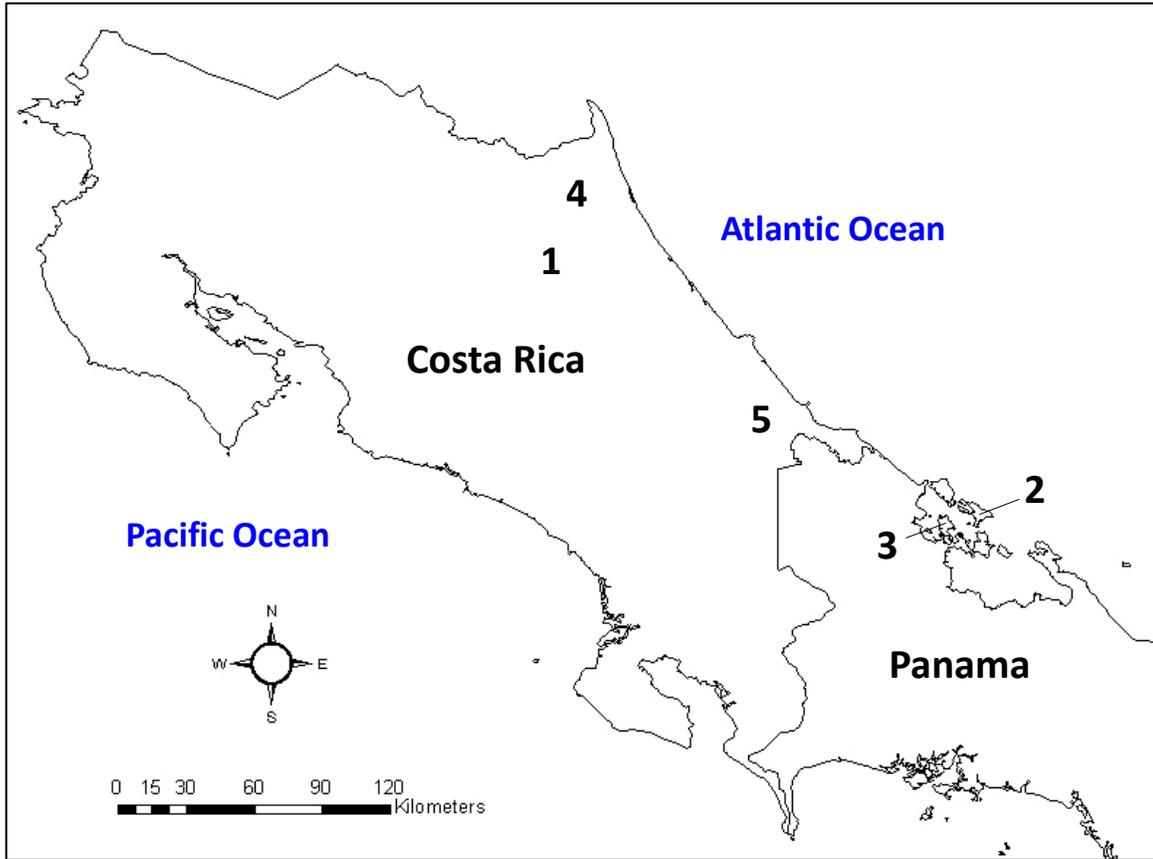
Figure 1. Map of *Oophaga pumilio* localities in Costa Rica and Panama. (1) La Selva Biological Station; (2) Isla Solarte; (3) Isla San Cristobal; (4) Tortuguero; (5) Puerto Viejo.
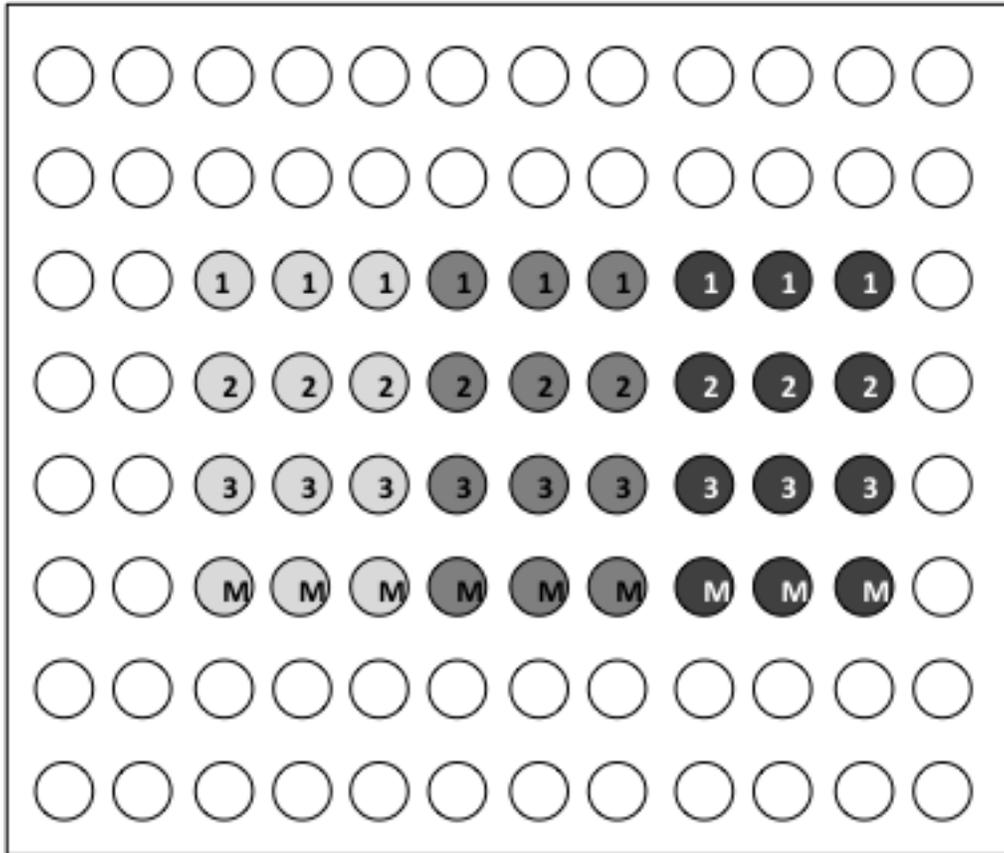
Figure 2. Arrangement of alkaloid treatments per population on 96 well plate. Numbers denote individual specimens: (1) frog 1; (2) frog 2; (3) frog 3; (M) methanol control. Additionally, each shaded region represents a specific alkaloid volume. Light shaded region represents 1.6 μL wells; medium shaded region represents 3.2 μL; darkest shaded region represents wells that received 4.8 μL of alkaloid.
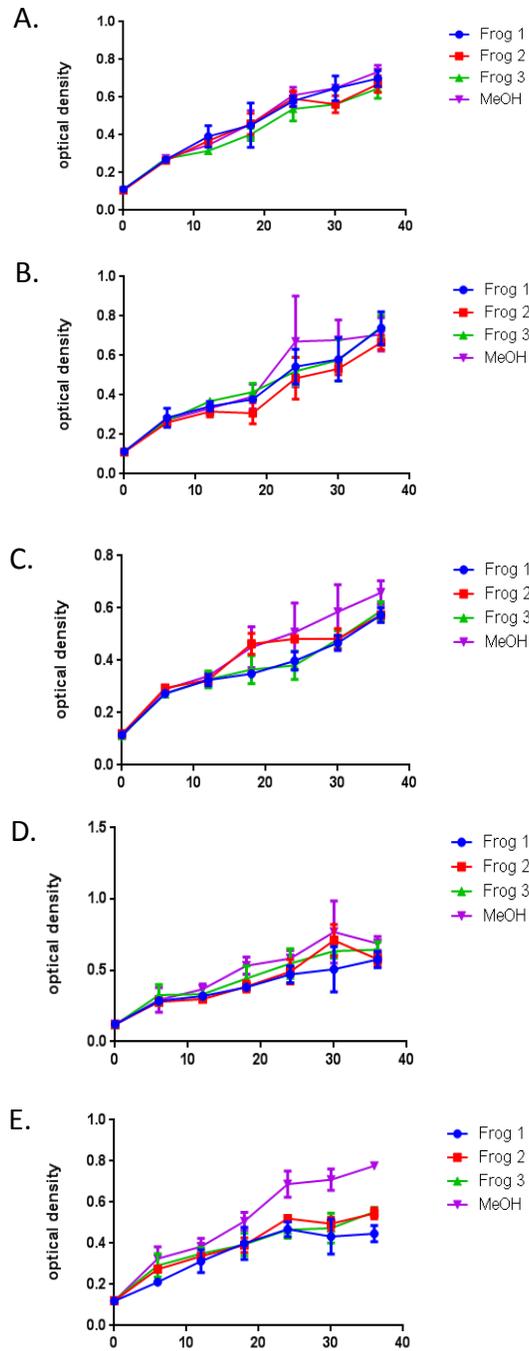
Figure 3. Mean optical density growth curves of *Oophaga pumilio* populations at 4.8 µL of alkaloid against *Escherichia coli* (+/- 1 S.E.). Data was averaged from all individuals within a population in addition to methanol controls. (A) La Selva; (B) Isla Solarte; (C) San Cristobal; (D) Tortuguero; (E) Puerto Viejo.
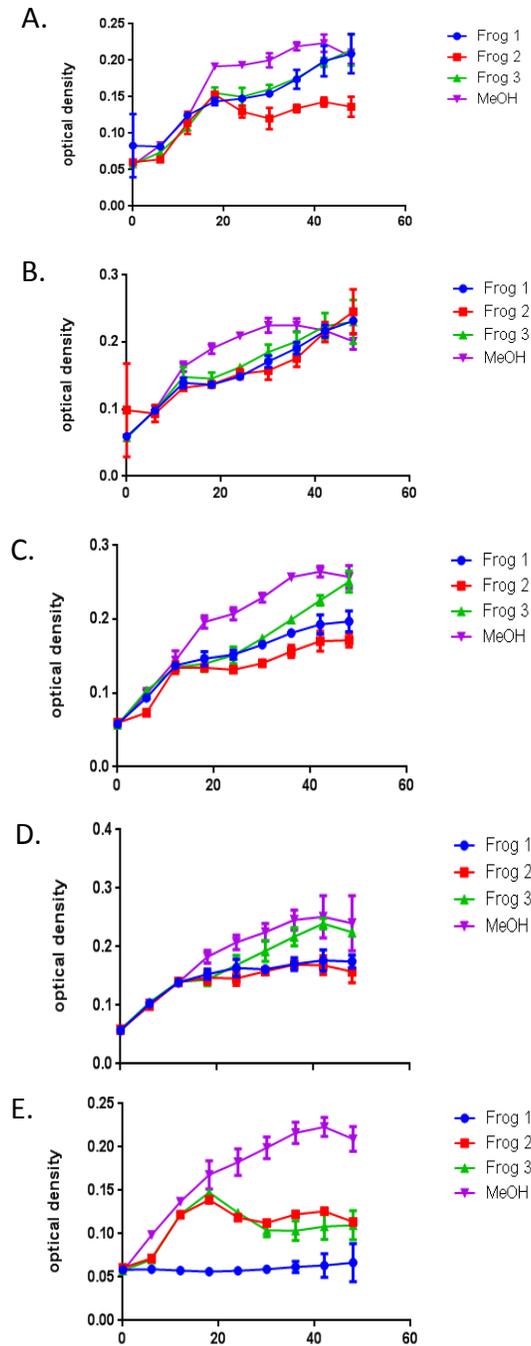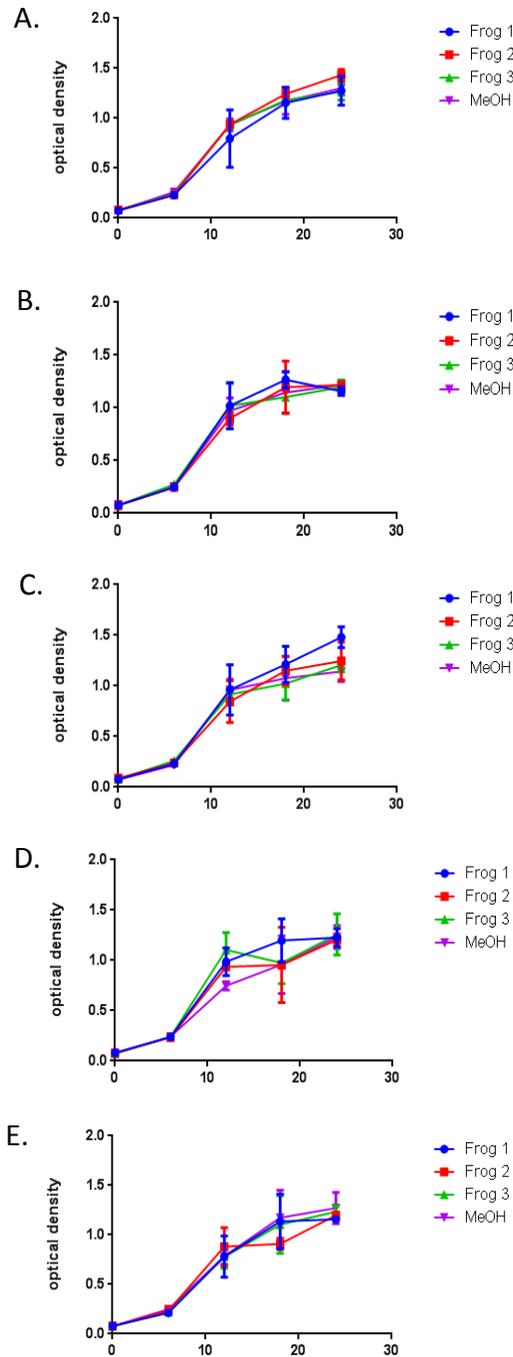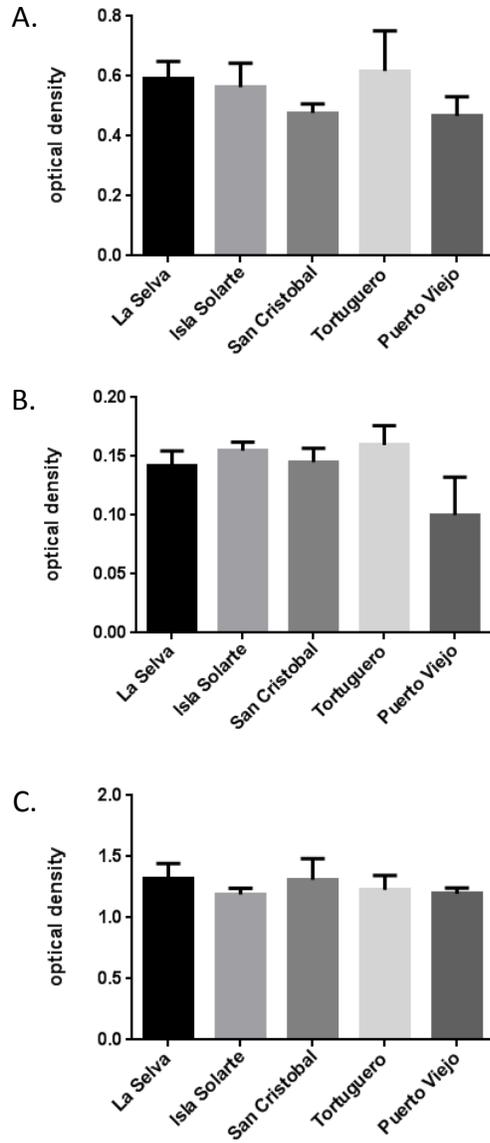
Figure 4. Mean optical density growth curves of *Oophaga pumilio* populations at 4.8 μL of alkaloid against *Bacillus subtilis* (+/- 1 S.E.). Data was averaged from all individuals within a population in addition to methanol controls. (A) La Selva; (B) Isla Solarte; (C) San Cristobal; (D) Tortuguero; (E) Puerto Viejo.

Figure 5. Mean optical density growth curves of *Oophaga pumilio* populations at 4.8 μL of alkaloid against *Candida albicans* (+/- 1 S.E.). Data was averaged from all individuals within a population in addition to methanol controls. (A) La Selva; (B) Isla Solarte; (C) San Cristobal; (D) Tortuguero; (E) Puerto Viejo.

Figure 6. Mean optical densities of *Oophaga pumilio* populations at 4.8 μL of alkaloid (+/- 1 S.E.). Data was averaged from all individuals within a population (n=3). (A) *Escherichia coli*; (B) *Bacillus subtilis*; (C) *Candida albicans*.
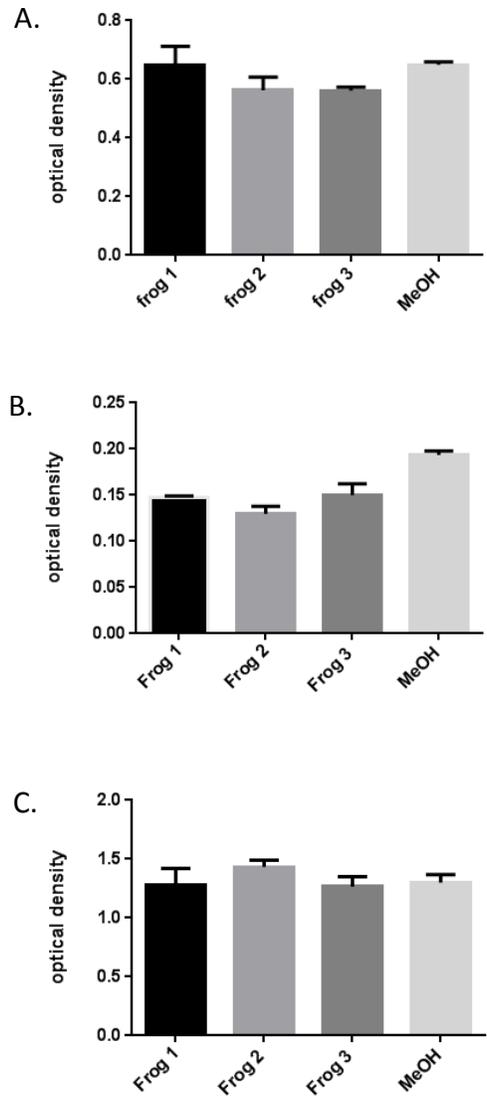
Figure 7. Mean optical densities of *Oophaga pumilio* specimens from the La Selva population at 4.8 μL of alkaloid (+/- 1 S.E.) (A) *Escherichia coli*; (B) *Bacillus subtilis*; (C) *Candida albicans.*
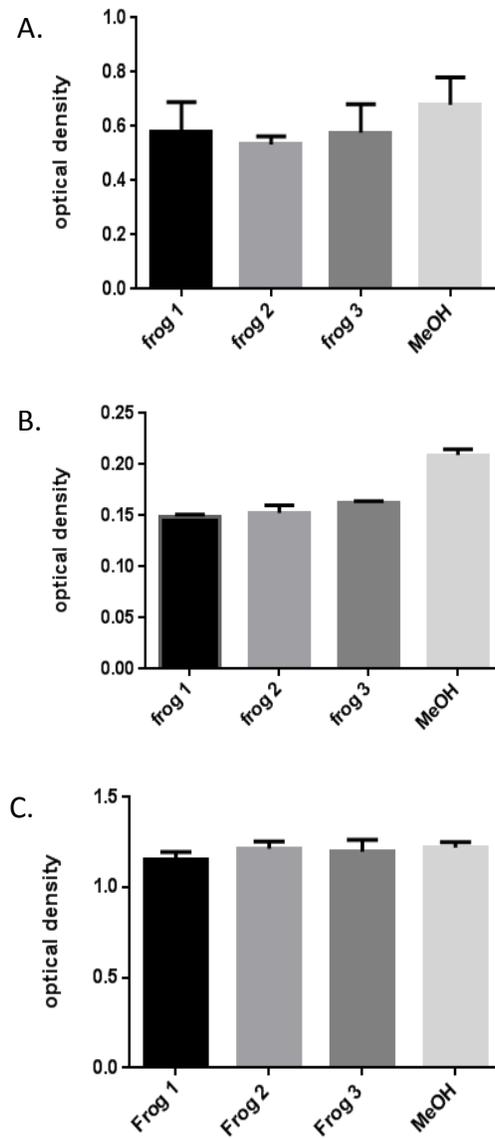
Figure 8. Mean optical densities of *Oophaga pumilio* specimens from the Isla Solarte population at 4.8 µL of alkaloid (+/- 1 S.E.) (A) *Escherichia coli*; (B) *Bacillus subtilis*; (C) *Candida albicans*.
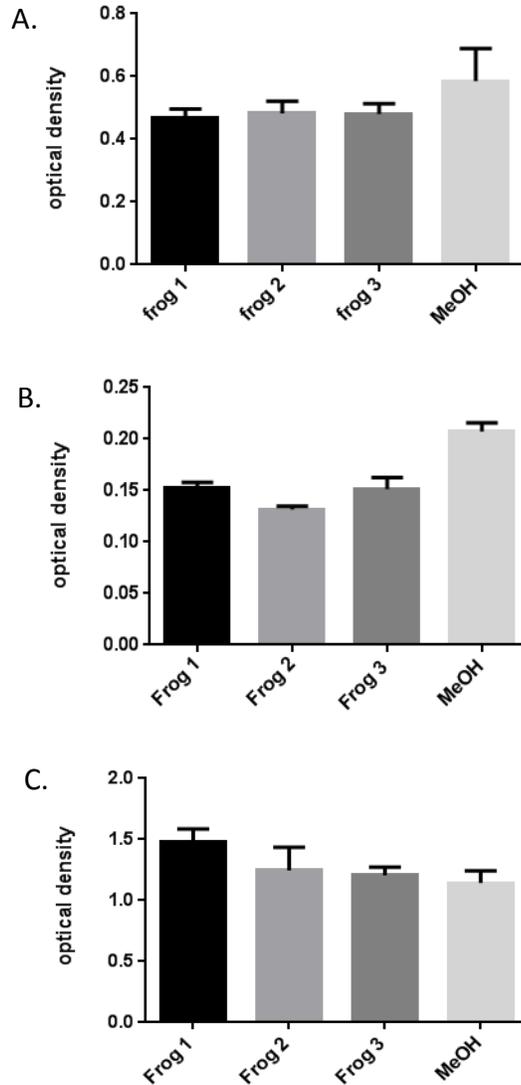
Figure 9. Mean optical densities of *Oophaga pumilio* specimens from the San Cristobal population at 4.8 μL of alkaloid (+/- 1 S.E.) (A) *Escherichia coli*; (B) *Bacillus subtilis*; (C) *Candida albicans.*
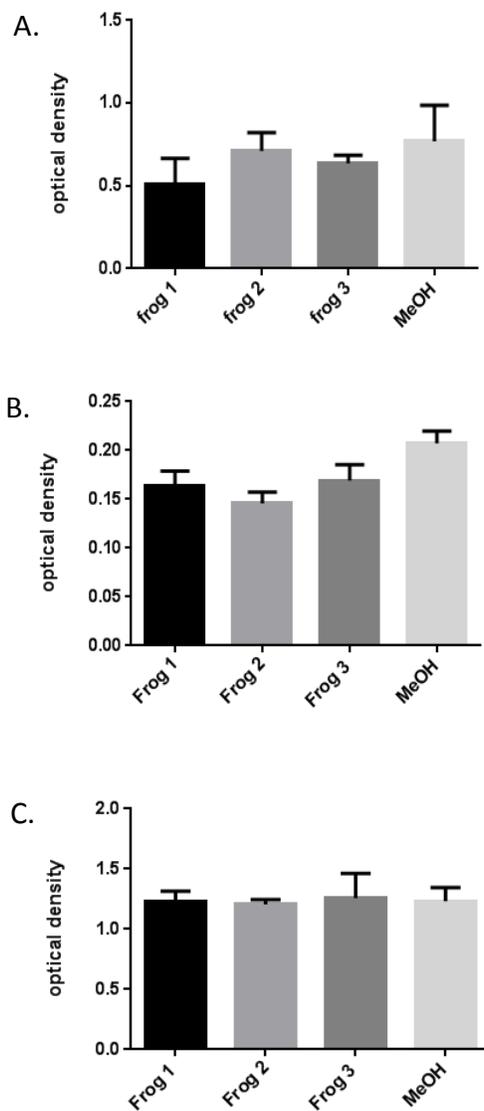
Figure 10. Mean optical densities of *Oophaga pumilio* specimens from the Tortuguero population at 4.8 μL of alkaloid (+/- 1 S.E.) (A) *Escherichia coli*; (B) *Bacillus subtilis*; (C) *Candida albicans*.
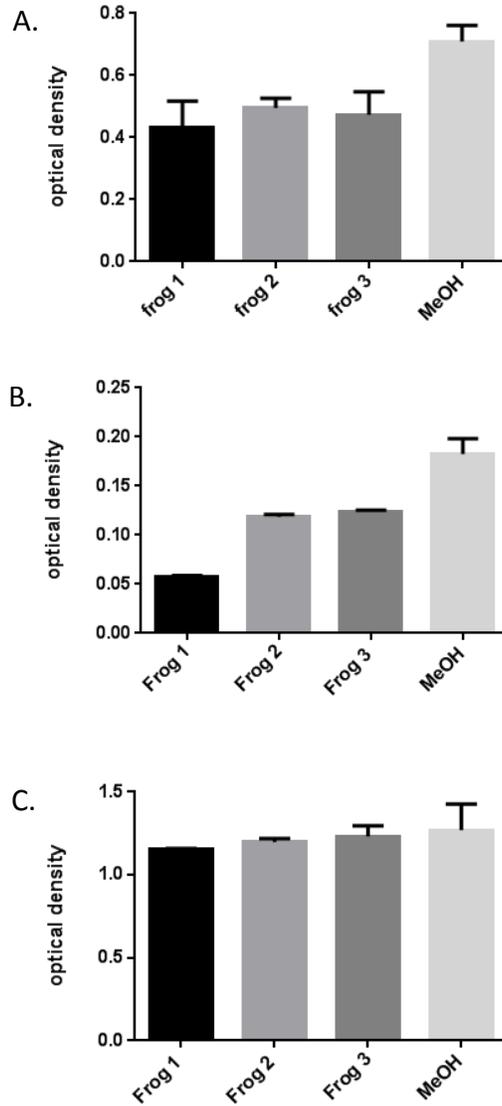
Figure 11. Mean optical densities of *Oophaga pumilio* specimens from the Puerto Viejo population at 4.8 μL of alkaloid (+/- 1 S.E.) (A) *Escherichia coli*; (B) *Bacillus subtilis*; (C) *Candida albicans*.