CAUSES AND CONSEQUENCES OF WARNING COLOR VARIATION IN A POLYTYPIC POISON FROG

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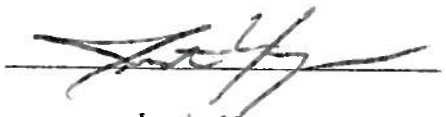
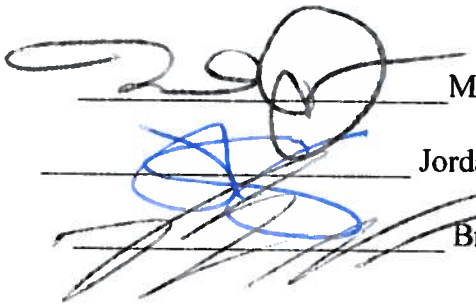


Justin Yeager

APPROVED: Corinne J. Zawacki Corinne Richards-Zawacki, Ph.D.

Director



Michael Blum, Ph.D.

Jordan Karubian, Ph.D.

Brice Noonan, Ph.D.

ABSTRACT:

Understanding the mechanisms that promote rapid phenotypic divergence in adaptive color-patterns affords valuable insights into understanding patterns of biodiversity. My research has emphasized divergence in coloration and patterning by focusing primarily on aposematic species where color-pattern is not only used as an anti-predatory signal, but has also been coopted to influence female mate choice and male/male conflict.

My dissertation research was grounded firmly in behavioral ecology, studying the evolutionary ecology of adaptation. I focused on two main scales of interactions between poison frogs and their local habitats. The first looked at the fine-scale influence of the frog's microhabitat in shaping aposematic signaling using a multi-trophic level approach. The second scale broadly sought to identify isolating barriers between phenotypically distinct populations.

ACKNOWLEDGMENTS:

Though a dissertation (by design) features only a single name, it is the product of a myriad of relationships between a wide variety of people. I am enormously grateful for the guidance, patience, and support of my 'boss', Corinne Richards-Zawacki. She taught me more than she'll know and pushed me to produce the highest quality products we could. Her influence will stick with me well beyond my dissertation. I also benefitted greatly from the mentorship of Mike Blum to whom I am indebted for teaching me nuances of this field and continually challenging both me and my work. To Brice Noonan and Jordan Karubian I owe a great debt for their mentorship, suggestions, friendship, and providing excellent role models. The entire Tulane EEBIO department are invaluable in terms of collaboration, assistance and friendship. Jack Leslie and Davi Batistella are the foundation of our ivory tower. I apologize to Davi, I feel like she's had to pull out a whiteout correction pen at some point on every piece of paperwork I've ever submitted, and to Jack for asking for countless "little favors". I would also like to thank my parents and sister for their immense support and occasional loans, and I would like to apologize for any incidental complaints due to stress. I would also like to thank my many collaborators whose credit is unfortunately not acknowledged on this document, though

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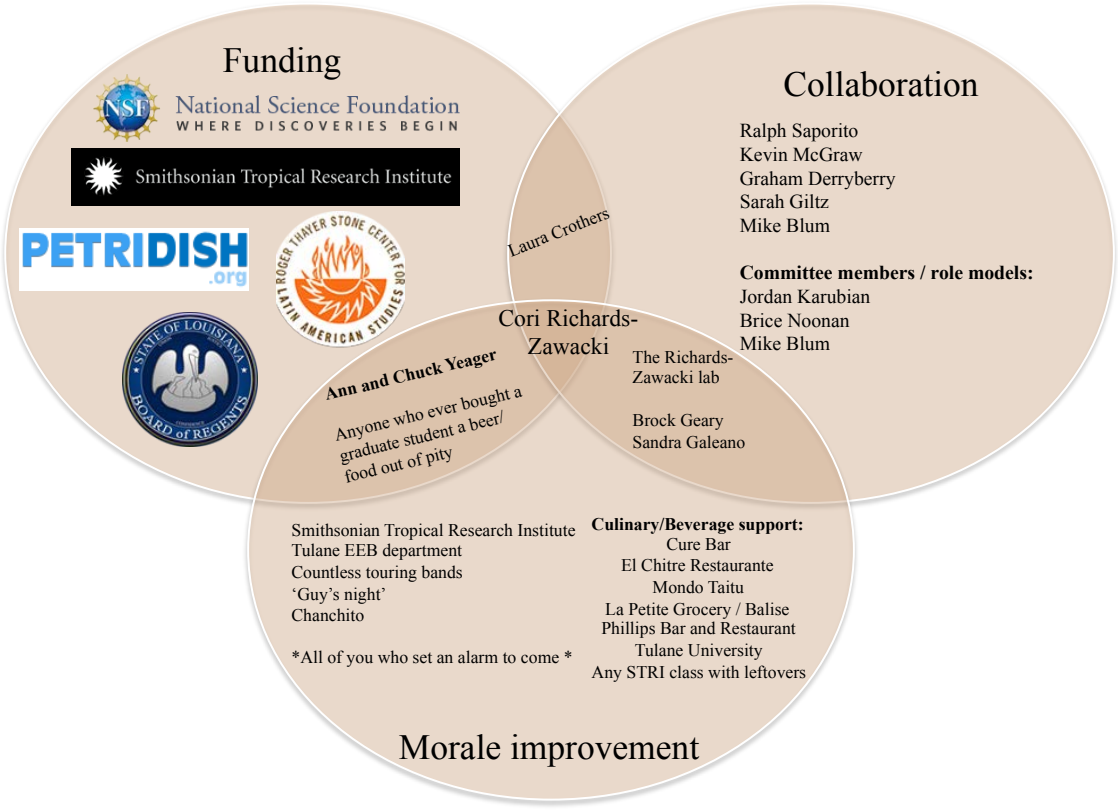

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Chapter 1

LOW RISK OF PREDATION ASSOCIATED WITH PHENOTYPIC DIVERSITY IN AN APOSEMATIC SPECIES

Abstract

Predators can be important sources of selection on prey phenotypes. Aposematism theory predicts that with high levels of predation, avoidance of familiar color patterns relative to novel (allopatric) phenotypes can act as a source of stabilizing selection to constrain prey phenotype. Here we leverage a natural transition zone between conspicuous aposematic and cryptic phenotypes of the polytypic poison frog *Oophaga pumilio* to test for differences in predator-mediated natural selection. Using a multi-year study, we compare predator attack rates on alternative model prey phenotypes to ask: 1) whether local prey populations are attacked less than foreign ones, 2) whether the ancestral (conspicuous aposematic) phenotype is universally avoided, and 3) whether fine-scale habitat differences influence attack rates. We find no support for the prediction that attack frequency differs among divergent phenotypes, regardless of whether the phenotypes are foreign or local. While we observed substantial heterogeneity in attack frequencies along transects within sites, this was not attributable to differences in canopy

cover. However, we do find a lower frequency of attacks in the area where *O. pumilio* is polymorphic than in adjacent monomorphic areas, suggesting that low predation may have enabled populations to diverge in phenotype, perhaps in response to other selective pressures.

Introduction

Phenotypic divergence between populations is often associated with reduced gene flow, a phenomenon that can ultimately lead to reproductive isolation (Coyne and Orr 2004). Prezygotic isolating mechanisms can limit gene flow between phenotypically distinct populations, for example if individuals chose mates assortatively based on divergent traits (Jiggins et al. 2001, Kirkpatrick and Ravigné 2002). Predation can also create prezygotic isolation by constraining the distribution of particular phenotypes and reducing secondary contact (Nosil et al. 2005, Rundle and Nosil 2005). Specifically, predation pressure is predicted to shape population boundaries if foreign prey phenotypes are subject to greater predation than local ones, and because of this, foreign individuals often do not often survive long enough to reproduce outside of their native range (immigrant inviability, Rundle and Nosil 2005). Ecological hybrid inviability can also contribute to reproductive isolation. This can happen when the likelihood of predation on the offspring resulting from mating between immigrant and native phenotypes is higher than predation on offspring of two native individuals due to selection against intermediate phenotypes (Nosil et al. 2005).

Aposematic species mitigate predation risk by coupling a signal that is easily identifiable to would-be predators with a defense that that renders them unpalatable or unprofitable as prey (Ruxton et al. 2004). Examples of aposematism are found across

numerous animal taxa including invertebrates (Ruxton et al. 2004) and vertebrates (Saporito et al. 2007, Mochida 2011). Experimental evidence for the efficacy of aposematism comes from field predation studies where replicas of conspicuous aposematic prey are attacked less than non-aposematic phenotypes (e.g., snakes: Brodie 1993, salamanders: Kuchta et al. 2005).

While the coloration of aposematic taxa seems likely to be subject to natural selection, natural selection and sexual selection often act on the same traits, and sometimes in opposing directions (Rudh et al. 2011). In the case of aposematism, elements of the warning signal can be subject to both sexual and natural selection, but in this case phenotypic divergence within and among populations is expected to be minimal as such signals are predicted to be under stabilizing natural selection.

In poison frogs, the combination of conspicuous coloration (Maan and Cummings 2012) and diet-acquired skin alkaloids (Myers and Daly 1976, Santos et al. 2003, Saporito et al. 2006) are thought to act as an aposematic signal (Summers and Clough 2001). This hypothesis is supported by studies that have shown that frog replicas colored to match local conspicuous phenotypes are attacked less frequently than cryptic or foreign aposematic forms (Saporito et al. 2007, Noonan and Comeault 2009, Chouteau and Angers 2011; but see Hegna et al. 2012, Richards-Zawacki et al. 2013). Aposematism theory similarly predicts that the movement of animals bearing locally adapted warning phenotypes outside their native range will result in increased predation risk and reduced fitness for immigrant individuals (Mallet and Barton 1989). This can lead to reproductive isolation via immigrant inviability (Funk 1998, Nosil 2004), which is driven by divergent habitat characteristics such as predator communities, and can act on

contemporary timescales, becoming established in as little as dozens of generations (Hendry et al. 2007 and references therein).

Phenotypic divergence in aposematic species is paradoxically abundant. Examples include ring species like the *Ensatina eschscholtzii* complex of salamanders (Wake et al. 1986), or in the context of mimicry, like co-mimic *Heliconius* butterfly species (Mallet and Joron 1999). Despite the use of color in anti-predatory signaling, populations of the strawberry poison frog, *Oophaga pumilio,* are polytypic, and are thought to have diverged in coloration rapidly in and around the Bocas del Toro archipelago of Panama (Summers and Amos 1997, Wang and Shaffer 2008, Cummings and Crothers 2013). Coalescent simulations suggest that selection, as opposed to purely neutral evolution, is required to explain this phenotypic divergence (Brown et al. 2010). Both natural selection via predation (Saporito et al. 2007) and sexual selection via female mating preferences (Reynolds and Fitzpatrick 2007, Maan and Cummings 2008, Richards-Zawacki and Cummings 2011) have been proposed to explain the divergence of Panamanian *O. pumilio* populations. However, the role of natural selection in the phenotypic divergence of *O. pumilio* remains the least studied of these alternatives (Cummings and Crothers 2013).

Studies have found innate avoidance by avian predators of prey species bearing long wavelength colors such as reds, oranges and yellows (Smith 1975, Schuler and Hesse 1985, Pegram and Rutowski 2014). There is also some evidence for this sort of generalized avoidance by predators of the ancestral (red) phenotype of *O. pumilio*. On Isla Colon in the Bocas del Toro archipelago, models representing an allopatric red phenotype were attacked less frequently than models of a local green morph (Hegna et al.

2012). In this species, the red (with blue legs) phenotype is thought to be ancestral (Wang and Shaffer 2008), but whether frogs bearing that phenotype are better protected than those bearing the derived (and often less conspicuous) phenotypes found in other areas of Bocas del Toro remains unclear. Habitat heterogeneity also remains an unexplored facet of predation studies in poison frogs, where forest traits such as canopy cover may influence the perception of aposematic signals and influence phenotype-specific attack frequencies.

Here we aim to clarify the role of natural selection in the phenotypic divergence of *O. pumilio* populations by taking advantage of a natural phenotypic transition zone. Specifically, we test the following predictions: 1) prey phenotypes differ in their frequency of attack and local phenotypes experience fewer attacks than foreign ones, consistent with aposematism theory and immigrant inviability 2) the ancestral conspicuous (aposematic) phenotype is attacked less frequently than other morphs in all localities, and 3) spatial heterogeneity in attack rates can be attributed to fine-scale environmental heterogeneity in canopy cover. We address these predictions using a multi-year field study in three sites spanning a phenotypic transition zone, including two areas where *O. pumilio* is monomorphic and a polymorphic area situated between them (Figure 1). We use clay replica frogs that capture this variation to measure the frequency of attack across phenotypes and localities and test our predictions about the role of natural selection in this system.

Materials and Methods

Study Populations: Our study took place in three sites on mainland Panama, adjacent to the Bocas del Toro archipelago, where frogs are abundant and vary in coloration. No

obvious geographic barriers to dispersal are present between these sites (Figure 1). At one of our study sites frogs are monomorphic and red with blue legs (Almirante, 09°19’16.3”N, 82°29’49.5”W), at another frogs are monomorphic and blue (Aguacate Peninsula, 09°10’37.9”N, 82°16’00.4”W), and at the third, frogs are polymorphic, ranging in coloration from uniformly blue to intermediate (brown), to red with blue legs (Dolphin Bay, 9°13'15.7"N, 82°13'5.6"W). This level of polymorphism, where *O. pumilio* morphs span the continuum from putatively cryptic (blue and brown) to conspicuous (red with blue legs) appears to be unique to the Dolphin Bay region as previous studies with sampling throughout the archipelago have not described a similar situation (e.g. Rudh et al. 2007, Wang and Shaffer 2008, Hauswaldt et al. 2011). One insular population on Isla Bastimentos exhibits polymorphism, though all phenotypes in that case are thought to be highly conspicuous (Richards-Zawacki et al. 2013). Almirante and Aguacate sites were chosen for their proximity to the polymorphic population and because their local *O. pumilio* phenotypes represent the extremes of the phenotypic continuum displayed in Dolphin Bay.

Clay Models: Clay frog replicas were constructed using silicone molds following Yeager et al. (2011). Briefly, we heated Van Aken™ modeling clay in small crockpots and poured liquid clay into silicone molds resulting in models that were similar in size (20mm snout-vent length, SVL) and shape to adult *O. pumilio* and matched to the dorsal and extremity coloration of frogs from our focal populations. We assessed the similarity of the coloration of our clay models to frogs from our focal populations using the visual modeling approach of Endler and Mielke (2005). We compared the color of our models to frogs from our focal populations under a tetrachromatic avian visual system using the

program AVICOL (Gomez 2006, Figure 2). Four spectral reflectance measurements were taken and averaged from the dorsum of 15 male *O. pumilio* from each of Almirante (red with blue legs), Aguacate (blue) and Dolphin Bay (brown) using an Ocean Optics Jaz portable spectrometer (Ocean Optics, Inc., Dunedin, FL) with an internal Jaz-PX pulsing xenon light source and a QR400-7-SR-BX reflection probe. A WS-1 white standard was used to account for lamp drift between every sample. Absolute irradiance measurements were taken using the Ocean Optics Jaz with a QP400-2-UV-VIS fiber with a CC-3-UV-S cosine corrector. Down welling absolute irradiance measurements were taken on a sunny morning in June 2011 from a closed canopy (low light) forest during the period of peak *O. pumilio* activity. The spectrometer was calibrated when switching between reflectance and irradiance measurements using an Ocean Optics LS-1-CAL tungsten halogen lamp. To estimate how each frog and model phenotype would be perceived by a potential predator (bird), we used the visual model developed by Endler and Mielke (2005) as implemented in the program AVICOL (Gomez 2006). Inputs into the model included (1) dorsal spectral reflectance measurements of male *O. pumilio*, (2) irradiance measurements from *O. pumilio* habitat, and (3) absorbance spectra of the four cone classes of an avian visual system (Blue tit, *Cyanistes caeruleus* from Hart and Vorobyev 2005) with weber fractions (v_i) for from Siddiqi et al. (2004). We chose a bird visual system because birds have been observed predating on *O. pumilio* (Alvarado et al. 2013, Lenger et al. 2013) and represent a significant proportion of attacks in previous clay frog predation experiments (Saporito et al. 2007, Noonan and Comeault 2009, Hegna et al. 2012, Richards-Zawacki et al. 2013).

Whereas brown models have been previously used to represent palatable species of frogs that commonly co-occur with *O. pumilio*, (e.g., Saporito et al. 2007, Noonan and Comeault 2009), brown is a naturally occurring *O. pumilio* phenotype in our Dolphin Bay study site. While we did not analyze the skin chemistry of these frogs, studies of other *O. pumilio* phenotypes from the Aguacate Peninsula (where Dolphin Bay is located) have demonstrated appreciable levels of toxicity (Myers and Daly 1976, Maan and Cummings 2012, Yeager et al. *in prep.*), leading us to believe this brown morph is also chemically defended. By placing clay models of three phenotypes (blue, brown and red with blue legs) at each of our three study sites, we were able to test for differences in attacks between phenotypes both within and outside their native ranges.

Field trials: At each study site, model sets were placed two meters apart along a single 250m transect through secondary forest (typical *O. pumilio* habitat, Pröhl and Berke 2001) where frogs were abundant. In each site we placed 1512 models, which were comprised of 504 per morph in each site. Each 'model set' consisted of three clay models, one of each color, placed in random order and with even spacing between them along a 1m line perpendicular to the main transect (Figure 3). We placed models in this way, rather than singly along the main transect, to increase the likelihood that potential predators that saw one frog phenotype saw all three before deciding which to attack. We placed 126 models of each phenotype along each transect twice (at least 14 days apart) in June and July of 2011 and 2012 (4 replicates for each transect). When placing the models, we cleared leaf litter and other organic debris from immediately around the models (cleared area = 1m x 0.3m) to prevent visual obstruction while maintaining all other attributes of the surrounding habitat.

We placed models directly on the cleared ground between 0900-1100h, and left them in place for 48h. Upon collection, each model was visually inspected for attack marks. Attacks were identified as indentations in the clay characteristic of predator morphology (e.g. "v" shaped bird beaks, paired rodent incisors, long sharp grooves from crab claws; Richards-Zawacki et al. 2013) and missing models were excluded from analyses (following Brodie 1993, Noonan and Comeault 2009).

Canopy Cover: Canopy cover is known to influence the perception of conspicuous signals such as via variation in absolute irradiance (Endler 1993). Recent evidence using 'human predators' has suggested that poison frogs can be more readily detected in open canopy gaps than closed forests (Rojas et al. 2014). During year two of our experiment only, we estimated canopy cover every 10m along each of our transects using a concave spherical densiometer, and asked whether canopy cover predicted attack in the 300 model sets directly under these measurements (Figure 3).

Statistical analyses: We used a generalized linear mixed model in which we entered the binary response of model attack (yes/no) as the dependent variable. We included site (i.e., population), model color, year and all two- and three-way interactions as fixed effects. We included the random effects of transect replicate (1–4, coded uniquely for each site) nested within year, and 'position' nested within site. Because of the low overall attack rate (7.9%), defining 'position' as a model set (i.e., group of 3 models) proved uninformative, as all variation was assigned to that random effect. So instead, we considered models placed in the same position on the transect for both transect replicates of that year as part of a 'position' (n = 6 models/position).

An effect of model color would suggest that frog phenotype predicts attack frequency across sites, while an effect of site would indicate that predation differs between locations. The model color by site interaction term specifically tests the prediction that the effect of model color differs among sites, and here we specifically predicted that colors would be attacked less frequently where they are local. The effect of year as well as interactions including this term would indicate that predator effects differed over time. The random effects of transect replicate within year and model group address the potential non-independence of predation events in close proximity in time and/or space (Brodie 1993, Saporito et al. 2007). The removal of non-significant terms ($p > 0.05$) did not influence the significance of other terms in the model.

In year two, we collected canopy cover data every 10m along our transects, and re-ran the analyses above and the added fixed effects of canopy cover and all 2- and 3-way interactions. We were interested in whether this form of environmental variation influenced attack risk, and whether this risk depended on color (Rudh et al. 2011). Only frog model sets within 2m of our canopy cover measurements were included in this analysis (Figure 3). We used Proc GLIMMIX in SAS (Version 9.2) for all analyses, and degrees of freedom for fixed effects were calculated using the Kenward-Roger approximation.

Many clay model studies have chosen to specifically focus on avian attacks, as birds are visual predators that can perceive and perhaps use coloration when selecting prey (Siddiqi et al. 2004, Saporito et al. 2007). While our aforementioned analyses included all attacks (avian or not), and we feel that this approach is justified based on our limited knowledge of the community of potential predators at our study sites (Lenger et

al. 2014), to be consistent with other poison frog studies we additionally analyzed the subset of avian attacks separately. Due to the small numbers of avian attacks in our dataset (n = 12), we lacked sufficient statistical power to test for differences across sites and phenotypes, but we tested for an effect of canopy cover on avian attack frequency using a logistic regression in SPSS (Version 20).

Results

Clay model attack data: Of the 4536 models we placed, 7.9% (n= 360) were attacked. There were differences in overall attack frequency between sites (Table 1, Figure 4). The frequency of attacks in our polymorphic site (Dolphin Bay, 4.23%) was also lower than in either of the monomorphic sites (Almirante 11.8%, Aguacate 7.8%, Binomial exact test: $P < 0.001$). However, we found no effect of model color or the interaction between site and model color (Table 1). There was no main effect of year, although there was a significant interaction between site and year (Table 1). The random effects of transect replicate and position explained significant variation in attack frequency (chi-square: 91.7, df = 3, $p < 0.001$), with position explaining ~25 times as much variation as transect.

We incorporated canopy cover measures along the 2012 transects in an effort to explain this heterogeneity in attack rates across transects. In our analysis of 2012-only data, in which we included canopy cover a covariate, we found no effect of model color, location, canopy cover or any interaction on attack risk (Table 2). Pooling data across sites and transects, canopy cover was not a significant predictor of avian attack risk ($p = 0.141$)

Discussion

Despite the fact that our focal sites were chosen to maximize the likelihood of detecting selection against non-native phenotypes, we found no support for the prediction that *O. pumilio* morphs would suffer increased predation outside their native range. These results differ from previous studies in Costa Rica, where brown *O. pumilio* models were attacked more frequently than the ancestral red with blue legs morph (Saporito et al. 2007), but are similar to a recent study spanning multiple phenotypes across Costa Rica and Panama (Dreher et al. 2015). Together, these findings suggest that that foreign *O. pumilio* phenotypes are not attacked any more frequently than those found locally, and conspicuous (aposematic) phenotypes do not necessarily gain an advantage in terms of reduced risk of predation where they are local (contrary to Saporito et al. 2007, Noonan and Comeault 2009).

As an alternative to a local phenotype advantage, we tested the prediction that the model with the ancestral (Wang and Shaffer 2008) conspicuous (red with blue legs) phenotype might incur fewer attacks than the derived (and less conspicuous) blue and brown phenotypes. We based this prediction on other studies that have found innate avoidance of some prey colors (e.g., by motmots, a potential predator of *O. pumilio*, Alvarado et al. 2013). For snake prey, colors that have been shown to be innately avoided by avian predators include red, black and yellow (Smith 1975), and birds have shown a lower tendency to consume brightly colored prey (Schuler and Hesse 1985, Pegram and Rutowski 2014). On Isla Colon in the Bocas del Toro archipelago, models representing a foreign red *O. pumilio* phenotype were attacked less frequently than models of a local green morph (Hegna et al. 2012). In the present study, we find no evidence that the ancestral aposematic (red with blue legs) phenotype is better protected than the more

cryptic, derived morphs. Further study is needed to address the extent to which predatory decisions are based on familiarity (learned avoidance) or innate (naïve) avoidance.

Given the variation we observed in attack frequencies along transects within each site, we also investigated whether such heterogeneity might be attributable to variation in canopy cover. Lower ambient light under canopy cover may have made it more difficult for predators to detect our models and to distinguish between model phenotypes (Rudh et al. 2013, Rojas et al. 2014). If so, attack rates along each transect might be lower in areas of high canopy cover and attack rates may differ more across model phenotypes in areas of low canopy cover. We found no support for an effect of canopy cover on attacks regardless of whether all predators or just avian predators were considered. However, additional work is needed to determine whether variation in other environmental factors, such as ground cover, could affect predation risk, or how ongoing anthropogenic habitat modification could alter signaling environments and subsequently affect attack rates (Yeager et al. *in prep.*).

It has been proposed that the Bocas del Toro Archipelago may have lower predation rates than the mainland (Hegna et al. 2013), which may correlate with the presence of polymorphic populations. However, there is mixed support for this idea as attack rates across morphs in one polymorphic *O. pumilio* population (and a monomorphic population adjacent to it) on Bastimentos Island did not support this prediction. In that study, the attack rate was higher in the polymorphic site than in the monomorphic one (Richards-Zawacki et al. 2013). However, polymorphism in that case was limited to two putatively aposematic phenotypes (red and yellow) and no difference in attack rate was observed between models representing these two phenotypes. In the

present study, where the polymorphism includes both conspicuous and more cryptic phenotypes, the overall frequency of attack in the polymorphic region (4.23%) was among the lowest found in other similar studies on poison frogs we are aware of (Table S2). This includes a study on *O. pumilio* in Costa Rica (14.75% attacked, Saporito et al. 2007) where coloration is less variable, several in Bocas del Toro (12.4 – 12.7% attacked, Hegna et al. 2011, Paluh et al. 2013, Richards-Zawacki et al. 2013) where the species is polytypic, and one study that included sites in both Costa Rica and Panama (overall predation 6.82%, Dreher et al. 2015), among others (Table S2). Finding that attack frequencies in our polymorphic site are lower than in any poison frog population studied is consistent with the prediction that diversification in coloration may have been permitted by low predation risk and a relaxation of stabilizing natural selection. Reduced predation frequency could be due to lower predator abundance or predator learning. The multiple phenotypes of chemically defended frogs may have promoted generalized avoidance by predators of these similarly sized anurans if their phenotypes gradually diverged (Ruxton et al. 2008). Individuals in the Aguacate Peninsula, which encompasses the polymorphic region as well as monomorphic blue phenotypes, have also been shown to have higher levels of chemical defense relative to areas of the mainland where the frogs are monomorphic and red with blue legs (Daly and Myers 1967, Yeager et al. *in prep.*). The level of chemical defense may partially explain the low rate of predator attacks when comparing Almirante with Aguacate and Dolphin Bay.

Several clay model studies have now been conducted for *O. pumilio*, permitting a comparison of attack frequencies on conspicuous vs. cryptic (e.g., brown) phenotypes in different parts of this species' range. In one study that took place in mainland Costa Rica

where frogs exhibit the ancestral phenotype, cryptic models were attacked more frequently than models representing the local conspicuous form (Saporito et al. 2007), though subsequent studies have not replicated this result (Dreher et al. 2015, Table S2). However, in the present study and two other studies conducted in the Bocas del Toro region, conspicuous (red/yellow) and cryptic (brown/green/yellow) models were attacked with similar frequency (Hegna et al. 2013, Dreher et al. 2015). Additional studies are needed to clarify whether the reduced attack frequency for conspicuous *O. pumilio* phenotypes, first demonstrated by Saporito et al. (2007), is limited to a portion of the species' geographic range (e.g., mainland Costa Rica). If bright, conspicuous coloration is no longer serving an aposematic function in Bocas del Toro, coloration may have been able to diverge in response to other forces, including, as has been previously proposed, sexual selection (Summers et al. 1997, Maan & Cummings 2009, Richards-Zawacki & Cummings 2011).

Other studies suggest that predation pressure can vary temporally (e.g., Mappes et al. 2014). It is possible that predator communities have changed over time, for example due to increasing anthropogenic influence in the region (Richards-Zawacki et al. 2013, see also Summers et al. 2003). However, recent observations have confirmed that predators do indeed attack *O. pumilio* (Master 1999, Saporito et al. 2007, Hegna et al. 2011, Hegna et al. 2013, Alvarado et al. 2013), including in Bocas del Toro (Richards-Zawacki et al. 2013, Lenger et al. 2014). Though we find no evidence for it in the present study, these observations suggest that predation remains a potential mechanism by which population boundaries could be reinforced.

Relaxed selection has been shown in some cases to be a precursor to the evolution of phenotypic plasticity (e.g. *Solenopsis*, Hunt et al. 2011), behavioral (Coss 1999 and references therein) and/or phenotypic divergence (Chouteau and Angers 2012). Cummings and Crothers (2013) proposed that there may be generalized predatory avoidance of new phenotypes of *O. pumilio* via stimulus generalization, which is consistent with our findings. We additionally propose that a relaxation in predator-mediated stabilizing selection on local phenotypes could have permitted phenotypic divergence in response to other sources of selection, such as sexual selection. Behavioral assessments of sexual selection have addressed female preferences for variation in dorsal and ventral coloration (reviewed in Cummings and Crothers 2013). In some instances females prefer males with colors other than their own (Maan and Cummings 2008, Richards-Zawacki et al. 2012), which, combined with a relaxation of natural selection pressures, could facilitate phenotypic divergence.

There is mixed evidence regarding the efficacy of aposematic signals outside their native range (reviewed in Rojas et al. 2015). Insectivorous passerine bird species did not avoid a novel aposematic true bug, despite it having a similar phenotype to a local (avoided) species (Veselý et al. 2013). Both the present study and two previous studies of *O. pumilio* in the Bocas del Toro region (Hegna et al. 2013, Dreher et al. 2015) show that allopatric phenotypes may be at least equally as effective in deterring predators as local color patterns. Clarifying whether predators show innate or learned avoidance of *O. pumilio* will be important to improve our understanding of the role of natural selection in this system.

The complex interaction between biogeography, population dynamics (e.g. Gehara et al. 2013), sexual selection and natural selection in this system will make identifying the mechanism(s) that contributed to the initial diversification of coloration in this species challenging. Tazzyman and Iwasa (2010) provide a compelling argument for the role of "coupled drift" whereby female mating preferences initially arise via drift and sexual selection subsequently acts to promote phenotypic divergence. As we fail to detect stabilizing natural selection on coloration, sexual selection, perhaps coupled with genetic drift, would appear to be a better candidate than predator-mediated natural selection for explaining the diversity of coloration in Bocas del Toro *O. pumilio*.

Variation in fitness landscapes driven by natural selection have long been considered important to explaining the distribution of adaptive phenotypes, such as aposematic signals (Wright 1932, Coyne et al. 1997, Mallet and Joron 1999). While our results do not suggest an active role of natural selection in maintaining phenotypic variation in *O. pumilio*, we cannot exclude the possibility that a relaxation of predation pressure has facilitated phenotypic divergence by reducing or removing the constraint of stabilizing natural selection on coloration. A clearer understanding of the presence and/or strength of color-assortative mating, and the connectivity of phenotypically distinct populations will help shed light on other aspects of the fitness landscape that may have influenced this impressive example of phenotypic diversity.

Figure 1-1: Frog images and labels denote sites where clay models were placed. In two sites *O. pumilio* is monomorphic (red with blue legs: Al = Almirante, blue: Ag = Aguacate) and in the third the frog is polymorphic (red, blue and brown: DB = Dolphin Bay), scale bar = 15km. The inset shows the location of the study area on a map of Panama.

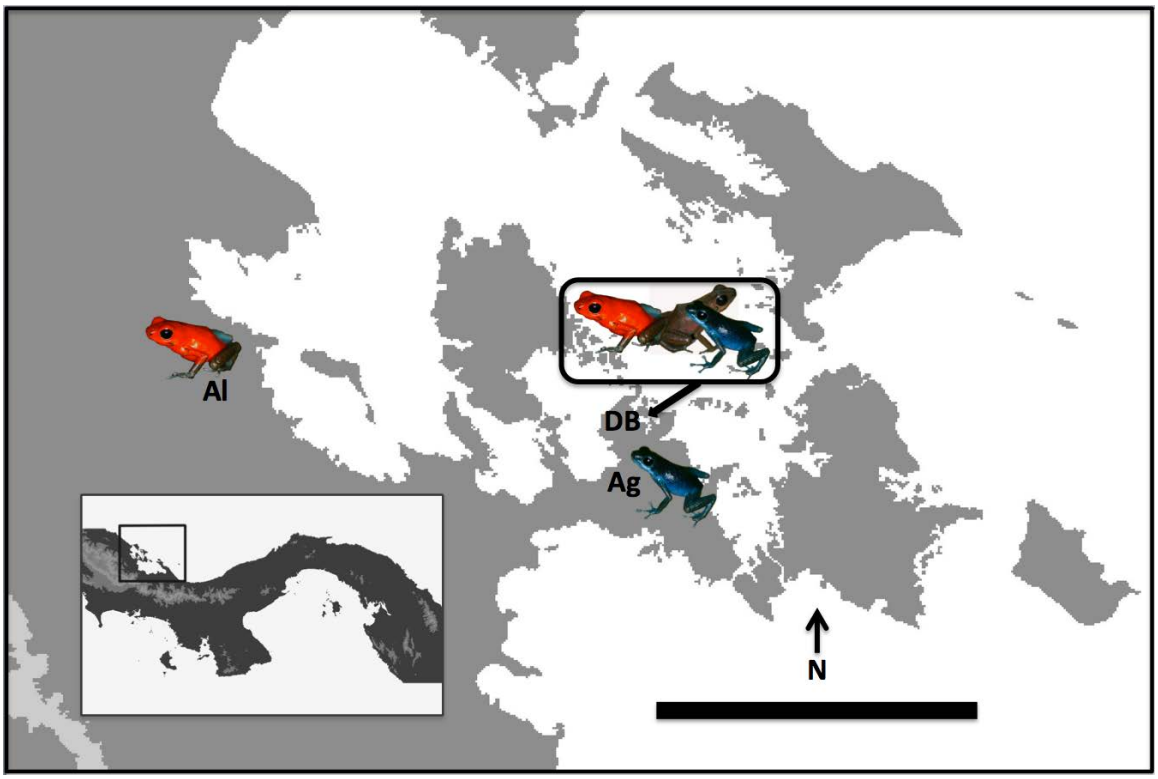


Figure 1-2: Coloration of 15 *O. pumilio* males (circles) and clay models (squares) representing each of the red with blue legs (Almirante), blue (Aguacate) and brown (Dolphin Bay) phenotypes in an avian tetrachromatic visual space. See supplemental methods for details of the visual modeling procedure.

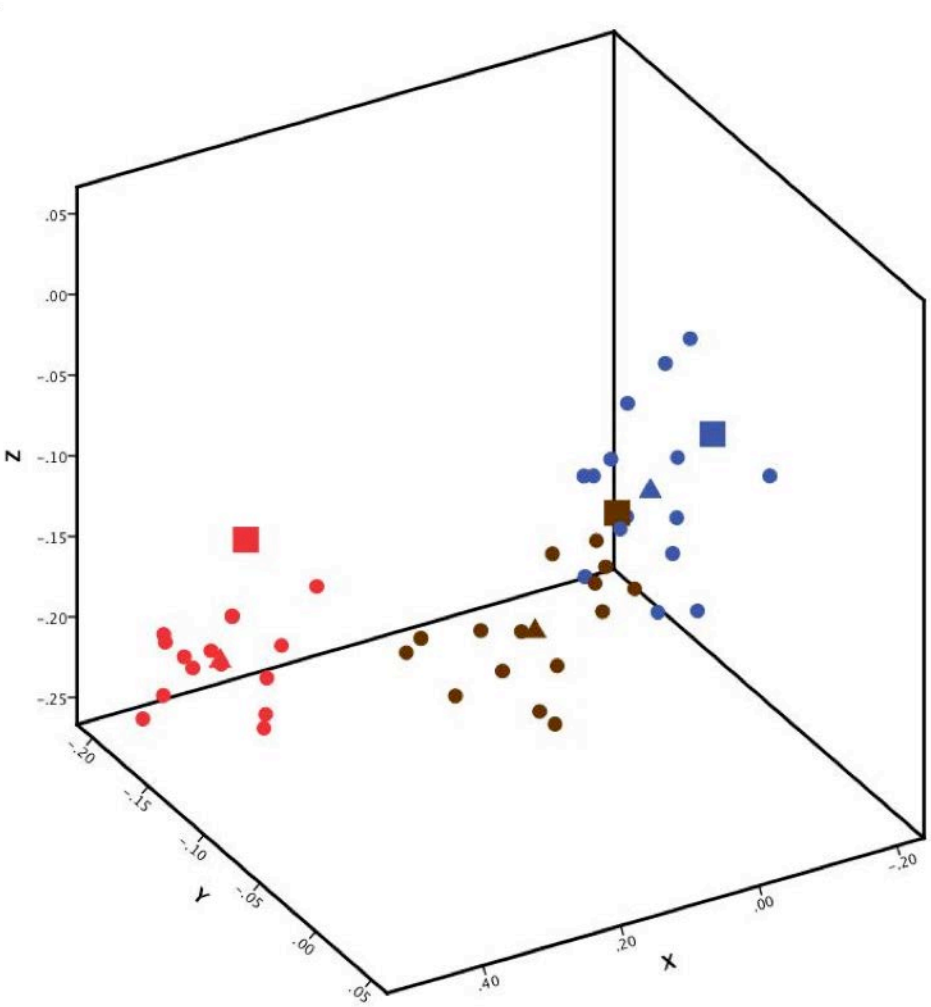


Figure 1-3: Illustration of the placement of clay models along transects. Shaded circles represent regions where canopy cover was measured (10m increments). Brackets denote model sets included in the mixed model for 2012 that included canopy cover as a fixed effect.

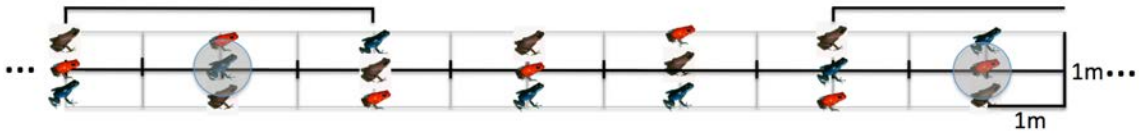


Figure 1-4: Number of models attacked across field sites. Stacked columns are colored according to attacks per model color, photos represent the local *O. pumilio* phenotype at each location.

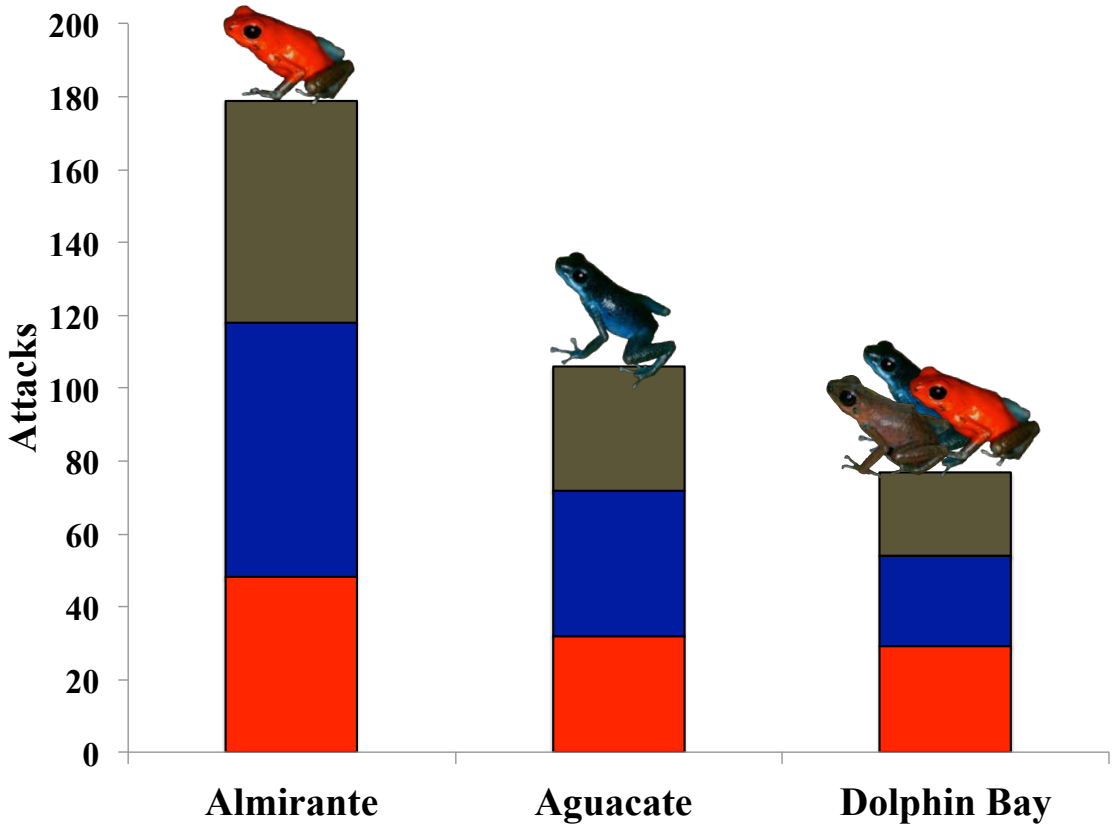


Table 1-1*:* Results of a generalized linear mixed model in which the binary response of attack (y/n) was entered as the dependent variable and model color, site, year and all two and three way interactions were entered as fixed effects. Num DF = degrees of freedom for the numerator, Den DF = df for the denominator.

Effect	Num DF	Den DF	F	P
Color	2	4518	1.04	0.3543
Site	2	1254	17.33	<0.0001
Site * Color	4	4518	0.68	0.6063
Year	1	2.801	0.28	0.6363
Year * Color	2	4518	1.33	0.2651
Site * Year	2	1254	5.11	0.0062
Site * Year * Color	4	4518	1.79	0.1283

Table 1-2: Results of a generalized linear mixed model in which the binary response of attack (yes/no) was entered as the dependent variable and model color, site, canopy cover and all two and three way interactions were entered as fixed effects. This analysis only includes models deployed in 2012. Num DF = degrees of freedom for the numerator, Den DF = df for the denominator.

Effect	Num DF	Den DF	F	P
Color	2	1350	1.24	0.2895
Site	2	115.3	0.58	0.5638
Site×Color	4	1350	1.28	0.2765
Canopy Cover	1	1097	0.13	0.7220
Canopy Cover × Site	2	1014	1.79	0.1679
Canopy Cover × Color	2	1350	1.21	0.2972
Canopy Cover × Site×Color	4	1350	1.43	0.2213

Table 1-3: Overview of poison frog predation studies including phenotypes studied, location of studies and a breakdown of attack frequency between cryptic and aposematic phenotypes.

Authors and Year	Species studied	Location	Morphs included	Cryptic and conspicuous?	Aim of study	Overall Predation frequency	Percent Cryptic attacks	Local Conspicuous	Foreign Conspicuous
Saporito et al. 2007	*Oophaga pumilio*	Costa Rica (mainland)	Red, Brown	Cryptic and Conspicuous	Cryptic vs. Conspicuous	12.40%	19%	11.00%	
Comeault and Noonan 2011	*Dendrobates tinctorius*	French Guiana	Yellow/black x2, brown	Cryptic and Conspicuous	Local vs. allopatric with frequency dependency (low/high)	2.36%	1.50%	1.65%	3.91%
Noonan and Comeault 2009	*Dendrobates tinctorius*	French Guiana	Solid yellow, striped yellow, brown	Cryptic and 2 Conspicuous	Local vs. allopatric with cryptic control	11.03%	7.14%	10.95%	15.00%
Chouteau and Angers 2011	*Ranitomeya imitator*	Peru	Yellow, Green, Brown	Cryptic and 2 Conspicuous	Local vs. allopatric with cryptic control	12.72%	11.67%	7.33%	19.17%
Hegna et al. 2011	*Oophaga pumilio*	Costa Rica (mainland)	Red	Conspicuous only (Spotted vs. Solid Color)	Patterned vs. solid color	12.38%		12.38%	
Hegna et al. 2012	*Oophaga pumilio*	Panama (insular/Bocas)	Red, brown, green	Cryptic(Brown/Green) and Conspicuous	Local vs. allopatric with cryptic control	7.47%	0.25%	3.45%	0.99%
Paluh et al. 2013	*Oophaga pumilio*	Costa Rica (mainland)	Red, Brown	Cryptic and Conspicuous	Moving/stationary (cryptic vs. conspicuous as well)	12.67%	13.33%	12.00%	
Richards-Zawacki et al. 2013	*Oophaga pumilio*	Panama (insular/Bocas)	(Red vs. Yellow) vs Red	Conspicuous only	Polymorphic vs. monomorphic	12.56%		14.36%	7.20%
Willink et al. 2014	*Oophaga granulifera*	Costa Rica (mainland)	Red, Yellow, Green	Cryptic, Intermediate, Conspicuous	Cryptic vs. Conspicuous, Local vs. Allopatric	3.05%	4.83%	1.75%	4.83%
Dreher et al. 2015	*Oophaga pumilio*	Costa Rica and Panama	Green, Blue, Yellow, Red	Cryptic (Blue/Green) and Conspicuous (Red/Yellow)	Cryptic vs. Conspicuous, Local vs. Allopatric	6.82%	7.36%	6.72%	6.14%
Rojas et al. 2015	*Adelphobates galactonotus*	Brazil	Orange, Blue, Brown	Cryptic and 2 Conspicuous	Cryptic vs. Conspicuous, Local vs. Allopatric	11.28%	2.95%	8.78%	7.87%
Current study	*Oophaga pumilio*	Panama (mainland/Bocas)	Red, Blue, Brown	Cryptic and Conspicuous	Polymorphic vs. monomorphic	7.94%			
					Almirante (red local)	11.77%	12.90%	9.52%	
					Aguacate (blue local)	7.80%	7.74%		7.94%
					Dolphin Bay (polymorphic)	4.23%	4.56%	3.57%	

Chapter 2

INSIGHTS INTO THE EXISTENCE OF POLYMORPHISM IN APOSEMATIC PREY FROM A PREDATOR LEARNING EXPERIMENT

Abstract

Prey species that use aposematic (warning) signals rely on their ability to quickly and effectively evoke avoidance behaviors in potential predators. Phenotypic polymorphism in defended prey species is predicted to impede predator education and is predicted to be rare. However, a number of examples of aposematic polymorphism exist in nature, offering an opportunity to investigate this so-called 'polymorphism paradox' to understand how polymorphism impacts predator education and learned avoidance of alternative prey phenotypes. We used live model predators (domestic chickens) to ask how polymorphism in an aposematic prey species, the poison frog *Oophaga pumilio*, affects the process and outcome of predator learning. Specifically, we tested the prediction that prey polymorphism slows predator education, and assessed how prior experience with one phenotype influenced predators' subsequent interactions with alternative prey phenotypes. Our study mimics the education process that would-be

predators presumably experience in the Bocas del Toro Archipelago of Panama where *O. pumilio* populations are either monomorphic or polymorphic in coloration. While we found some support for learned avoidance, the attack frequency and rate of learning did not differ among chickens exposed to differently colored frogs. Furthermore, chickens educated on one frog phenotype do not avoid that phenotype more than other frog phenotypes with which they have no prior experience. To the extent that this model predator's behavior is similar to natural predators, our results suggest that predation is unlikely to exert stabilizing selection on coloration in *Oophaga pumilio*, leaving this phenotype free to diverge in response to other evolutionary forces.

Introduction

Coloration is oftentimes an ecologically relevant signal in prey species, acting to mitigate predation risk. Predators have been shown to use prey phenotypes as cues in their predation decisions (Mappes et al. 2005) and predator learning can affect selection on prey phenotypes. For example, some predators have been shown to develop a search image for cryptic prey, thereby increasing foraging efficiency (Pietrewicz and Kamil 1979, Dukas and Kamil 2001). Alternatively, aposematic prey species may manipulate the foraging behaviors of predators in order to avoid being attacked by using a combination of conspicuous coloration and a defense that renders them unpalatable (Wallace 1889, Mappes et al. 2005). Predators may learn to associate unprofitable experiences with a prey's conspicuous aposematic phenotype to form a learned avoidance response (Ihalainen et al. 2008).

Poulton (1890) first proposed that warning displays would be involved in the retention of memories related to predators' unprofitable experiences with prey species.

Key tenets in aposematism theory suggest that predators can 1) readily identify a prey species as defended, 2) learn to avoid it, and 3) retain that knowledge for a period of time (Ruxton et al. 2004). Though wariness to novel phenotypes (e.g., via neophobia or dietary conservatism) can be important factors in predatory decisions (reviewed in Mappes et al. 2005), here we focus on how previous experience with defended prey (e.g. learning) shapes attack decisions based on differences in prey phenotype.

Deviations from the local, aposematic phenotype within a defended prey population are expected to be minimal and infrequent, as stabilizing natural selection likely acts on the aposematic signal to ensure recognition by predators (Joron and Mallet 1998, Ruxton et al. 2004, Rowland et al. 2010). However variation in aposematic prey phenotypes has been described frequently (Mallet et al. 1998, Mochida 2009, Brown et al. 2011). Most intraspecific variation in aposematic coloration occurs between geographically isolated populations, and often in the context of mimicry (Wake et al. 1986, Joron and Mallet 1998, Symula et al. 2001, Harper and Pfennig 2007). Perhaps the rarest form of intraspecific aposematic variation is within-population polymorphism, where multiple aposematic phenotypes occur in sympatry. Strong purifying selection is predicted to limit diversity in prey phenotypes (Joron and Mallet 1998), as polymorphism in prey may dilute the strength of predatory avoidance by slowing education, or 'confusing' predators (Servedio 2000). This may result in higher attack frequencies in polymorphic populations than for monomorphic aposematic prey, though this prediction remains largely remains untested (Ham et al. 2006).

A clearer understanding how prey phenotype affects the process of predator education will help to inform how natural selection pressures act to shape prey

phenotypes. Predation attempts on unpalatable prey presumably bear a fitness cost for the would-be predator in terms of search effort wasted, the predator revealing itself to other prey, and the potential need for recovery time after sampling the unprofitable prey (Ruxton et al. 2004). The economics of aposematism in predator-prey interactions have received considerable interest in recent years, with mathematical models providing valuable insights into how varying levels of defense and conspicuousness impact predator decisions (Speed and Ruxton 2007, Speed et al. 2010). However, empirically measuring the responses of live predators (as opposed to the use of mathematical models) allows for direct assessment of which prey phenotypes influence attack decisions while also taking into account individual variation in predator behavior (Mappes et al. 2005), the sum of which can influence prey populations.

The poison frog *Oophaga pumilio* is an aposematic (Saporito et al. 2007) species that displays high levels of phenotypic divergence across the Bocas del Toro region of Caribbean Panama (Summers et al. 2003, Wang and Shaffer 2008). The question of how this phenotypic divergence came about has received considerable attention in recent years, with some support being found for sexual selection via female mate choice as a potential driver of diversification (Summers et al. 1999, Maan and Cummings 2008, Maan and Cummings 2009, Richards-Zawacki and Cummings 2010, Tazzyman and Iwasa 2010). The contribution of natural selection in promoting or maintaining diverse phenotypes has received notably less attention (Cummings and Crothers 2013), with the bulk of natural selection studies focusing on the attack decisions of predators towards differently colored model frogs in field predation studies (Saporito et al. 2007, Hegna et al. 2011, Hegna et al. 2013, Paluh et al. 2014). Natural selection due to predation has

been shown to be important to maintaining geographic boundaries in other polytypic poison frogs (Chouteau and Angers 2011, Comeault and Noonan 2011), though in Western Panama predators do not appear to attack allopatric *O. pumilio* phenotypes more frequently than native ones (Hegna et al. 2013, Dreher et al. 2015), and the tendency of local predators to avoid attacking multiple poison frog phenotypes (generalized avoidance) can not be ruled out (Yeager et al *in prep.*).

Studies featuring model prey and wild predators provide essential insights into natural selection pressures, though they lack the ability to explicitly address the underlying mechanisms that shape the learning and decision process that predators undergo. Field observations have confirmed that avian predators attack poison frogs (Master 1998), including *O. pumilio* (Alvarado et al. 2013). Here we take advantage of naturally occurring phenotypic variation between contiguous populations of *O. pumilio*, which includes monomorphic red and blue populations separated by a polymorphic region where frog coloration spans a continuum from red to brown to blue, to elucidate the role that variation in prey phenotype may play in the education of naïve predators. We used the chicken (*Gallus gallus domestius*) as visually-oriented model avian predators, following Darst and Cummings (2006), to infer how predator learning could contribute to predation decisions. As their color vision is relatively well understood (Osorio et al. 1999), multiple studies have used chicken as a model predator for assessing predation risk on poison frogs (Darst and Cummings 2006, Amézquita et al. 2013, Stuckert et al. 2014). One study demonstrated greater hesitancy towards novel phenotypes of poison frogs relative to similarly-sized palatable brown frogs, suggesting generalization of a learned avoidance to a shared mimetic aposematic phenotype

(Stuckert et al. 2014). However, it remains unclear how predators are influenced by variation in prey phenotype where defended prey range in phenotype from cryptic to conspicuous. We specifically compared the rate of education for chickens allowed to interact with red, blue or multiple frog phenotypes (red/blue/brown) and their subsequent attack behaviors towards all three frog phenotypes after education. Understanding how prey phenotype influences both predator education and attack decisions will provide valuable insights into how wild predators can affect the geographic organization of divergent prey phenotypes.

Methods

Chickens: Domestic broiler chickens were purchased three days after hatching from an agricultural supply store in Changuinola, Panama. They were fed cracked corn feed twice daily and allowed continuous access to clean water. Chickens were randomly assigned to one of three identical outdoor screened pens, each measuring 1.5 x 3 x 3m (LWH) and containing sawdust as bedding. Individual chickens were identified by unique leg bands.

Behavior arena: The arena used for both education and assessment trials (details below) measured 1 x 1 x 1m with the walls and bottom constructed of non-reflective black fabric. A sparse covering of leaf litter was added to the floor. During all trials, the arena was situated in lowland tropical forest under partial canopy cover to replicate the ambient irradiance of habitats where the frogs would be viewed by potential predators in the wild. Chickens were then introduced into the arena via a door in one side. Both education and assessment trials were scored live, and also recorded with an overhead camcorder outfitted with a wide-angle lens. Video recordings were used to validate the live scores, and in cases of discrepancies, video-validated scores were used for analysis.

Education trials: Chickens were randomly assigned to one of three treatment groups representing the frog phenotype(s) they would be exposed to during education trials: red, blue, or polymorphic (red, blue and brown frogs, alternating in sequential trials) to assess the rate of predator education between prey phenotypes. Education trials were initiated once chickens were observed to exhibit foraging behaviors for insects in their pens (~ 4d of age). Just prior to the start of each trial, an adult male *O. pumilio* was tethered to a small stick at the arena's center using dark-colored floss and covered by a clear 10cm diameter acrylic dome. The trial began when a chicken was placed into the behavior arena through the door in the arena's side. When the chicken first pecked at the dome, or after one minute had elapsed (whichever occurred first), the dome was lifted vertically off the frog and out of the arena using a clear monofilament line. For two minutes after the dome was removed the chicken was free to interact with the unprotected frog and the number of pecks at the frog by the chicken was recorded. Chickens in the red and blue treatments each participated in 16 education trials, each time with a different male frog of the same phenotype. Chickens in the 'polymorphic' treatment each participated in 48 education trials, 16 with each of blue, red and brown *O. pumilio* males, and again a different male frog was used for each trial. Polymorphic trials provided equal exposure to each frog phenotype, and the order of presentation of frog phenotypes in the polymorphic treatment group was randomized. Chickens were returned to their pens for a minimum of three hours between education trials. The maximum number of education trials that a chicken participated in on a single day was three, although on most days only two trials were performed. Due to time constraints, some chickens in the red treatment group were educated three times daily for four days (trials 1-12), and two times daily for two days

after that (trials 13-16). Education trials for the polymorphic treatment were conducted twice daily for the first 18 days (trials 1-38), and increased to three times daily for the final five days of education (trials 39-48).

Frogs: All frogs used in this study were collected from the same polymorphic population (Dolphin Bay Preserve: 9°13'17.77"N, 82°13'5.60"W) where *O. pumilio* dorsal phenotypes span a continuum from blue to brown to red with ventral coloration varying from pure red to mottled red/blue to pure blue. Phenotypic extremes in Dolphin Bay resemble adjacent monomorphic populations in the Aguacate Peninsula (blue dorsal/ventral) and Almirante (red dorsal; red, blue or red/blue mottled ventral) areas of Bocas del Toro province. An individual frog was used for up to 16 trials per day (< 2hrs per day) and although frogs were used more than once, they were replaced with new wild-caught individuals every 14 days.

Statistical analysis for education trials: For education trials, we were interested in knowing whether the number of pecks directed at a frog by a model predator was attributable to effects of frog color (treatment, stimulus frog color) or number of exposures (trial number). A significant interaction effect of treatment * trial number (for all three treatments) or stimulus frog color * trial number (for the polymorphic treatment only) would indicate differences in learning over time between stimulus frog phenotypes. Including trial per day in our analysis allows us to ask whether multiple exposures per day results in differences in the likelihood of pecking. A significant interaction effect of trial number * trial per day would indicate pecking behaviors are affected by repeated exposure to focal frogs both within a day and over time.

Age: Husbandry space and experimental design limitations led to chickens beginning training at three different ages (4, 14 and 23 days old), which also led to an uneven distribution of ages among treatment groups (numbers beginning at 4/14/23 days: blue 17/15/0, red 7/14/10, polymorphic 30/0/0). Before pooling subjects of all ages, we tested for an effect of chicken age on the number of pecks in the red stimulus group, the only treatment in which chickens of all three ages were included. We assessed the effect of age in a model (described in detail below) containing the fixed effects of i) age, ii) trial number (1–16) and iii) trial per day (1–3), iv) the quadratic term trial number * trial number, v) the trial number * trial per day interaction, and vi) the trial number * trial number * trial per day interaction; we included random effects as described below. Ages did not differ significantly in this full model ($F_{2,480}$=2.72, p=0.067), and sequential removal of non-significant effects never resulted in a significant ($p<0.05$) effect of age.

First 16 education trials: To assess how our treatments (interaction with red, blue, or alternating red, blue and brown 'polymorphic' frogs) influenced chicken behavior, we used a generalized linear mixed model in which we entered the number of pecks to uncovered frogs as the dependent variable; because mean and variance were unequal, we specified a negative binomial error distribution. Our model included the fixed main effects of i) treatment, ii) trial number (1–16; trial numbers > 16 for the polymorphic group were excluded in this analysis) and iii) trial per day (1–3). We included the R-side random effect of subject, specifying an autoregressive (first order) covariance structure and the G-side random effect of batch (age class). We began by constructing a model that included the quadratic effect of trial number * trial number as well as all interactions between fixed effects, but this model did not converge. We sequentially removed effects,

beginning with complex interactions and effects for which we could make no *a priori* predictions. The first model that converged contained the three main effects, i) the quadratic term trial number * trial number, the two-way interactions between ii) trial number * trial per day and iii) treatment * trial number, and the three-way interactions iv) trial number * trial number * treatment and v) trial number * trial per day * treatment. In this model, there was a significant main effect of trial per day ($F_{1,1469}$=14.5, $p<0.001$) and a significant interaction between trial number and trial per day ($F_{1,1469}$=13.2, $p<0.001$) where pecks decreased for second trial/day over time (Figure 2); because we were primarily interested in trial number and the interaction between trial number and treatment, we opted to split the data by trial per day for further analysis.

Once we split the data, our initial model included the fixed effects of i) treatment, ii) trial number (1–16), iii) the treatment * trial number interaction, iv) the quadratic term trial number * trial number and v) the interaction between this quadratic term and treatment. When the quadratic term was non-significant, we removed it and the interaction that included it. Because we made *a priori* predictions about the two main effects (treatment and trial number) and their interaction, we present these results as our final model; further removal of non-significant ($p<0.05$) terms did not influence the significance of the terms of interest.

Polymorphic trials: To assess how the color of the stimulus frog influenced predator interest in the polymorphic stimulus treatment group, we again used a generalized linear mixed model in which we entered the number of pecks to uncovered frogs as the dependent variable; because mean and variance were again not equal, we specified a negative binomial error distribution. Our model included the fixed main effects of i)

stimulus frog color, ii) trial number (1–48) and iii) trial per day (1–3). We included the R-side random effect of subject, specifying an autoregressive (first order) covariance structure and the G-side random effect of batch. We began by constructing a model that included the quadratic effect of trial number * trial number as well as all interactions between fixed effects, but this model did not converge. We sequentially removed effects, beginning with complex interactions and effects for which we could make no *a priori* predictions. The first model that converged contained the three fixed main effects, iv) the quadratic term trial number * trial number and the interaction terms v) trial number * trial per day and vi) stimulus frog color * trial number. In this case, the effects of trial per day ($F_{2,1428}$=2.7, p=0.070) and the interaction between trial number and trial per day were marginal ($F_{2,1428}$=2.6, p=0.072). Because we were primarily interested in trial number and the interaction between trial number and stimulus frog color, and because inspection of least square means suggested that this effect largely arose from an unbalanced design (3rd trials per day appeared only in days 19-23 encompassing trials 39-48), we again split the data set by trial per day. Once we split the data, our initial models included the fixed effects of i) stimulus frog color, ii) trial number (1–48), iii) the stimulus frog color * trial interaction and iv) the quadratic term trial number * trial number. Degrees-of-freedom for fixed effects were calculated using the between-within method (Schluchter and Elashoff 1990).

Assessment trials: We used assessment trials to determine the behavioral consequences of previous experience (education trials) on chickens' propensity to attack familiar (red, blue or 'polymorphic') and unfamiliar frog phenotypes. The day after chickens completed education trials (16 trials for red and blue treatment groups, 48 for polymorphic) they

were presented with all three frog phenotypes (one adult male of each of blue, red and brown, covered by 10cm clear acrylic domes) in two assessment trials. Both assessment trials for a given chicken were conducted on the same day, a minimum of 3 hours apart. To begin each trial, the stimulus frogs were placed in the centers of three of four equally sized 0.25m^2 quadrants of the behavior arena. The chicken was then placed into the arena through the door in the side of the arena, which was located in the fourth, empty quadrant. We recorded the number of times the chicken i) approached or ii) pecked at a dome covered frog, and iii) the duration of time the chicken spent in the same quadrant (association time) with each frog for two minutes. As with education trials, behaviors exhibited by the chickens during assessment trials were scored in real-time, but the trials were also video-recorded from an overhead position. Videos were used to validate the live scores and in cases of discrepancies, video-validated scores were used for analysis. *Statistical analysis for assessment trials:* We used two generalized linear mixed models with the number of pecks, and number approaches, respectively, as dependent variables. Association time with each frog phenotype was assessed using a generalized linear model. For each model we included the fixed main effects of i) treatment (education treatment group: red, blue or 'polymorphic'), ii) stimulus frog color and iii) the quadratic effect of treatment * stimulus frog color where individual (chicken) was entered as a random effect and we specified a variance components covariance structure.

A significant main effect of treatment would indicate that the phenotype of frog that chickens were educated on influenced their pecking behaviors, number of approaches or time spent in proximity to frogs. A main effect of stimulus frog color would indicate that chickens behaved (pecked, approached, or associated more or less often) with some

frog phenotypes more than others during the assessments. Similarly a treatment * stimulus frog color interaction would indicate that chicken behavior toward different frog phenotypes is affected by their prior experience (during education trials).

Results:

Education trials

First 16 trials: In the first trial of the day, neither the quadratic term of trial number * trial number ($F_{1,712}$ =2.65, p=0.104) nor its interaction with treatment ($F_{2,712}$ =0.69, p=0.502) were significant predictors of peck number, and so we removed them from the model. In the resulting simpler model, peck number did not differ among treatments ($F_{2,715}$ =0.65, p =0.520), it was not associated with trial number ($F_{1,715}$ =0.09, p=0.766), and there was no interaction between these effects ($F_{2,715}$ =0.16, p=0.852, Figure 2). In the second trial of the day, neither the quadratic term of trial number * trial number ($F_{1,712}$ =0.04, p=0.838) nor its interaction with treatment ($F_{2,712}$ =2.04, p=0.130) were significant predictors of peck number, and so we removed them from the model. In the resulting simpler model, peck number did not differ among treatments ($F_{2,715}$ =0.24, p=0.788), and the interaction between treatment and trial number was non-significant ($F_{2,715}$ =0.20, p=0.821). Pecks on the second trial of the day, however, were negatively associated with trial number ($F_{1,715}$ =24.66, p<0.0001; intercept±SE = -0.95±0.51, β±SE = -0.20±0.07) indicating support for learned avoidance via reinforcement over time. Only a subset of the red treatment group experienced three trials per day during the first 16 trials. In these trials, there was no relationship between pecks and trial number ($F_{1,37}$ =0.80, p =0.377).

Polymorphic trials: In the first trial of the day, frog stimulus color was not a significant predictor of pecks to uncovered frogs ($F_{2,652}$ =0.59, p=0.554). There was a marginally

significant effect of trial number ($F_{2,652}$ =3.32, p=0.069; intercept±SE = -1.97±1.05, β±SE = -0.11±0.07) and a marginally significant interaction between frog stimulus color and trial number ($F_{2,652}$ =2.74, p=0.065), but no quadratic effect of trial number * trial number ($F_{2,652}$ =2.03, p=0.155) on the number of pecks to uncovered frogs (Figure 2). For the second trial of the day, pecks were significantly predicted by trial number ($F_{1,652}$ =13.9, p=0.0002; intercept±SE = -0.91±0.58, β±SE = -0.20±0.06) and the quadratic effect of this term ($F_{1,652}$ =7.2, p=0.0077; β±SE= 0.003±0.001), but not by stimulus frog color ($F_{2,652}$ =1.2, p=0.314) or the interaction between trial number and stimulus frog color ($F_{2,652}$ =0.54, p=0.583). In the third trial of the day, no model containing stimulus frog color converged. The most complicated model that did converge contained the non-significant effects of trial number ($F_{1,116}$=0.3, p=0.600) and the quadratic term trial number *trial number ($F_{1,116}$=0.3; p=0.539). Removing the quadratic term had little effect on the estimate for trial number ($F_{1,117}$=0.2; p=0.637).

Assessment trials

The overall number of chickens that pecked at dome-covered frogs during assessment trials was low (29%, Figure 3). There was no effect of education treatment ($F_{2,87}$=0.35, p=0.7022), stimulus frog color ($F_{2,174}$=0.22, p=0.8049) or their interaction ($F_{4,174}$=0.39, p=0.8178) on the number of pecks (Figure 3). This suggests prior experience with different frog phenotypes has no effect on the likelihood that a chicken will attack different frog phenotypes. This result was robust when we split assessment data by trial number (1st or 2nd), and when we considered chicken pecks as binary (y/n). The number of times chickens approached frogs also was unaffected by treatment ($F_{2,87}$=2.20, p=0.1169), stimulus frog color ($F_{2,174}$=0.54, p=0.5824) or their interaction

($F_{4,174}$=1.90, p=0.1127, Figure 3). Finally, the time spent in proximity with frogs was also not influenced by prior experience (treatment) ($F_{2,261}$=1.58, p=0.2073), stimulus frog color ($F_{2,261}$= 0.59, p=0.5541) or their interaction ($F_{4,261}$=1.95, p=0.1024).

Discussion

We investigated to what extent the phenotype of unpalatable prey species can influence learning in naïve predators, and tested whether the accumulation of prior unprofitable experience with one prey phenotype will influence the frequency of attacks towards alternative prey phenotypes. If attack frequencies are lowest for phenotypes with which predators have prior experience (relative to novel phenotypes), predator education could be a mechanism by which natural selection can produce ecological isolation between phenotypically distinct prey populations. To test this we used model predators and educated them on frogs representing adjacent *O. pumilio* populations that are either monomorphic (red or blue), or polymorphic (red, blue and brown) in coloration. By using live frogs from a single locality containing all three of these phenotypes we were able to keep all but anti-predatory phenotype the same between treatments.

We asked whether frog coloration influences the frequency of attacks during the education process by first comparing pecks toward frogs (first 16 trials) during the education process, which mimics the experience predators would have in either one of two monomorphic populations (red or blue) on mainland Bocas del Toro, Panama, or the polymorphic population (red/blue/brown) between them. We found no evidence that frog phenotype affects the rate of education between chicken treatment groups. However, we did find evidence for learned avoidance (a decrease in attacks over the course of the education trials), which was strongest for the second presentation within a training day.

Predators may decrease pecks on the second presentation due to the reinforcement of unprofitable experiences (Ruxton et al. 2004). Randomly presenting multiple phenotypes did not appear to slow the rate of education or affect the overall frequency of attacks to uncovered frogs during training (as compared to chickens presented with all red or all blue stimulus frogs). The polymorphic trials mimicked predator experiences in polymorphic frog populations, or on the edge of two phenotypically distinct populations and specifically tested the 'polymorphism paradox' by determining if multiple prey phenotypes results in higher attack frequency.

In our assessment trials we asked how prior unprofitable experiences with one prey phenotype would affect predator behavior towards novel prey phenotypes. Chickens have been shown to discriminate between novel objects and those with which they have experience, often showing hesitation towards approaching novel objects even in familiar environments (Dawkins 2002). The frequency of attacks was not lower for known-unprofitable phenotypes as compared to novel ones in this study; all *O. pumilio* phenotypes were pecked with equal (low) frequency, as would be predicted by generalized avoidance. Chickens in the three training treatment groups also showed no difference in the number of approaches towards frogs or the time they spent in association with one, two common metrics for estimating interest in an experimental subject. These results also support the findings in a field experiment where the responses of wild predators to model frogs were assessed, and no difference in the frequency of attacks was found among these same phenotypes (red, brown and blue; Yeager et al. *in prep.*). While surprising, these findings contribute important insight into the role (or lack thereof) of natural selection in prey phenotype evolution, particularly where prey

phenotype is variable. Specifically, predators may more readily generalize avoidance of unprofitable prey than previously thought, which may allow for greater divergence in prey phenotype.

Search images are thought to play a large role in prey detection and subsequent predatory decisions. Studies have shown predator attention is limited and search images can streamline the foraging process and increase efficiency (reviewed in Punzalan et al. 2005). Though typically thought of as a means of searching for cryptic prey, the same benefit in terms of foraging efficiency could be applied to prey rejection, where a search image could expedite rejection. Aposematic signaling may also be multimodal in *O. pumilio*, where color pattern may be combined with an olfactory component (Fritz et al. 1981, Yeager 2013). A growing body of evidence suggests that predator avoidance decisions may place less emphasis on coloration and more emphasis on other cues in various species of poison frogs as several studies have failed to show support for the common prediction of greater predator avoidance of local phenotypes over allopatric ones (Hegna et al. 2013, Dreher et al. 2015, Rojas et al. 2015, Yeager et al. *in prep.*). Predators may also decouple the color component of the aposematic signal and use size or shape as an early avoidance cue. Throughout our education trials, frequent reinforcement was needed to promote avoidance and attacks never completely ceased. This result could be interpreted as the strength of chemical defense of the frogs being enough to encourage avoidance, though not strong enough to completely mitigate attack risk.

Understanding how prey manipulate predator behaviors and decisions by means of anti-predatory signals affords us further insights into how selection can act to promote

and maintain phenotypic divergence in prey species. We previously hypothesized that a relaxation in natural selection could permit phenotypic divergence in prey species (Yeager et al. *in prep.*). As we find no evidence for phenotype-specific bias in predator education rates or subsequent attack biases, this study's results are consistent with the idea that the absence or relaxation of natural selection can influence the evolution of prey color patterns. Similarly, wariness towards novel prey has been proposed as a method by which rare phenotypes may increase in frequency (reviewed in Mappes et al. 2005). Aversion to one prey phenotype may be generalized towards alternative prey phenotypes (Ruxton et al. 2004). Generalized avoidance behaviors can subsequently be amplified where prey are abundant/gregarious (Gagliardo and Guilford 1993), such as polymorphic populations where multiple defended prey phenotypes are found.

Although natural selection may function as a mechanism by which population boundaries are maintained and can function as a source of pre-zygotic reproductive isolation, our findings do not support this prediction for *O. pumilio*. If predators are not exerting stabilizing natural selection, this may allow prey population boundaries to be less rigid than previously thought. This supports previous studies in wild populations of poison frogs in the Bocas del Toro archipelago where no difference in attack rates were found between divergent frog phenotypes (Richards-Zawacki et al. 2013; Hegna et al. 2013; Dreher et al. 2015). These studies, and our present findings cast doubt onto the likelihood that natural selection is an important contemporary factor in the maintenance boundaries between phenotypically distinct *O. pumilio* populations. By comparing the role of natural selection in shaping phenotypic divergence between populations, we are better able place into context previous mate choice experiments (Maan and Cummings

2008, Richards-Zawacki and Cummings 2011, Crothers and Cummings 2015), which suggest that sexual selection may be a driver of phenotypic diversity in this species. Given the lack of evidence for predator-driven natural selection and the unlikeliness of drift to explain this divergence (Brown et al. 2010), it appears that sexual selection is a more likely mechanism by which *O. pumilio* population boundaries are maintained in the Bocas del Toro region of Panama.

Figure 2-1: Frog populations represented by the experiment (Almirante, Al; Aguacate, Ag; Dolphin Bay, DB). All frogs used in education trials came from the polymorphic population DB. Scale bar = 15 km.

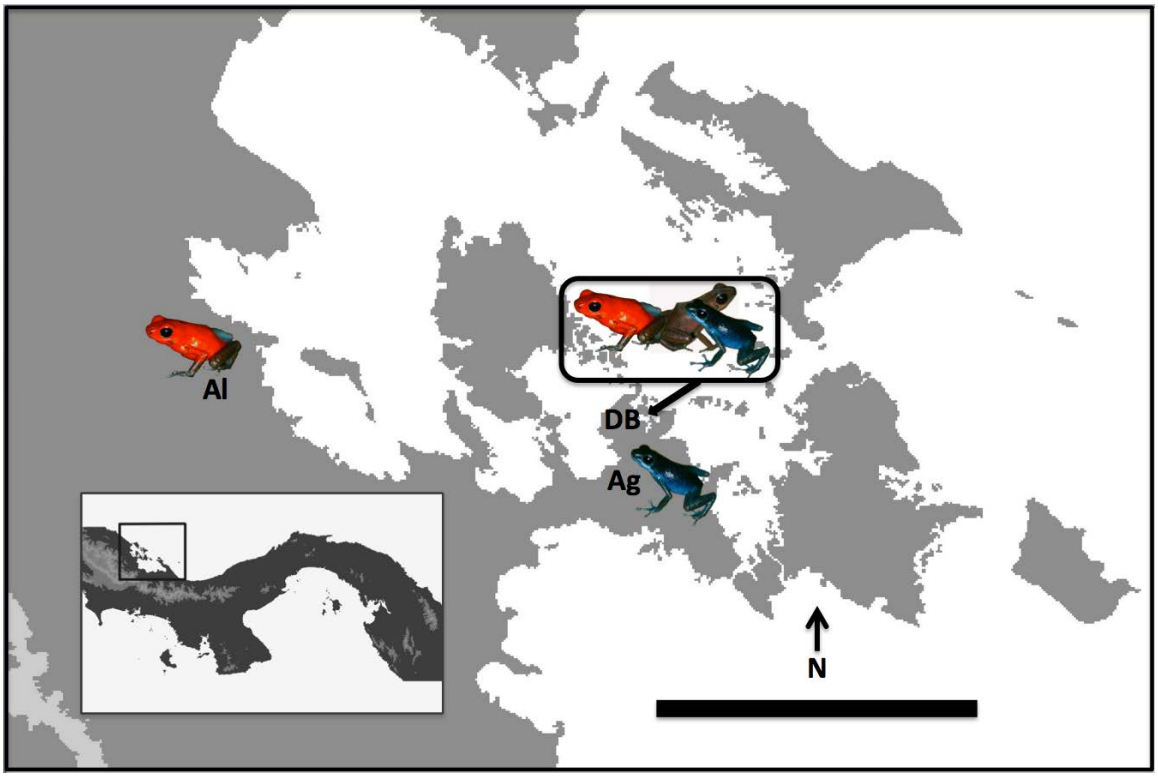


Figure 2-2: Frequency of pecks to uncovered frogs during the first 16 education trials for chickens educated on red frogs (A), blue frogs (B), and multiple phenotypes of frogs (polymorphic, C) and during all 48 education trials (D) for chickens from the polymorphic treatment. Line color indicates trial per day: First = black, second = dark grey, third (red treatment only) = light grey.

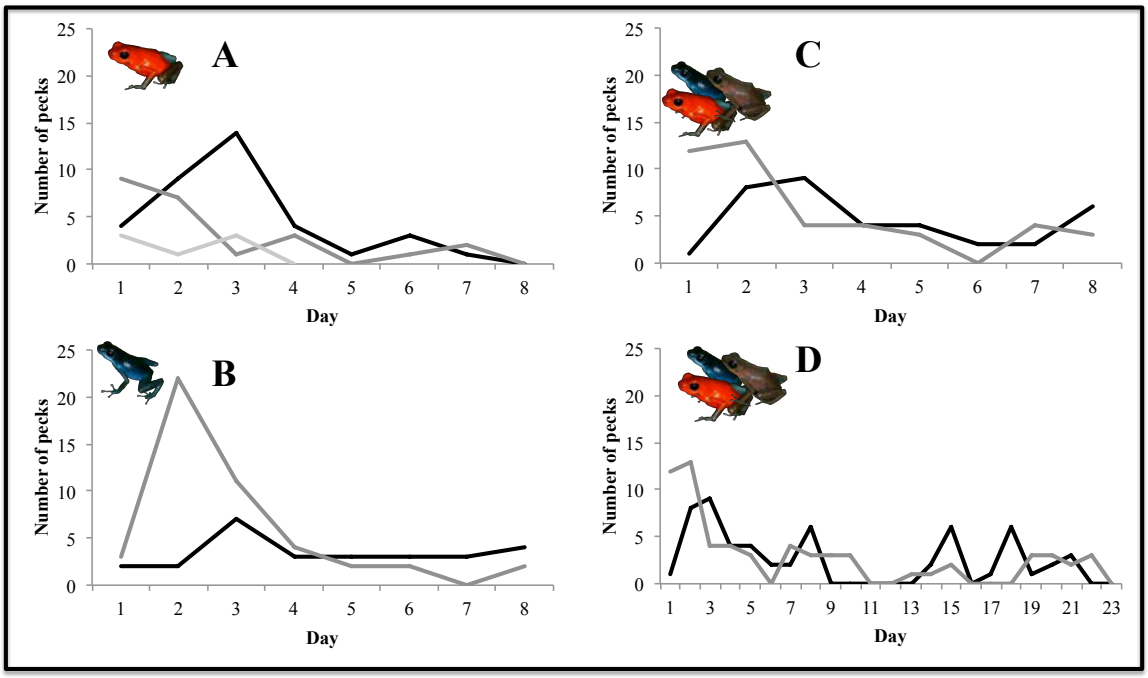
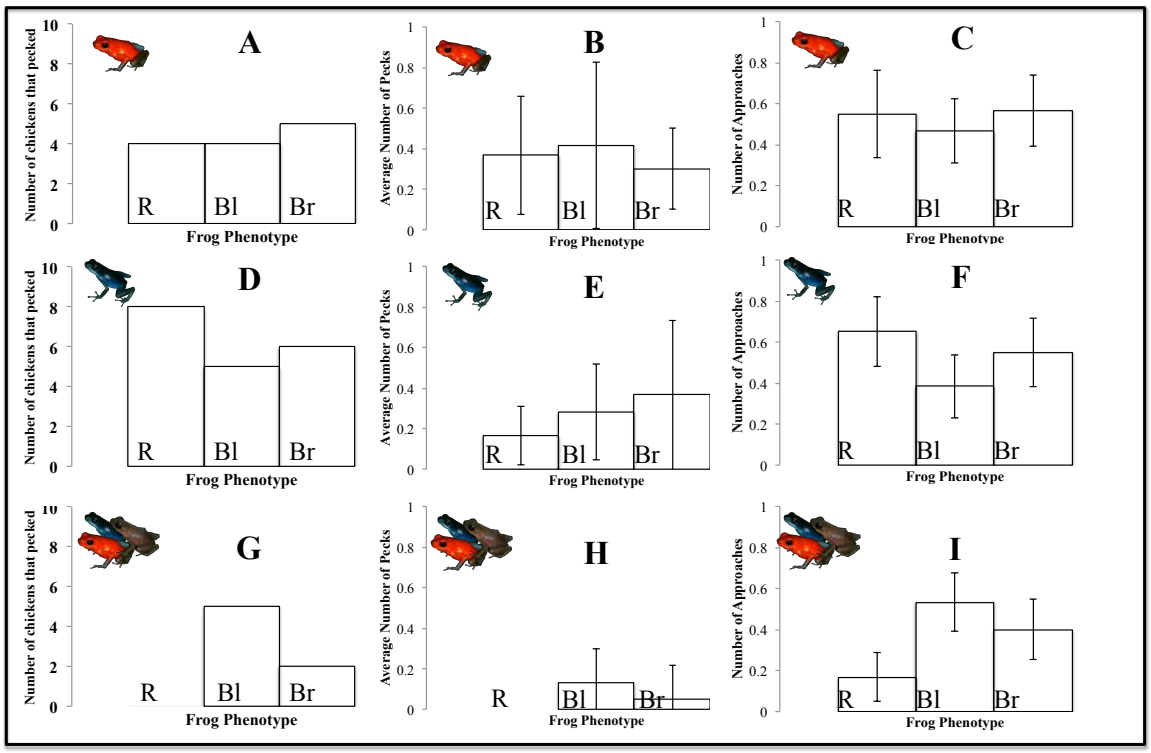


Figure 2-3: Results of chicken assessment trials. The phenotype of frog the chickens were educated on is denoted by icons, and the phenotype of the frog whose dome was pecked in the arena is denoted by letters (R= red, Bl= blue, Br= brown). A, D, and G show the number of chickens that pecked at a covered frog at least once. B, E, and H show the average number of pecks per chicken. C, F, and I show the average number of approaches per chickens to each frog phenotype. Error bars are standard error of the mean.



Chapter 3

MULTI-TROPHIC IMPACTS OF HABITAT QUALITY ON AN APOSEMATIC SIGNAL

Abstract

An active dynamic exists between animal phenotypes and local habitats in which they are found. Over time, selection can bring about local adaptation - changes in phenotype that increase fitness under local conditions. However, variation among local habitats can mediate animal phenotypic changes in other ways as well. The local habitat not only serves as the setting in which animal phenotypes are perceived, but can also serve as the source of environmental resources that shape the expression of animal traits. Thus altering habitats, such as occurs due to anthropogenic disturbance, has the potential to change not only the signaling environment occupied by a species, but also the animals ability to develop and maintain a salient signal as well. The strawberry dart frog *Oophaga* (*Dendrobates*) *pumilio* displays an impressive variety color patterns across a relatively small geographic range in Panama. The often bright and conspicuous colors these frogs display has been proposed to be important not only in the context of mate choice, but also as an aposematic warning signal. The exogenous resources that

contribute to these warning signals include defensive alkaloids and fitness and color-influencing carotenoids. We hypothesized that habitat disturbance would limit the availability of these exogenous resources, leading to a reduction in the effectiveness of the aposematic signal. To test this, we used a multi-trophic level study of resource availability and color and toxicity components of the frogs' phenotype that included replicates of disturbed and adjacent undisturbed habitats. We found evidence that anthropogenic disturbance results in dietary shifts in two populations, which also correlate with shifts in carotenoid and alkaloid profiles. Visual models suggest that the effect of habitat quality on color phenotype affects how frog phenotypes would be perceived by potential predators and mates.

Introduction:

A species' fitness landscape is dictated by a combination of biotic and abiotic factors that can result in fitness peaks and valleys. Habitat differences can influence the availability of limited resources, which in turn contributes to the topography of a species fitness. Fine scale differences between local habitats can have dramatic implications for intra- and interspecific interactions, such as predator-prey dynamics (Heck Jr. and Crowder 1991). The structure of forest structure can also influence the filtration of lighting to alter traits like phenotypic signals (Endler 1993), or the availability of resources such as carotenoids that influences both conspicuous animal coloration and mating success (Kodric-Brown 1989). Some conspicuous color patterns, or readily recognizable phenotypes are coupled with an appreciable degree of defense and are thought to act as an aposematic or warning coloration to deter would-be predators (Wallace 1867). Examples of aposematic coloration are most commonly found in

invertebrates (Ruxton et al. 2004) though it has been described with increasing frequency in recent years in amphibians (Kuchta 2005, Saporito et al. 2007, Mochida et al. 2013) A relatively unexplored facet of predator prey dynamics of aposematic coloration is how ecological variation, such as habitat quality, influences the evolution and maintenance of aposematic signals. Chemical defenses in aposematic species are often acquired from dietary sources (Ruxton et al. 2004). This can occur during larval stages such as caterpillars acquiring defensive compounds such as cyanogenic glucosides, linamarin, or lotaustralin from plants (Nahrstedt and Davis 1985), maternally provisioned like in the poison frog *Oophaga pumilio* (Stynoski et al. 2014), or acquired as adults such as in many anurans (Daly 1998) where the territories occupied by individuals dictates the bioavailability of chemical defensive compounds.

Variation in habitat quality may particularly influence species that rely on aposematic coloration as diet-acquired biomolecules can influence the level of chemical defense, or alter characteristics of aposematic phenotypes (Crothers et al. *in review*), as well as impact the signaling environment in which individuals are viewed. Because aposematic signals are predicted to be under predator-mediated natural selection differences in signal may be readily perceivable by predators and negatively impact fitness, thus habitat differences may influence interactions on multiple trophic levels.

Dietary resources can also include fitness-influencing biomolecules, such as pigments that contribute to color displays. Carotenoids are one such example that are commonly sequestered from dietary sources and used to influence animal coloration (Kodric-Brown 1989, Hill et al. 2002). In the poison frog *O. pumilio*, carotenoids are likely involved in honest signaling of mate quality, and have been shown to influence

reproductive fitness (Dugas et al. 2013). In addition to influencing animal coloration, maternal provisioning of carotenoids can positively affect offspring fitness (McGraw et al. 2005) and can also play a role in fine-tuning color discrimination through their deposition in the eyes of many species (Vorobyev and Osorio 1998, Vorobyev 2003).

A series of recent experiments on guppies (*Poecilia reticulata*) provides a framework for understanding how habitat variation can affect animal phenotypes. Female guppies display a preference for carotenoid-influenced conspicuous orange spots on the males, which has arisen due to a feeding sensory bias (Rodd et al. 2002). Carotenoid availability is sex-biased; males are more limited than females (Grether et al. 1999) and suffer asymmetrical consequences of reduced carotenoid consumption (Grether et al. 2004). Male ornamentation is further constrained by the abundance of predators which can more easily locate and consume brightly colored males, which can constrain coloration resulting in a reduction in traits such as conspicuous orange patches in guppies (Kemp et al. 2009). Predator abundance also correlates with attributes of the habitat, specifically canopy cover, where predator density increases in areas where ambient light is on average higher than in low predation environments (Reznick et al. 2001). Guppy ornamentation is therefore directly influenced by both local resource abundance and predators, where predatory abundance also correlates back with the local environmental conditions.

Resources such as carotenoids and alkaloids do not necessarily vary independently. Organisms that sequester alkaloids for chemical defense are thought to bear significant oxidative stress, including stress incurred in the storage and/or modification of alkaloids which may be assisted by antioxidant pigment molecules such

as carotenoids (Ahmad 1992, McGraw 2005, Blount et al. 2009, Santos and Cannatella 2011). Crothers et al. (*in review*) found a positive correlation between tricyclic alkaloid quantity and beta-carotene in *O. pumilio*. Carotenoids may also correlate with toxicity in an honest aposematic signal, where more conspicuous coloration means more potent chemical defense, though this remains controversial in theoretical models (Blount et al. 2009, Maan and Cummings 2012).

Here we investigate the effects of anthropogenic disturbance, a common form of local habitat variation, on the development and expression of the aposematic signal of the strawberry poison frog *Oophaga* (formerly *Dendrobates*) *pumilio*. A growing body of evidence supports the hypothesis that phenotypic traits such as dorsal coloration may serve a role in both natural and sexual selection in neotropical poison frogs, and particularly in *O. pumilio* (Maan and Cummings 2008, Richards-Zawacki and Cummings 2010, Richards-Zawacki et al. 2012, Richards-Zawacki et al. 2013). Throughout most of its range *O. pumilio* phenotype is largely homogeneous with slight variations on a central theme of a red body with blue legs. However, in Western Panama *O. pumilio* displays at least 15 distinct color patterns on and around the Bocas del Toro island archipelago (Daly and Myers 1967, Wang and Shaffer 2008). Inter-population phenotypic divergence is highest in this area, although there are also examples of striking intra-population variation, where two or more color phenotypes are found sympatrically (Richards-Zawacki et al. 2013, Yeager et al. *in review*). Sexual dichromatism has also been documented, with male coloration being brighter than female coloration in one insular population (Maan and Cummings 2009, Crothers et al. 2011).

Multiple origins have been proposed for the evolution of conspicuous coloration

in poison frogs (Santos et al. 2003, Vences et al. 2003, Santos and Cannatella 2011). The initial evolution of conspicuous coloration is thought to be costly. Finding appropriate foods, transportation of defensive molecules across the gut, concentration/biotransformation of molecules, and intraspecific competition over resources are several potential costs of sequestering toxins (Ruxton et al. 2004). Many poison frogs are dietary specialists (Darst et al. 2005), and dietary specialization is thought to be linked with the evolution of conspicuous aposematic coloration in the poison frog family Dendrobatidae (Santos et al. 2003, Darst et al. 2005). Toxicity in poison frogs is acquired through the bioaccumulation and sequestration of alkaloids from prey species (reviewed in Saporito et al. 2012), and these biomolecules are stored in and expressed from granular glands in the dorsal skin (Saporito et al. 2010). Perhaps because toxicity is acquired from their diet, variation in alkaloid profiles is extensive. Distinct alkaloid signatures are found among geographically isolated populations (Daly and Myers 1967, Saporito et al. 2006, Maan and Cummings 2012), and intra-population variation between sexes has also been found (Saporito et al. 2010). Populations also vary in alkaloid profiles seasonally (dry/wet), demonstrating that chemical defenses of *O. pumilio* can change rapidly with regards to changes in available resources (Saporito et al. 2006). Maan and Cummings (2012) hypothesize that the differences in toxicity between geographically separated populations may arise from: i) alkaloid availability differences owing to geographic location, ii) homogenous alkaloid availability but differing foraging strategies between populations, or iii) differing abilities of populations to modify, synthesize or sequester the toxins.

Conspicuous aposematic signals may also be salient signals to conspecifics in the context of sexual selection (Jiggins et al. 2001, Maan and Cummings 2008, Nokelainen et al. 2011, Crothers and Cummings 2015). When costly signals diverge as a result of traits favored by mates, they are thought to be honest signals of an individual's fitness (Schluter and Price 1993). In dung beetles female-preferred male secondary sexual characteristics are positively influenced the environment by means of larval food availability (Moczek and Emlen 1999). Local environments can also have negative influence on sexual selection, recent evidence has shown that eutrophication caused by anthropogenic disturbance can have negative sweeping effects which results in lowering the strength of sexual selection and can result in a reversal of speciation (Seehausen et al. 1997, Vonlanthen et al. 2012). Local habitats may dictate the availability of important biomolecules for *O. pumilio*, which may become limited or patchy due to disturbance, thereby affecting the frogs' ability to maintain effectiveness of their aposematic signal.

Because *O. pumilio* are highly territorial (McVey et al. 1981) differences in their habitats are likely to translate into differences in several distinct biomolecules utilized for inter- and intraspecific signaling as well as chemical defense. Using a multi-trophic level approach, here we ask how variation in habitat quality influences prey availability, diet, skin pigmentation and chemical defense of four phenotypically distinct populations of *O. pumilio*. We chose to focus our investigation on carotenoid skin pigments and alkaloid skin toxins because both of these classes of biomolecules are derived from the frogs' diet. Alkaloid and carotenoid profiles together influence frogs' aposematic signals, which is thought to be under natural selection leveraged by predators.

We chose populations for which we could make *a priori* predictions about the potential relative importance of disturbance on one or more of our quantified traits (Figure 1). We hypothesized carotenoid availability to be limiting to the development of longwave color patterns (red and orange) more than intermediately conspicuous (yellow/green) or cryptic (blue) populations. In terms of aposematic signaling we predicted both sexes would be similarly affected by disturbance, though in sexual selection female preference for brighter males has been demonstrated for a wide variety of populations (Maan and Cummings 2009). In some instances male brightness has been shown to be an honest signal of male quality, correlating with male advertisement calling behavior (Crothers et al. 2011) and agonistic interactions (Crothers and Cummings 2015) in which case sex-specific predictions for male coloration may be justifiable.

We predicted dietary limitation of alkaloid-rich prey would negatively affect all populations, though particularly those with conspicuous aposematic coloration, as these would be predicted to suffer most from a mismatch between color and toxicity. Previous studies have shown high between-population variation in chemical defense among *O. pumilio* populations using mouse injections (measured in units of relative lethality) and a general (though not perfect) correlation between brightness and toxicity (Daly and Myers 1967, Maan and Cummings 2012). Changes in alkaloid profiles due to resource limitation could negatively impact the fitness of frogs and reductions in toxicity would be predicted to be more costly to bright, conspicuous populations than more cryptically colored ones. Changes in *O. pumilio* toxicity could be especially important when predation risk is not completely mitigated by aposematic coloration, or variation in predator suits exists (Mappes et al. 2005, Yeager et al. *in prep.*).

Finally, we infer how selection may act on phenotypic variation in *O. pumilio* populations produced by habitat modification. To address how phenotypes would be perceived in their respective microhabitats, we use visual modeling techniques to predict how frog coloration is perceived both by conspecifics and by potential predators. By using a bottom-up approach, we are able to assess the impact of habitat disturbance on availability of prey, diet composition, and biomolecule sequestration, and infer how these effects might trickle up to affect how frogs signals are interpreted by potential predators and potential mates.

Methods:

Site Selection: We chose our study sites to represent four phenotypically distinct populations of *O. pumilio* (Figure 2). During June and July of 2011 and 2012, we sampled frogs at each site from adjacent but distinct habitat areas, which we predicted would differ in resource availability. At the same time, we measured attributes of those study areas to quantify habitat variation. Each pair of sampling areas at a site included one with marked anthropogenic influence to the land (hereafter "disturbed", e.g., monoculture, gardens, manicured landscapes) and one where human influence was minimal (hereafter "undisturbed", e.g., mature secondary growth forest). Disturbed areas were selected only if they were in close proximity (within 140-580m) to an undisturbed area where frogs were similarly abundant. Our study sites/populations (Figure 1) included red frogs with blue legs (Almirante: disturbed 09°19'33.6" N, 82°29'42.1"W; undisturbed 09° 19'16.3"N, 82°29'49.5"W, 580m between sampling areas), solid orange frogs (Isla Solarte: disturbed 09°19'59.0"N, 82°13'10.9"W; undisturbed 09°19'54.8"N, 82° 13'07.8"W, 161m between sampling areas), yellow/green frogs with black dorsal

spots (Isla Colon: disturbed 09°24’14.9”N, 82°15’0.0”W; undisturbed 09°24’12.0”N, 82°15’04.7”W, 169m between sampling areas), and dark blue frogs (Aguacate Peninsula: disturbed 09°10’40.8”N, 82° 15’56.9”W; undisturbed 09°10’37.9”N, 82°16’00.4”W, 140m between sampling areas).

Quantifying habitat variation: Each study area (disturbed or undisturbed) was circular and 40m in diameter, centered on a spot where frogs were abundant. We measured canopy cover using a concave spherical densiometer (averaging four readings, one taken facing each cardinal direction) from each of five locations within each study area: in the center and 10 m from center in each cardinal direction. We measured ground cover by recording (1) percent composition in four categories: bare soil, shrubs, trees and leaf litter, and (2) the number of morphologically distinct plant types present in 1 m^2 quadrats placed at the center and 10 m from center in each cardinal direction within the study area. We used a principal components analysis (PCA) to reduce the habitat variables (canopy cover, percent trees, percent shrubs, percent leaf litter, and number of vegetative species) to a set of linearly uncorrelated composite variables (with Eigen values > 1). We then used nested ANOVAs for each principal component with fixed factors of site and sampling area, and including a site by sampling area interaction to quantify differences between study areas and sites.

To compare arthropod diversity and abundance between sites and study areas, we used a combination of sampling strategies. We first collected all leaf litter and detritus from three 1 m^2 quadrats distributed randomly around the center of each study area. These samples were pooled and placed in Berlese funnels. Each funnel was made from a 19 L water bottle, above which we suspended a 40 W incandescent light bulb and below

which we placed a collection cup containing 70% ethanol. Because few arthropods were recovered using this method, we also used pitfall trapping. We randomly embedded fifty 0.14 L plastic cups in the ground throughout each plot so that their rims were flush with the surrounding soil. We then covered the cups with plastic plates. We replicated this sampling design twice in each study area, yielding a total of 100 samples per study area. Pitfall trap cups were left empty to avoid any sampling bias that could result from using baits or preservatives (Underwood and Fisher 2006). Arthropods were removed from traps after 24h and immediately stored in 70% ethanol. Within each site, samples were pooled by study area (disturbed vs. undisturbed). Because samples were pooled, we used analysis of similarity (ANOSIM) to compare diversity between study areas (disturbed vs. undisturbed) among all sites.

Quantifying frog traits: In each site and study area, we hand captured frogs (10 male, 10 female) between 0900 and 1100 h, the time of peak activity for *O. pumilio* (Graves 1999), and placed them individually in 0.05 L deli cups for transport to the Smithsonian Tropical Research Institute's Bocas del Toro Research Station (hereafter, STRI). Immediately after capture, we took a downwelling absolute irradiance measurement at the exact point where each frog was first observed using an Ocean Optics Jaz portable spectrometer (Ocean Optics, Inc., Dunedin, FL) with a QP400-2-UV-VIS fiber and a CC-3-UV-S cosine corrector. A small sample of the substrate the frog was perched upon when first sighted was also collected. At STRI, we took reflectance measurements of each of two skin areas of the frogs (mid-dorsum and mid-venter) and of each substrate sample using the integrated Jaz-PX pulsing xenon light source and a QR400-7-SR-BX reflection probe, held at a 45 degree angle and at a distance of 3 mm to the specimen. We used the

average of three measurements from each skin surface and the average of two measurements of each substrate in statistical analyses. Measurement of a WS-1 white standard was used to account for lamp drift between every sample. During each transition from reflectance to irradiance measurements, the Jaz spectrometer was re-calibrated using an Ocean Optics LS-1-CAL tungsten halogen lamp. After skin reflectance measurement, we euthanized the frogs by freezing and used double-pithing as a secondary method of euthanasia to ensure death. We then removed the dorsal and ventral body skin and digestive tract from each animal. We divided the dorsal skins into left and right halves; one half was preserved in 100% methanol for alkaloid analyses and the other immediately frozen using liquid nitrogen for carotenoid analyses. We opened the stomachs and intestinal tracts on a petri dish and flushed them with 70% ethanol to collect all gut contents and preserve prey consumed by each frog. The rest of the corpse was preserved in ethanol for future studies. All samples were transported to Tulane University for further analysis.

Stomach contents: Due to maceration and digestion inside the gut prior to euthanasia, gut arthropod specimens were degraded and often disarticulated. As a result, we counted head capsules as individuals for abundance estimates and assigned taxonomic classifications based on visible characters of the head and similarity in appearance with intact organisms represented in the sample. Arthropods were identified to the lowest taxonomic level possible using appropriate keys. We were able to identify most insect specimens to genus but many non-insect arthropods were only able to be assigned to higher-level taxonomic groups (e.g. Acari, Araneae, Isopoda and Diplopoda). Although mites were very abundant in many of our samples, constraints on our taxonomic expertise

with these taxa necessitated the lumping of all mites into the subclass Acari. We visualized variation among digestive tract samples using non-metric multidimensional scaling (nMDS). In the nMDS plots, the distance between two points is a representation of the dissimilarity between the gut sample profiles, taking into account abundance and diversity of taxa among individual frogs. We scaled the diameter of each circle by the quantity of Formicine ants, which are known to be an alkaloid-rich part of the diet of this species (Saporito et al. 2004). We used a one-way ANOSIM to test the hypothesis that the difference between study areas (disturbed vs. undisturbed) for each site exceeds within-group variation in both diversity and abundance. The test statistic for an ANOSIM (R-value) can range from +1 to -1, with positive values indicating greater between-group differences than within-group differences. Significance of R values was determined by permutation of group membership with 10,000 replicates. We used a Bray-Curtis dissimilarity matrix and implemented the ANOSIM in PAST 3.0 (Hammer et al. 2001).

Carotenoid analyses: We stored frog skins on liquid nitrogen while in Panama, on dry ice for transport to Arizona State University, and in a -80 °C freezer at that university prior to analysis. We weighed individual skins to the nearest $1x10^{-4}$ g and extracted carotenoids from ~0.1g samples of frog skin using a micronizer in the presence of a 1.4 mL hexane (tert butyl methyl ether, 1:1 v/v) solvent. The tissue and solvent were then centrifuged and the resulting supernatant was recovered and dried for carotenoid analysis. Samples were analyzed using a Waters 2695 (Waters, Milford MA) high pressure liquid chromatography (HPLC) instrument. We followed the protocol described in McGraw et al. (2006) with the following modifications, which were necessitated by the presence of

ketocarotenoids in our samples. The HPLC column (Waters YMC Carotenoid column, 5 mm, 4.6 mm #250 mm) was pretreated with a 1% orthophosphoric acid in methanol for 30 min at 1 mL/min. Next, the solvent composition and flow rate were altered to optimize separation of different ketocarotenoids. Using a constant flow rate of 1.2 mL/min, we first used an isocratic elution with 42: 42: 16 (v/v/v) methanol: acetonitrile: dichloromethane for 11 min, followed by a linear gradient up to 42: 23: 35 (v/v/v) methanol: acetonitrile: dichloromethane until 21 min. These conditions were held until minute 25, at which time we returned to the original isocratic conditions for minutes 25-29.5. Carotenoid types were identified by comparison to authentic standards from CaroteNature (Ostermundigen, Switzerland) using external standard curves to quantify the concentration of each carotenoid type found in the skins. Total carotenoid values were reported in ng/g of tissue.

Alkaloid analyses: We stored frog skin samples for alkaloid analysis in methanol immediately after dissection and until alkaloid analysis. We analyzed individual methanol skin extracts for alkaloids using gas chromatography-mass spectrometry (GC-MS) on a Varian Saturn 2100T ion trap MS coupled with a Varian 3900 GC with a 30 m x 0.25 nm inner diameter Varian Factor Four VF-5ms fused silica column. Alkaloid GC separation was achieved using a temperature gradient program from 100 to 280° C at a rate of 10 °C per minute using helium as the carrier gas (1 mL/min). We analyzed each alkaloid fraction with both electron impact MS and chemical ionization MS using 100% methanol as the chemical ionization (CI) reagent. Individual alkaloids were identified according to their MS properties and GC retention times and compared against previously reported anuran alkaloids (Daly et al. 2005). We assigned individual alkaloids code

names consisting of a number that corresponds to the nominal mass, in addition to a letter to distinguish different alkaloids of the same mass. Individual alkaloids were quantified in μg per frog (full skin).

Carotenoid/alkaloid statistical analyses: We tested for differences in total quantities of carotenoids and alkaloids using general linear models (GLMs) performed in SPSS (Version 20). Our models included site, with sampling area (disturbed vs. undisturbed) nested within site, and sex as fixed factors and all 2- and 3-way interactions. Because alkaloid and carotenoid quantities were non-normally distributed, the models were run on log-transformed carotenoid and square root-transformed alkaloid data. We also tested for differences in carotenoid and alkaloid diversity (number of chemical species present) using generalized linear models. We used the Proc GENMOD command in SAS (Version 9.2) with a Poisson distribution with site, study area (disturbed vs. undisturbed) and sex as fixed factors as well as all 2- and 3-way interactions. An effect of site in these models would suggest that carotenoid/alkaloid concentrations/diversities differ among differently colored *O. pumilio* populations. An effect of study area would indicate that skin chemical properties differ among habitat types (disturbed vs. undisturbed) whereas a significant site by study area interaction would suggest that the effect of habitat disturbance on skin chemistry differs across the studied populations. We visualized differences in skin chemical profiles among sites and study areas using non-metric multidimensional scaling (nMDS) and scaled the diameter of each circle to represent variation in the total quantity of alkaloids/carotenoids recovered. We used a one-way ANOSIM to test the hypothesis that the difference in the composition and quantities of biochemicals between sites and study areas exceeds within-group variation. We again

used a Bray-Curtis dissimilarity matrix and conducted the analysis in PAST 3.0 (Hammer et al. 2001).

Viewer-specific visual modeling: To estimate the effect of microhabitat differences among study areas on the perception of frog coloration, we modeled the contrast (chromatic and achromatic) between each captured frog and the substrate upon which it was first observed using the visual model developed by Vorobyev and Osorio (1998) in the program AVICOL V6 (Gomez 2006). We ran independent models for each frog under both the *O. pumilio* visual system (trichromatic, Siddiqi et al. 2004) and a bird visual system (tetrachromatic blue tit, *Cyanistes caeruleus*, Hart and Vorobyev 2005). The models took into account the spectral reflectance of the frog's dorsum as well as the downwelling absolute irradiance and spectral reflectance of the substrate at the point where the frog was encountered on the study plot. The model's output is an estimate of the contrast between the frog and its substrate, reported as both chromatic (color contrast, deltaS) and achromatic (luminance or brightness contrast, deltaQ) components, in units of 'just noticeable differences' (JNDs). A JND value > 1 indicates that the frog is detectable by the viewer's visual system on its given background. For all models, we used the photoreceptor densities and Weber fractions from Siddiqi et al. (2004). The absolute irradiance recorded at the encounter location for some frogs (n = 20) was lower than the spectrophotometer was able to detect. In these cases we substituted the irradiance measure with the lowest detectable absolute irradiance collected from the same study area. The resulting deltaS and deltaQ values were square root transformed to achieve normality prior to statistical analysis.

We used General Linear Models in SPSS (Version 20) with the fixed effects of site (population), study area (disturbed or undisturbed), sex, and all 2- and 3-way interactions to test for differences in chromatic (deltaS) and achromatic (deltaQ) contrast between frogs and their chosen substrates under both their own visual system and an avian visual system. A significant site effect would suggest that frogs from different populations, all of which differ in coloration, also vary in their conspicuousness to conspecifics or potential predators, whereas an effect of study area would mean that conspicuousness differs between disturbed and undisturbed habitats. A significant site by study area interaction would suggest that the impact of habitat disturbance on frog conspicuousness differs among populations. Sexual dichromatism has been shown in one *O. pumilio* population (Isla Solarte: Maan and Cummings 2009). In our model, a significant main effect of sex would indicate conspicuousness varies either due to sexual dichromatism or microhabitat differences, whereas a sex by study area effect would reveal sex-specific differences in the effect of habitat disturbance on conspicuousness of frog coloration. A site by sex interaction would indicate that there is a sex-specific difference in conspicuousness between populations. A three-way interaction would indicate disturbance has a site specific impact on conspicuousness that is sex-specific.

Results:

Effects of anthropogenic disturbance: Three principal components (PCs) with eigenvalues greater than one explained 78.53% of variation in our measurements of habitat (canopy cover, percent trees, percent shrubs, percent leaf litter, and number of vegetative 'species', Table 1). PC1 explained 38% of variation and associated positively with the presence of leaf litter and understory plant diversity and negatively with the

percent of bare soil. PC2 explained 22% of the variance and associated positively with canopy cover and the presence of trees and bare soil and negatively with percent of shrubs. PC3 explained 18% of the variance and associated positively with canopy cover and shrubs and negatively with presence of trees. PCs 1 ($F_{3,32} = 7.222$, $p = 0.001$) and 3 ($F_{3,32} = 3.989$, $p = 0.016$), but not PC2 ($F_{3,32}=0.606$, $p =0.616$) differed among study sites. PCs 1 ($F_{1,32} = 113.435$, $p \leq 0.001$) and 2 ($F_{1,32}=12.278$, $p = 0.001$), but not PC3 ($F_{3,32} = 0.704$, $p = 0.408$) differed among study areas). Undisturbed sites had greater PC1 and PC2 scores than disturbed sites, indicating more canopy cover, more trees, more leaf litter and more understory plants in undisturbed sites than disturbed ones and more bare soil and more shrubs in disturbed sites. For PCs 2 ($F_{3,32} = 12.979$, $p \leq 0.001$) and 3 ($F_{3,32}=5.826$, $p =0.003$), but not PC 1 ($F_{3,32} = 2.085$, $p = 0.122$) there was a significant interaction between site and study area, suggesting the magnitude of the difference between disturbed and undisturbed habitat characteristics differed among our four sites.

Our combined (Berlese + pitfall) trapping effort to assess arthropod communities recovered 2,667 arthropods, 760 of which were individuals belonging to taxa also recovered from *O. pumilio* stomachs. Because we pooled all traps within a sampling site we were unable test for interacting effects, but we found no overall difference in the composition of arthropod species recovered between study areas (disturbed vs. undisturbed). This was true whether we included all arthropods trapped ($n = 8$, $R = 0.2083$, $p = 0.1746$), or reduced the dataset to only include taxa also recovered from frogs' stomachs ($n = 8$, $R = 0.1875$, $p = 0.1702$).

We were able to recover and identify 13,309 arthropods from the stomachs of 78 *O. pumilio*. Stomach contents were overwhelmingly dominated by Acari mites and

formicid ants, particularly of the genera *Nesomyrmex, Solenopsis, Strumigenys* and *Pheidole* (97.84%, Table 3). Formicid ants made up the majority of the prey items in the stomachs of all populations of *O. pumilio* (disturbed/undisturbed: Almirante 98.57%/99.15%, Aguacate 97.67%/95.56%, Isla Colon 93.27%/97.98%, Solarte 99.72%/99.36%). Aguacate (blue) frogs showed no difference in stomach content diversity between study areas whether all frogs were considered together ($n = 40$, $R = -0.00028$, $p = 0.4107$) or sexes were analyzed separately (males: $n = 20$, $R = 0.02233$, $p = 0.2975$; females: $n = 20$, $R = -0.06478$, $p = 0.826$). Almirante (red) frogs differed in stomach content diversity, with undisturbed sites having greater diversity whether all frogs were considered together ($n = 40$, $R = 0.2891$, $p < 0.0001$) or males ($n = 20$, $R = 0.2284$, $p = 0.004$) and females ($n = 20$, $R = 0.2491$, $p = 0.0048$) were analyzed separately. Isla Colon (yellow/green) frogs showed no difference in stomach contents among study areas whether sexes were lumped ($n = 38$, $R = 0.02242$, $p = 0.2147$) or analyzed separately (males: $n = 19$, $R = -0.01797$, $p = 0.5235$; females: $n = 19$, $R = -0.01344$, $p = 0.4770$). Solarte (orange) frogs differed in stomach content profiles with frogs from undisturbed habitats having higher diversity than disturbed habitats, when sexes were considered together and separately (overall: $n = 40$, $R = 0.5930$, $p < 0.0001$; males: $n = 20$, $R = 0.7914$, $p < 0.0001$; females: $n = 20$, $R = 0.4136$, $p < 0.0001$). Visual representation of stomach contents using nMDS (Figure 4) show overall consumed prey profiles as well as estimates of alkaloid rich formicid ants consumed (size of circles).

Carotenoid analyses recovered 24 unique major pigment peaks across HPLC runs (Table 4). Many pigments recovered were esterified leading us to recover both free and ester forms. A linear regression showed a positive relationship between the number of

carotenoid types and the total amount of carotenoid recovered from frog skins ($F_{1,78}$= 1411.047, $p < 0.001$, Figure 5). The quantity of carotenoids was not significantly different between sampling areas (disturbed vs. undisturbed), site by sampling area, or sampling area by sex, however the three-way interaction of site by study area by sex was significant (Table 5). There were no significant effects of study area, site by study area affecting the number of carotenoids (Table 6). Aguacate (blue) frogs had very few carotenoids both in terms of types and quantity. In this population there was a marginally significant difference in carotenoid diversity between study areas when all frogs were included (n = 19, R = 0.104, p = 0.0803), but not when split by sex (males n = 10, R = 0.304, p = 0.1346; females n = 9, R = -0.00625, p = 0.3848). When all frogs were considered together, Almirante (red) frogs in disturbed (16.30 ± 0.038 average number of types of carotenoids ± SE) habitat had a marginally greater number of carotenoids in their skin than frogs in undisturbed (15.50 ± 0.042) habitat (n = 20, R = 0.1058, p = 0.0798). When sexes were considered separately, there was no difference in carotenoid diversity among males from disturbed and undisturbed areas (n = 10, R= -0.064, p = 0.6576), but females in disturbed habitats had marginally higher carotenoid diversity than females in disturbed habitats (n = 10, R= 0.364, p = 0.0536). Isla Colon (yellow/green) frogs did not differ in carotenoid number or diversity among study areas (disturbed 9.2± 0.189, undisturbed 10.3± 0.104, n = 19, R= 0.05377, p = 0.167), though when considered separately, females from undisturbed habitats at this site had marginally greater carotenoid diversity than females from disturbed habitats (n = 9, R= 0.216, p = 0.0872), and males from undisturbed habitats had more diverse carotenoids in their skin than males from disturbed habitats (n = 10, R= 0.2687, p = 0.0403). Solarte (orange) frogs in

undisturbed habitats had marginally greater carotenoid number (13.6± 0.060) than frogs from disturbed habitats (12.7± 0.220, n = 19, R= 0.1267, p = 0.0755), which appears to be mainly driven by females (females n = 10, R= 0.392, p = 0.0484, males n = 9, R= -0.125, p = 0.752; Figure 6).

We recovered 142 different alkaloid compounds from the 48 *O. pumilio* we sampled (Table 7; alkaloid compounds recovered: Isla Colon = 77, Solarte = 34, Aguacate Peninsula = 43, Almirante = 55). As with carotenoids, the number of alkaloid types was positively related to the total quantity of alkaloids recovered from frog skins ($F_{1,46} = 69.180$, $p \leq 0.001$, Figure 7). There was no main effect of sampling area or site by sampling area, but there was a marginally significant sampling area by sex interaction (Table 5) on alkaloid quantity where females in undisturbed sites had more alkaloids (401.02 ± 61.65μg) than those in disturbed sites (250.51 ± 61.65μg), whereas males showed the opposite trend (undisturbed: 231.71±61.65μg , disturbed: 368.32± 61.65μg). There was no main effect of sampling area, however a marginally significant site by sampling area effect, and a significant site by sampling area by sex interaction was found for the number of alkaloids (Table 6). Aguacate (blue) frogs showed no difference in alkaloid diversity between disturbed and undisturbed habitats whether all frogs were considered together (n = 12, R = 0.03333, p = 0.3279), or when males (n = 6, R = 0.2963, p = 0.2986) were considered separately. However, females from disturbed habitats had marginally greater number of alkaloids than females from undisturbed habitats (n = 6, R= 0.4074, p = 0.0963). Almirante (red) frogs from undisturbed habitat had a greater diversity of alkaloids in their skin than frogs from disturbed habitat (n = 12, R = 0.5889, p = 0.0023). This difference was only marginally significant when the sexes were

considered separately (males n = 6, R = 0.7073, p = 0.0994; females n = 6, R = 0.5926, p = 0.0961). Frogs from Isla Colon (yellow/green) had marginally greater alkaloid diversity in undisturbed habitats than in disturbed habitats (n = 12, R= 0.1611, p = 0.0744), though the pattern did not hold when sexes were considered separately (males: n = 6, R = 0.3333, p = 0.1014; females n = 6, R = 0, p = 0.5013). Frogs from Solarte (orange) had greater alkaloid diversity in undisturbed habitats than disturbed habitats (n = 12, R= 0.2667, p = 0.0303), but again, the pattern did not hold when sexes were considered separately (males n = 6, R = 0.8889, p = 0.1019; females n = 6, R = 0.2222, p = 0.2051, Figure 8).

***Effects of site*:** The four populations of *O. pumilio* sampled differed in all measured variables (Tables 2, 5 and 6). Stomach contents profiles differed among sites (overall R = 0.208, p = 0.0001). The total quantity of carotenoids recovered per gram of skin samples differed among sites (Table 5). The Almirante (red) and Solarte (orange) populations had the greatest concentration of carotenoids (estimated marginal means ± standard error: Almirante 641.29 ± 44.34 ng/g, Solarte 480.57 ± 45.71 ng/g) which included red ketocarotenoids (and their esters), followed by the yellow/green Isla Colon (159.32 ± 45.71 ng/g) and blue Aguacate (3.296 ± 45.71 ng/g) populations. Carotenoid diversity also differed among sites (Table 6) with Almirante having (15.90 ± 0.143 average number of types of alkaloids ± SE) types than Solarte (13.16 ± 0.392), Isla Colon (9.74 ± 0.357), or Aguacate (1.11± 0.073).

Alkaloid quantity differed among sites (Table 5) with Aguacate frogs having greater quantities of alkaloids than Almirante, Isla Colon or Isla Solarte (estimated marginal means ± standard errors: Aguacate 625.90 ± 61.65 μg, Isla Colon 355.90 ± 61.65 μg, Isla Solarte 192.97 ± 61.65 μg, Almirante 76.80 ± 61.65 μg). Alkaloid

diversity also differed among sites (Table 6) with Isla Colon (24.50 ± 0.424 average number of types of alkaloids ± SE) having greater diversity of alkaloids than Aguacate (18.42 ± 0.362 alkaloids), Isla Solarte (11.75 ± 0.368 alkaloids), and Almirante (10.58 ± 0.420 alkaloids).

Visual modeling results: Model results are reported in two variables, chromatic (color, deltaS) and achromatic (~brightness, deltaQ) contrast. We report these JND values for both tetrachromatic avian visual models, representing frog conspicuousness to a potential predator, as well as trichromatic *O. pumilio* vision (Figure 3, Table 2). In the tetrachromatic (bird) visual system, chromatic contrasts between frog and substrate (deltaS, Table 2) did not differ between study areas. There was a significant effect of site by study area, and a marginally significant effect of study area by sex for tetrachromatic viewers where male frogs from undisturbed sites tended to be the brighter. Achromatic contrasts between frog and substrate also differed among sites and showed a significant site by study area interaction. There was a marginally significant effect of study area and a marginally significant study area by sex interaction where frogs (males specifically) from undisturbed sites were brighter than disturbed sites (deltaQ, Table 2).

In the trichromatic *O. pumilio* visual system chromatic contrasts (DeltaS, Table 2) between frog and substrate differed among sites and study areas, and there was a marginal main effect of sex, where males overall tended to be more contrasting. In Isla Colon and Solarte frogs were brighter in undisturbed sites, though in dark blue frogs from Aguacate frogs were brighter in disturbed sites.

Achromatic contrasts (DeltaQ) between frog and substrate differed among sites and there was a marginally significant effect of study area where undisturbed frogs were

more contrasting than disturbed frog populations (Table 2). The site by study area interaction was also significant with frogs from undisturbed sites having more contrast with their backgrounds, particularly in Isla Colon and Solarte (Figure 3, Table 2).

Discussion:

The diversity of phenotypes displayed by the poison frog *O. pumilio* has drawn considerable attention as a study system with the potential for disentangling the neutral and/or selective forces responsible for phenotypic divergence. Here we uniquely investigate how proximate environmental factors influenced by anthropogenic habitat modification affect the expression of skin color and toxicity, factors that together make up an anti-predator signal that is also used in mate choice. We investigated four phenotypically distinct populations of the polytypic poison frog *O. pumilio* to test the prediction that anthropogenic disturbance could affect one or multiple traits that are involved in multiple trophic level interactions. We find significant differences across geographically distinct populations (sites) for all traits measured, reinforcing the overall prediction that geographically isolated populations differ beyond color patterns alone.

To specifically address how disturbance affects frog populations we began by assessing the bioavailability of arthropods between sites and found no significant differences in the overall composition of arthropods between pairwise disturbed/undisturbed forests chosen. Even when we considered only the subset of the arthropods trapped that were also found in frog stomachs we found no significant differences between arthropods in disturbed versus undisturbed forests. In examining frog stomach contents, we found that frogs maintained high fidelity to specific types of prey, such as mites and ants, although the composition of stomach contents overall

differed between study areas. Male *O. pumilio* territories have been shown to be concentrated around ant mounds (Staudt et al. 2010), which likely promotes dietary conservatism. However, stomach contents in two of the four populations sampled (Almirante red and Solarte orange) showed significant differences (both in species diversity and quantity) between disturbed and undisturbed sites. Frogs from these two populations also differed in their skin alkaloid and carotenoid profiles where frogs from undisturbed sites had higher quantities, suggesting that dietary acquisition of biomolecules shows similar patterns of variation to that of the prey frogs consume, and that microhabitat differences likely affect these steps of the phenotype acquisition pathway.

Feeding behaviors in other poison frogs have been shown to vary seasonally, likely as a result of changes in territoriality during the dry season (Born et al. 2010). It is possible that in Almirante and Solarte frogs modify their feeding behavior in response to habitat differences. If so, this could explain why we see differences in stomach and skin contents between disturbed and undisturbed study areas within these sites but see no difference in the arthropod communities themselves. It is likely that our pitfall traps captured prey items that weren't available to frogs. However, even when we considered only the subset of arthropods that were also found in frog stomachs we found no differences between disturbed and undisturbed habitat arthropod diversity at these sites. Regardless, finding that carotenoid and alkaloid profiles in the skins of these frogs differed in concert with their stomach contents supports the idea that that local habitat variation, and specifically that related to human land use, have an impact on the accumulation of biomolecules important to the frogs' aposematic signals.

Carotenoid profiles in the skins of frogs from all four sites differed between disturbed and undisturbed sampling areas either when all frogs were considered together or for at least one sex when sexes were considered separately, where frogs from undisturbed sites tended to have more carotenoids. In contrast to guppies (Grether 2000), we find that carotenoid profiles in *O. pumilio* skin were most frequently dissimilar between disturbed and undisturbed habitats for females than for males. We predicted that longwave phenotypes (red/orange) would be more likely to be limited by the bioavailability of carotenoids. We find marginally significant differences in carotenoid composition for those populations (Almirante, red; and Solarte, orange) as well as sex-specific differences in the yellow/green population of Isla Colon. Specifically we find a larger dissimilarity in carotenoid profiles for females than males where in two of the three populations with differences (Isla Colon and Solarte) females have greater diversity in undisturbed sites. This could potentially be due to i) higher heterogeneity in carotenoid resources over their home ranges, ii) lack of defended territories with abundant sources of carotenoids, or iii) because females utilize carotenoids in reproduction. Increases in the consumption of dietary carotenoids in *O. pumilio* have been shown to increase reproductive fitness (Dugas et al. 2013), which suggests an important role of dietary carotenoids in females in addition to influencing coloration. In studying dermal carotenoids we are also afforded with valuable preliminary insights into the pigment based composition of poison frog coloration. In addition to structural color elements, we have shown that dermal pigments are universally present in frog skin, and abundant in medium and long wavelength phenotypes.

Alkaloid profiles have been shown to vary on multiple levels including geographically (Daly and Myers 1967), seasonally (Saporito et al. 2006), and between sexes (Saporito et al. 2010). Our aim was to determine if fine-scale differences in microhabitats (<1 km) could have effects on chemical defense based on the quality of forest the frogs inhabit. Describing the level of chemical defense or 'toxicity' is difficult without *a priori* knowledge of how specific alkaloid types deter predators either independently or synergistically, and whether these effects are robust across predator species. Thus, as a proxy for toxicity we quantified the overall alkaloid profiles in terms of numbers of alkaloid types and total quantities of skin alkaloids. Though we cannot be sure changes will negatively affect frog fitness, we consider significant reductions from the 'undisturbed' population alkaloid profiles as potentially detrimental, as predators have learned to associate a certain level of defense with the phenotype of that population. In three populations (Almirante, Solarte and Isla Colon) we find overall dissimilarity in alkaloid profiles among study areas, where undisturbed populations have more alkaloids than disturbed ones. Diet-acquired chemical defenses are used by all life stages of *O. pumilio*: not only do juveniles and adults sequester alkaloids from foraging, females also provide alkaloids to tadpoles via maternal provisioning of feeder eggs providing them with chemical defense (Stynoski et al. 2014), so finding less alkaloids in females from disturbed sites could impact not only their survival, but also their reproductive fitness, similar to carotenoids. Alkaloids may additionally be important for preventing pathogen growth on frog skins (Mina et al. 2015).

The aposematic phenotype (warning color + defense) in *O. pumilio* likely to be acted upon by both sexual selection and natural selection. We used individual 'snapshot'

visual models to infer whether habitat and skin color variation affects the perception of frog phenotypes. These models take into account variation in the substrate of local signaling environments, variation in frog coloration, as well as in the ambient lighting where frogs were encountered. Models of conspecific vision addressed whether phenotypic differences attributable to habitat and phenotypic variation are perceivable to other *O. pumilio*, which could have impacts for sexual selection via male-male competition or female mate choice. In the *O. pumilio* visual system, both chromatic and achromatic contrasts differed between disturbed and undisturbed forests. For chromatic contrast, frogs from undisturbed study areas were generally more contrasting with their backgrounds than frogs from disturbed study areas, with the exception of the more cryptic blue population of Aguacate where frogs from the disturbed area were more contrasting with the background. Achromatic contrast also differed between disturbed and undisturbed areas. Here the direction of change was site specific where dark blue frogs from Aguacate were more conspicuous in disturbed sites, but yellow/green frogs from Isla Colon and orange frogs from Solarte were brighter in undisturbed sites. Sexual selection is thought to be a key force promoting phenotypic divergence across populations of *O. pumilio* where both mating and agonistic decisions are often based on phenotypic cues (reviewed in Cummings and Crothers 2013). Our visual models show habitat-specific differences in phenotypic conspicuousness at levels detectable to the frogs themselves. This suggests that habitat quality could impact intra- and intersexual encounters. Chromatic contrast can be an important component of sexually selected signals in heterogeneous environments. In fish (*Telmatherina*), individuals with more chromatic contrast with their environment have higher reproductive fitness (Gray et al.

2008). Though achromatic contrast may be less intuitively tied to sexual selection, evidence suggests it may be an important trait for female preference in bird plumage (Mennill et al. 2003), or in species and/or mate recognition in guppies (Cole and Endler 2015).

We used a tetrachromatic avian visual model to ask whether differences in habitat lead to perceivable differences in frog conspicuousness to a color-discriminating potential predator. Both chromatic and achromatic contrasts of frog coloration were found to be influenced by habitat, though the effect varied between geographic locations. For example, the cryptic dark blue phenotype of Aguacate frogs was more conspicuous in both chromatic and achromatic contrast in disturbed sites, whereas orange frogs from Solarte had higher chromatic contrast the undisturbed site. Cryptic *O. pumilio* morphs tend to also be more reclusive behaviorally, exhibiting reduced male aggression and less explorative foraging behaviors (Pröhl and Ostrowski 2011, Rudh et al. 2011, Rudh et al. 2013). An increased phenotypic contrast could be detrimental to more cryptic frogs, like those from Aguacate, if it increases the ability of predators to detect them.

Taken together, the analyses presented here demonstrate evidence of how fine-scale habitat variation can impact multiple trophic-level interactions. Using *O. pumilio* as an example, we show how habitat alteration translates to differences in the accumulation of important fitness-influencing biomolecules such as carotenoid pigments and defensive alkaloids, and suggest how these profile changes could influence frog fitness. We also model visual signals, showing that the frogs are likely to be perceived differently in disturbed versus undisturbed areas by both conspecifics and potential predators, with the potential to affect the processes of both natural and sexual selection.

Figure 3-1: Map showing the four phenotypically distinct *O. pumilio* populations (sites) sampled. Scale bar is 10 km. Abbreviations: IC = Isla Colon, IS = Isla Solarte, AL = Almirante, AG = Aguacate.

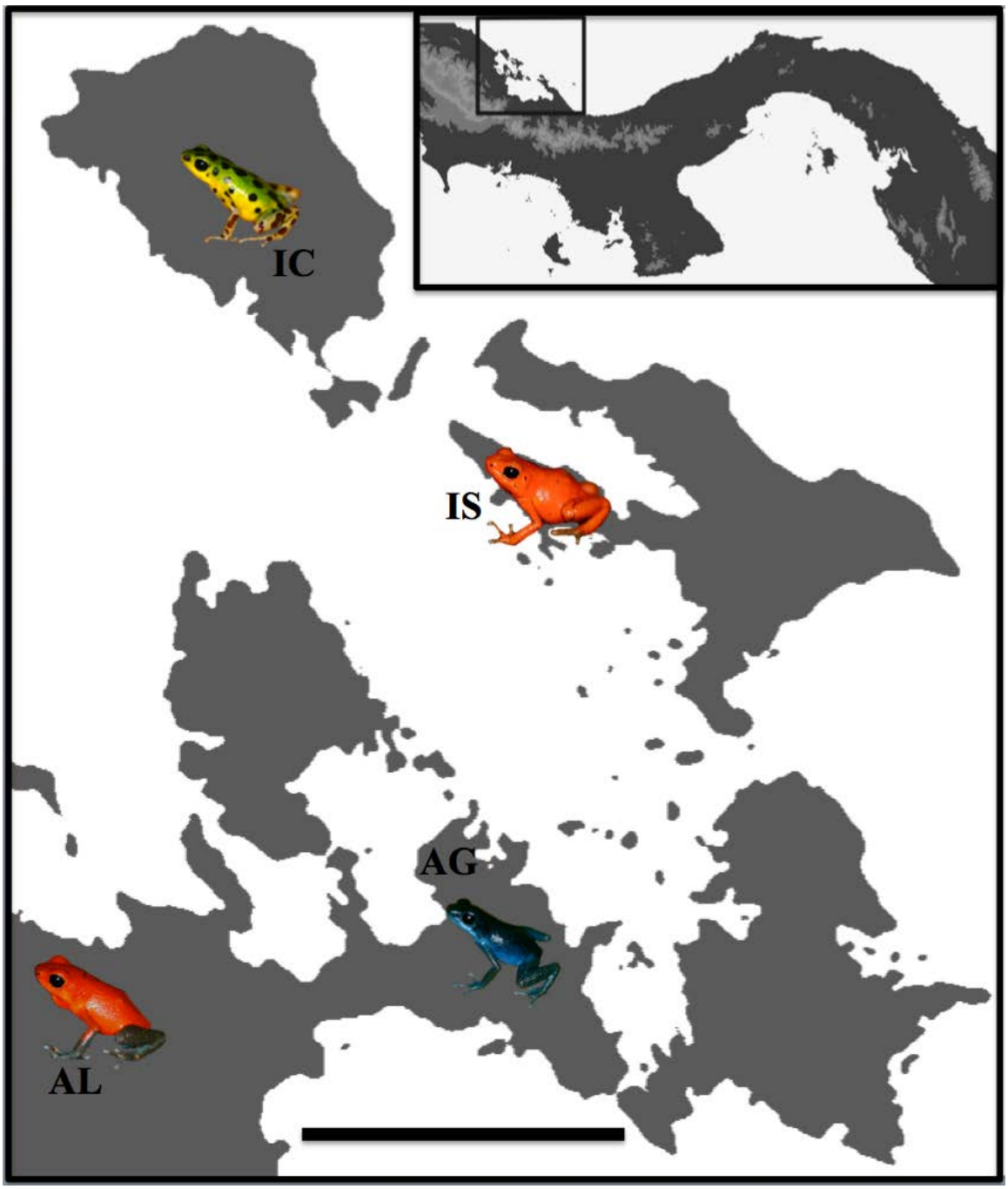


Figure 3-2: Illustration depicting trophic level interactions addressed by this study: differences in forest characteristics (A), abundance of arthropods and diversity of prey consumed by frogs (B), important biomolecules such as carotenoids and alkaloids (C), and sources of both natural and sexual selection (D).

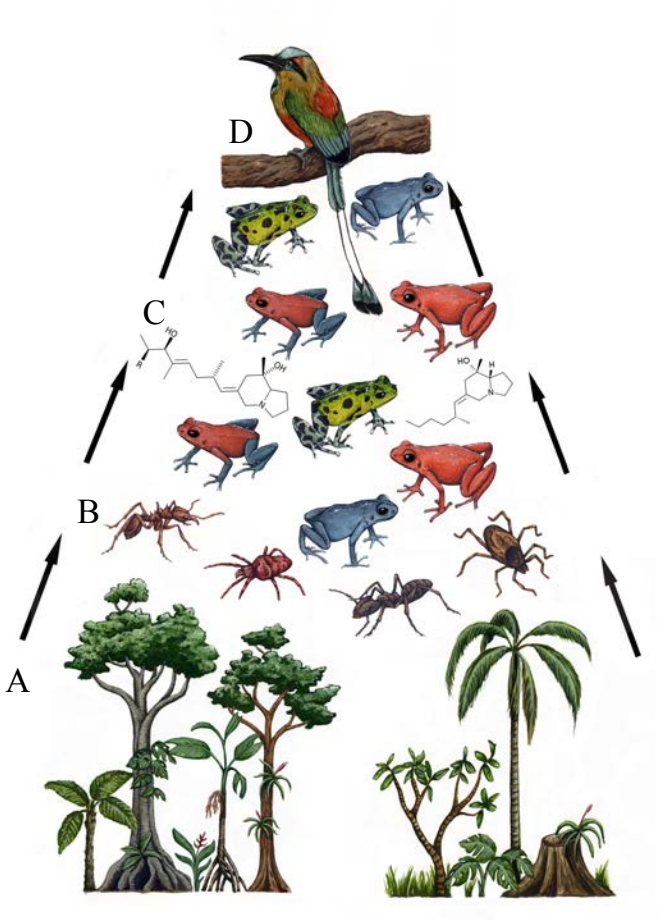


Figure 3-3: Population JND averages (± standard error) from visual models. Left panel: trichromatic *O. pumilio* visual system, right panel: tetrachromatic avian visual system. Filled shapes represent frogs from disturbed sites, unfilled represent undisturbed sites. Squares show male conspicuousness where circles show females.

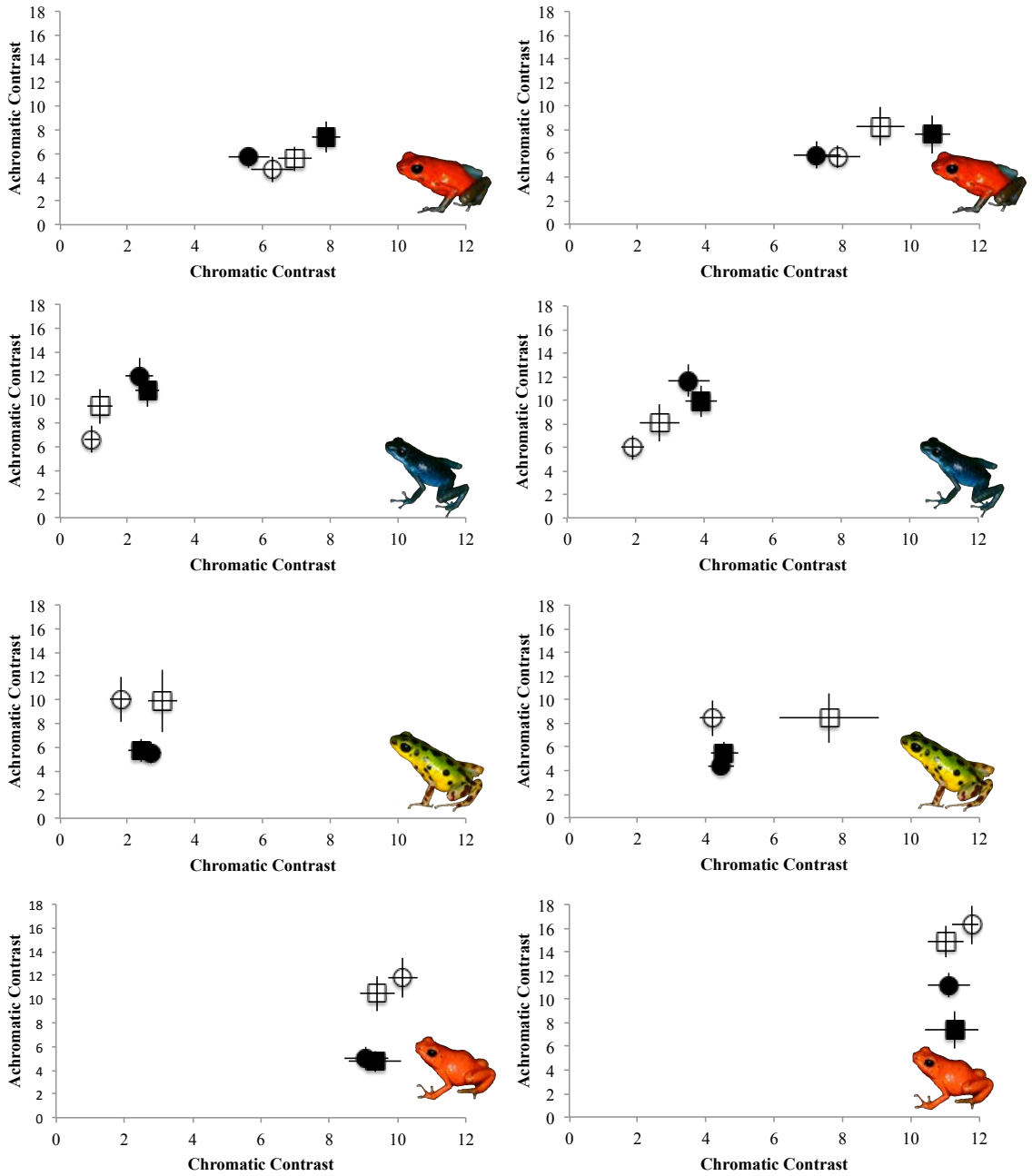


Figure 3-4: Stomach content nMDS all populations, shaded circles represent frogs from disturbed sites, unshaded represent frogs from undisturbed sites. The size of the circles represents the quantity of Formicine ants (see methods).

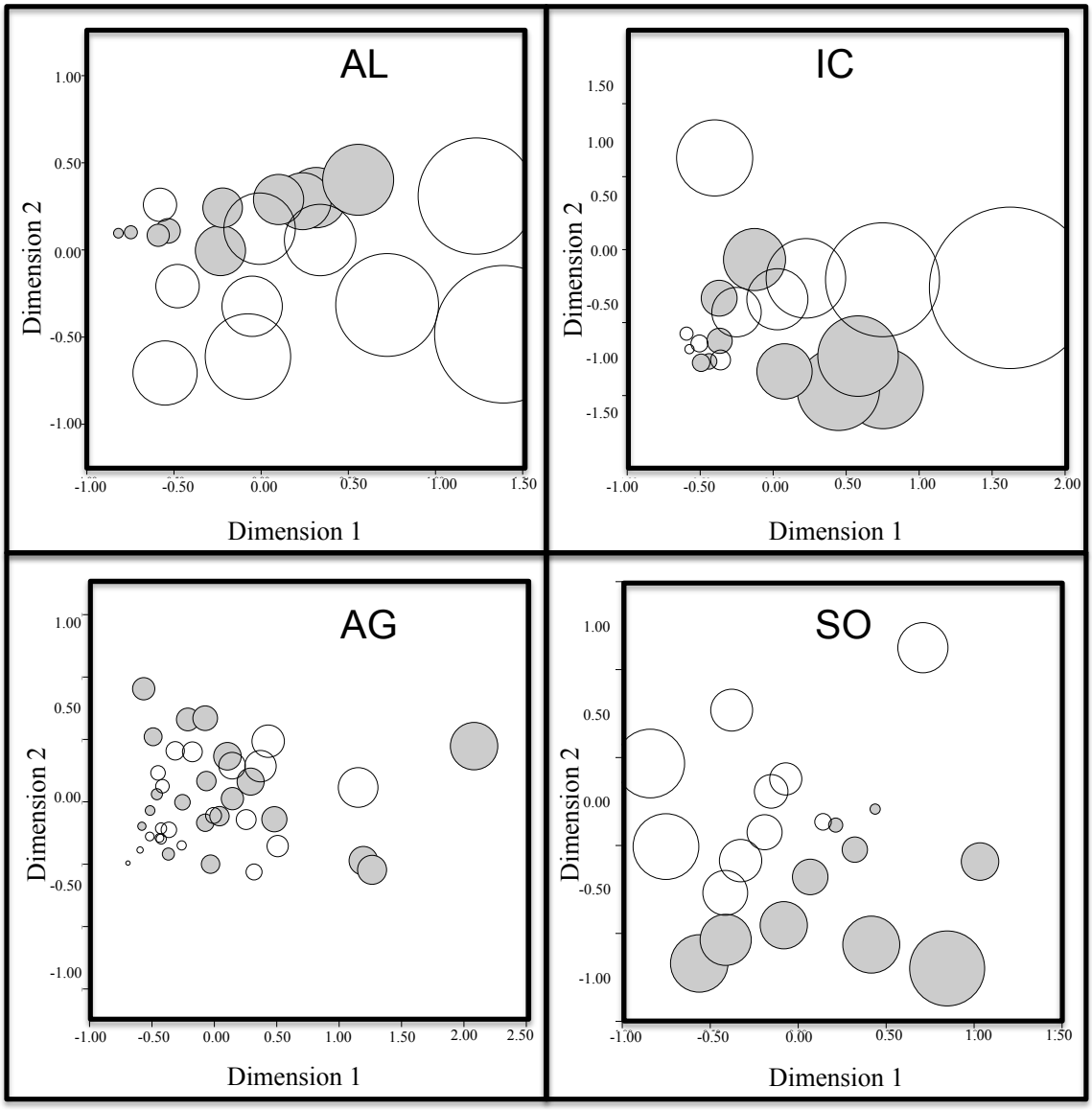


Figure 3-5: Relationship between carotenoid diversity and concentration in frog skin. Open shapes represent individuals from undisturbed sites, filled shapes are from disturbed habitats. Squares represent males and circles represent females, shapes are colored by frog phenotype (Almirante: red, Isla Solarte: orange, Isla Colon: green, Aguacate: blue).

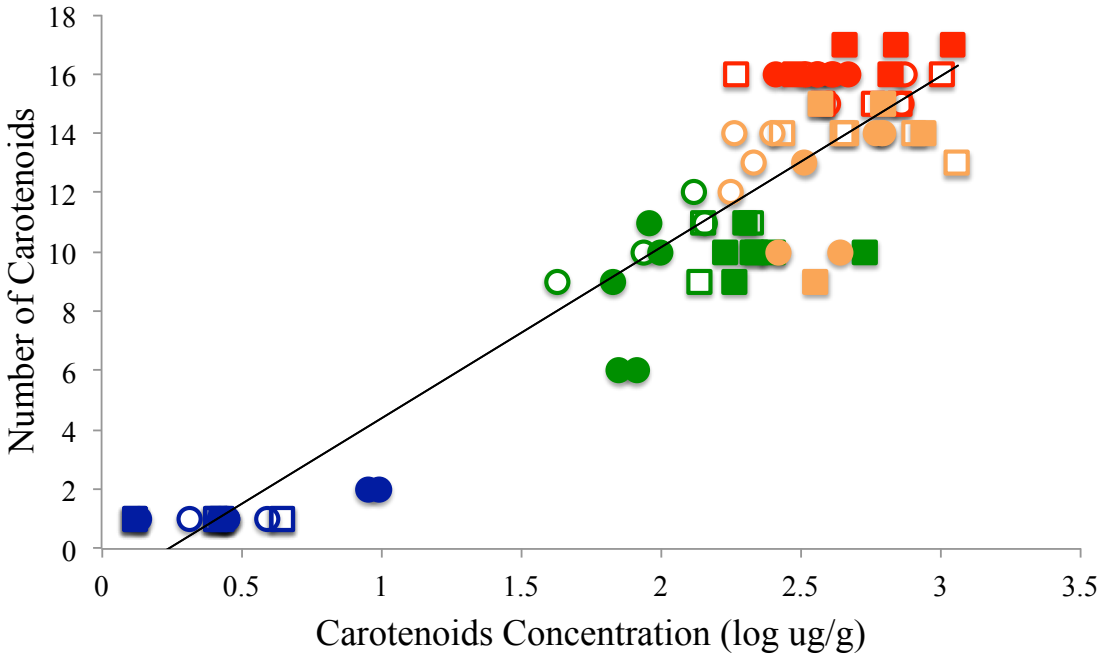


Figure 3-6: nMDS plots to visualize carotenoid profiles. Shaded circles represent frogs from disturbed sites, unshaded represent frogs from undisturbed sites. The size of the circles represent the total quantity of carotenoids for each individual.

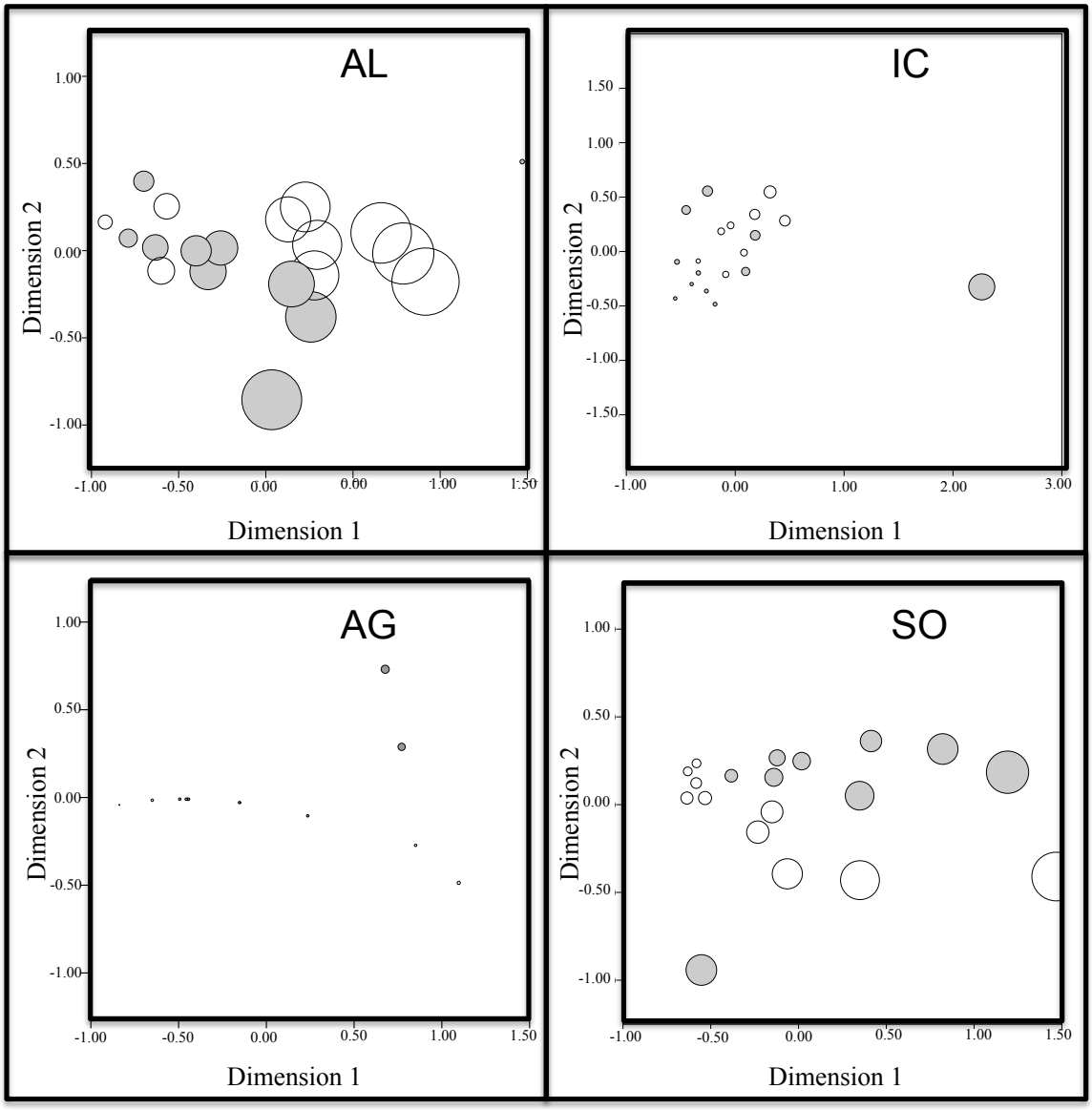


Figure 3-7: Linear regression of alkaloids. Unshaded shapes represent undisturbed sites, shaded disturbed sites. Squares represent males, circles females. Red: Almirante, Orange: Isla Solarte, Green: Isla Colon, Blue: Aguacate.

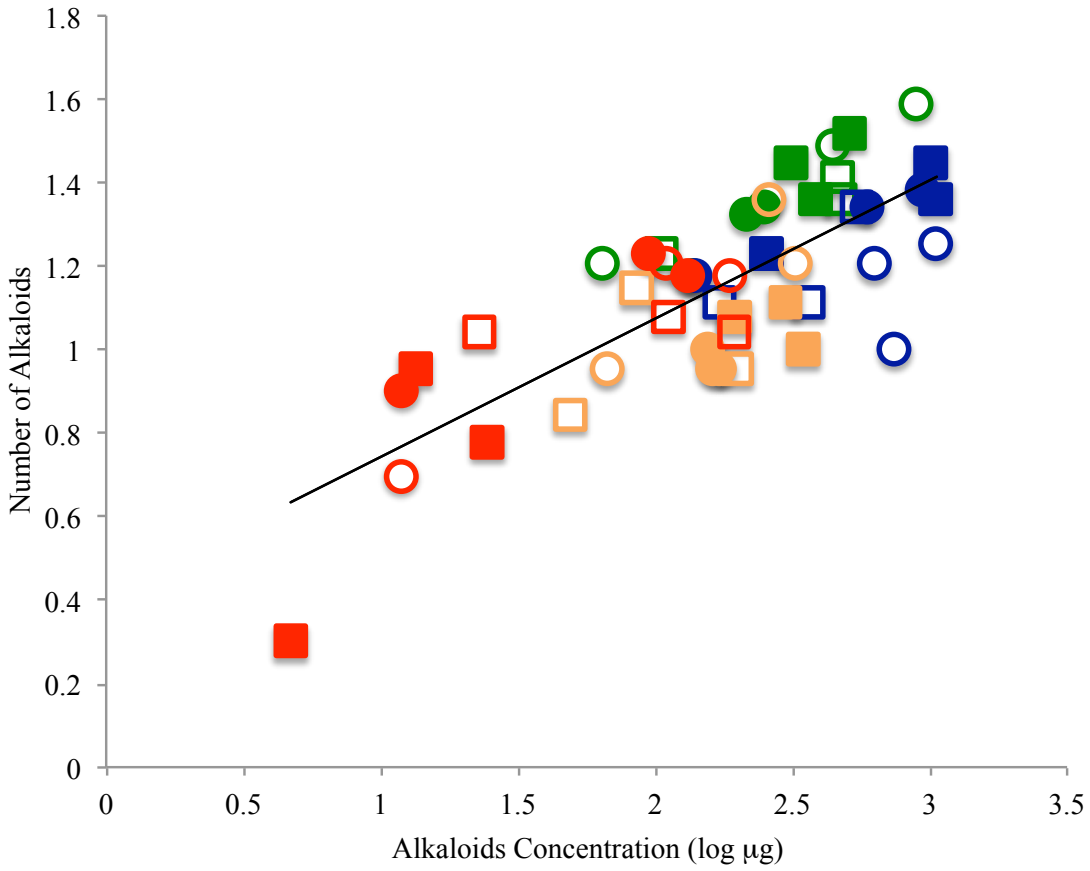


Figure 3-8: nMDS plots to visualize alkaloid profiles. Shaded circles represent frogs from disturbed sites, unshaded represent frogs from undisturbed sites. The size of the circles are the total quantity of alkaloids for each individual.

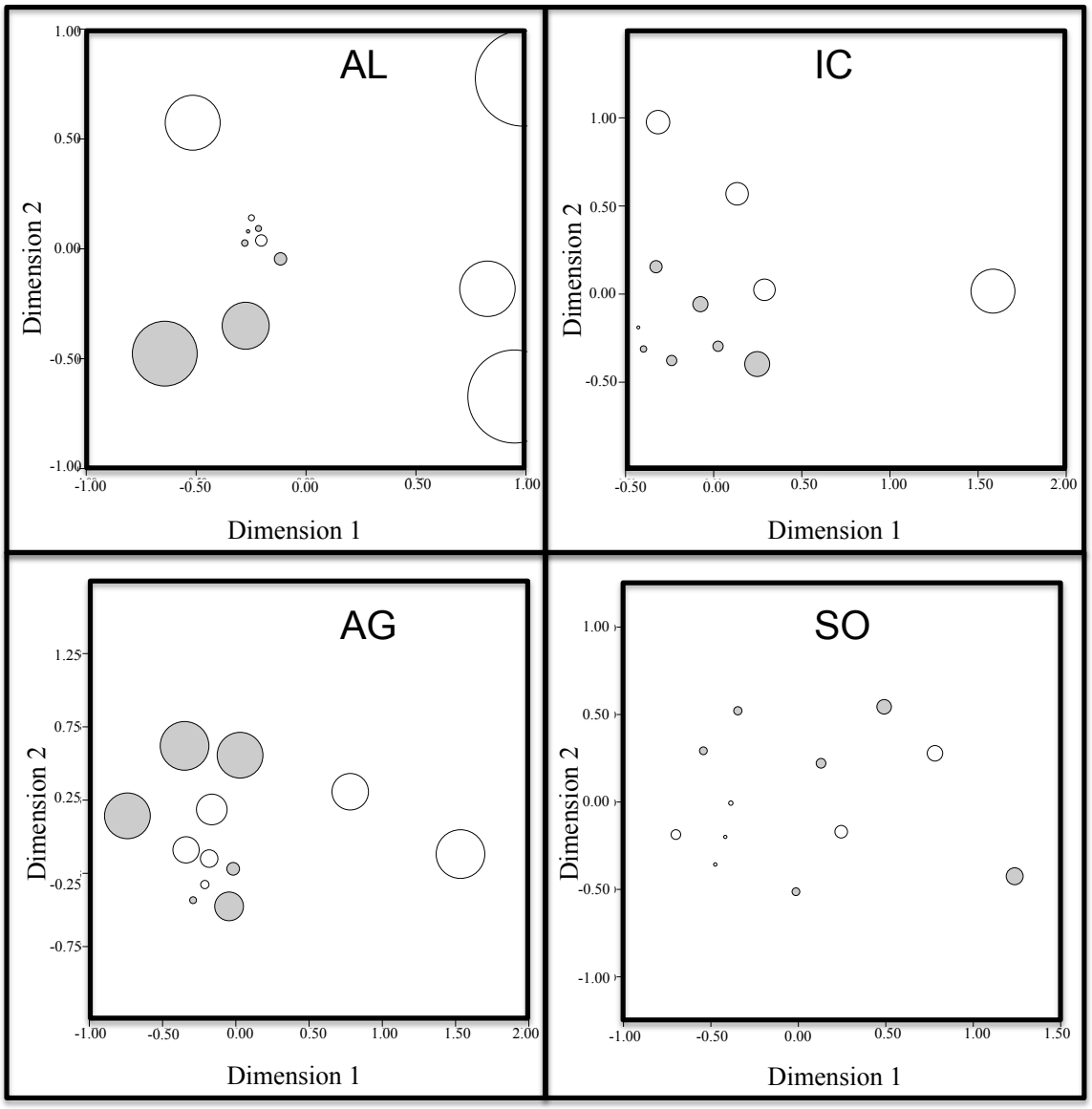


Table 3-1: Principal components analysis component loadings for forest traits measured.

Forest trait	PC1	PC2	PC3
Canopy cover	0.336	0.599	0.572
Percent bare soil	-0.666	0.575	0.297
Percent trees	0.495	0.49	-0.506
Percent shrubs	0.51	-0.599	0.503
Percent leaf litter	0.776	0.093	-0.291
Vegetative species	0.782	0.219	0.288

Table 3-2: General linear model results from visual models including chromatic (deltaS) and achromatic contrast (deltaQ) between frog and substrate modeled in *O. pumilio* trichromatic vision and tetrachromatic avian vision.

	Trichromatic Viewer Chromatic Contrast (deltaS)			Trichromatic Viewer Achromatic Contrast (deltaQ)			Tetrachromatic Viewer Chromatic Contrast (deltaS)			Tetrachromatic Viewer Achromatic Contrast (deltaQ)		
	df	F	Significance	df	F	Significance	df	F	Significance	df	F	Significance
Corrected Model	15	46.446	0.000	15	3.618	0.000	15	29.964	0.000	15	5.174	0.000
Intercept	1	4559.120	0.000	1	1635.890	0.000	1	6091.058	0.000	1	1741.563	0.000
Site	3	203.663	0.000	3	5.193	0.002	3	136.374	0.000	3	12.060	0.000
Sampling area	1	5.436	0.021	1	2.738	0.099	1	0.679	0.411	1	2.930	0.088
Sex	1	3.549	0.061	1	0.275	0.601	1	0.618	0.432	1	0.611	0.435
Site*Sampling Area	3	6.476	0.000	3	10.633	0.000	3	4.727	0.003	3	9.644	0.000
Site*Sex	3	1.598	0.191	3	0.465	0.707	3	5.771	0.001	3	1.814	0.145
Sampling Area*Sex	1	0.002	0.967	1	0.176	0.675	1	3.682	0.056	1	2.742	0.099
Site*Sampling Area*Sex	3	2.501	0.060	3	0.737	0.531	3	1.289	0.279	3	0.259	0.855
Error	224			224			224			224		
Total	240			240			240			240		
Corrected Total	239			239			239			239		

Table 3-3: Classification and number of arthropods recovered from frog stomachs. Male = M, Female = F.

						Aguacate				Almirante				Isla Colon				Isla Solarte			
						Disturbed		Undisturbed		Disturbed		Undisturbed		Disturbed		Undisturbed		Disturbed		Undisturbed	
Class	**Subclass**	**Order**	**Family**	**Subfamily**	**Genus**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Arachnida	**Acari**				**spp.**	158	295	127	217	417	702	542	923	410	663	435	664	207	416	494	432
Arachnida	**Araneae**				**spp.**	0	0	0	0	0	0	0	3	0	2	0	0	0	0	0	0
Arachnida	**Dromopoda**	**Pseudoscorpiones**			**spp.**	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Diplopoda	**Helminthomorpha**					0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Malacostraca	**Eumalacostraca**	**Isopoda**			**spp.**	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Insecta	**Pterygota**	**Blattodea**	**Termitidae**		**spp.**	0	0	3	0	0	0	16	0	0	0	0	25	3	0	0	0
		Coleoptera	**Anthribidae**		**spp.**	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
			Corylophidae		**sp.**	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
			Curculionidae	**Scolytinae**	**spp.**	0	0	0	0	0	0	0	0	0	0	3	5	0	0	0	0
				Unknown	**spp.**	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	3
			Histeridae		**spp.**	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
			Nitidulidae		**spp.**	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0
			Ptiliidae		**spp.**	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
			Staphylinidae	**Pselaphinae**	**Eupsenius**	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
				Pselaphinae	**spp.**	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0
				Scydmaeninae	**Euconnus**	1	0	0	1	0	0	0	0	0	0	0	3	0	0	0	0
				Unknown	**spp.**	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
			Silvanidae		**spp.**	0	0	0	1	0	0	0	0	0	0	0	2	1	0	0	0
			Unknown(adult)		**spp.**	3	2	1	2	3	0	0	1	0	0	0	0	0	0	0	0
			Unknown (larva)		**spp.**	0	8	5	2	1	1	0	2	45	0	3	0	0	0	1	1
		Diptera	**Phoridae**			0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
			Unknown (adult)		**spp.**	1	0	0	0	0	0	0	0	29	12	0	0	0	0	0	0
			Unknown (larva)		**spp.**	0	0	1	0	0	0	0	0	19	18	0	0	0	0	0	0
		Hemiptera	**Auchenorrhyncha**			0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
			Unknown (nymph)		**spp.**	0	0	0	0	4	1	0	1	0	0	0	0	0	0	0	0
		Hymenoptera	**Eupelmidae**		**spp.**	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Formicidae	**Amblyoponinae**	**Prionopelta**	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
				Formicinae	**Camponotus**	0	0	0	0	0	0	3	0	5	0	0	0	0	0	0	0
					Gnamptogenys	0	0	0	0	0	1	0	0	2	0	0	0	1	0	0	0
					Paratrechina	0	0	0	0	0	0	5	18	0	0	0	0	2	2	2	0
				Myrmicinae	**Aphaenogaster**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
					Apterostigma	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	3
					Crematogaster	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0
					Cyphomyrmex	4	1	0	3	6	5	14	9	10	7	0	1	1	22	0	0
					Eurhopalothrix	2	2	1	0	2	0	1	0	0	0	0	2	8	20	3	7
					Mycocepurus	0	0	0	0	0	0	1	2	0	0	0	0	0	1	0	0
					Nesomyrmex	22	37	55	41	49	30	146	141	138	57	157	61	0	0	0	0
					Pheidole	0	0	0	0	0	0	0	56	94	26	3	0	200	215	19	96
					Pyramica	0	0	0	0	2	0	0	0	0	0	0	0	10	0	0	0
					Solenopsis	198	123	126	121	69	73	541	451	198	181	196	508	0	0	0	0
					Strumigenys	2	20	4	14	7	7	17	15	5	4	0	2	140	159	458	347
				Unknown	**spp.**	10	4	0	1	5	6	20	1	1	0	11	1	6	8	2	0
			Proctotrupidae		**spp.**	0	0	0	0	1	0	0	0	1	1	0	0	5	4	2	0
		Lepidoptera	**Unknown (larva)**		**spp.**	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	2
		Thysanura	**Phlaeothripidae**		**spp.**	0	1	0	2	3	0	0	0	0	0	0	0	0	0	0	0
					#Individuals	10	10	10	10	10	10	10	10	9	9	10	10	10	10	10	10

Table 3-4: Average carotenoid concentrations (in ug/g) by sex, study area and site.

	Aguacate				Almirante				Isla Colon				Isla Solarte			
	Disturbed		Undisturbed		Disturbed		Undisturbed		Disturbed		Undisturbed		Disturbed		Undisturbed	
Carotenoid types	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
apocarotenoid	1.861	4.041	3.303	2.852	3.725	1.030	0.000	3.528	6.078	1.009	0.717	1.570	11.835	2.687	0.000	0.821
canary xanthophyll	0.000	0.000	0.000	0.000	43.932	26.664	38.017	38.768	7.624	0.226	0.486	0.921	20.714	13.959	24.011	24.168
canthaxanthin	0.000	0.000	0.000	0.000	24.375	11.767	15.912	16.252	0.000	0.000	0.000	0.000	9.597	2.945	11.763	5.239
lutein	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	8.077	0.778	1.978	1.675	0.000	0.000	0.000	0.000
xanthophyll	0.000	0.000	0.000	0.000	7.269	5.451	7.235	7.188	0.000	0.000	0.000	0.000	3.250	1.658	9.754	4.520
cis-ketocarotenoid	0.000	0.000	0.000	0.000	7.165	4.871	6.018	5.862	0.000	0.000	0.000	0.000	5.132	2.385	10.846	3.335
echinenone	0.000	0.000	0.000	0.000	33.077	14.585	15.005	11.168	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3-hydroxy-echinenone	0.000	0.000	0.000	0.000	45.742	29.515	36.408	37.326	0.000	0.000	0.000	0.000	44.489	25.504	58.757	24.168
B-cryptoxanthin	0.000	1.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
lutein ester(1)	0.000	0.000	0.000	0.000	26.143	19.185	25.801	21.466	0.000	0.000	0.000	2.398	40.649	34.237	29.756	9.245
cis-xanthophyll	0.000	0.000	0.000	0.000	26.906	19.638	4.053	0.000	0.000	0.000	0.000	0.000	12.336	0.000	22.588	8.684
canary xanthophyll ester(1)	0.000	0.000	0.000	0.000	69.923	38.180	72.373	63.332	23.880	7.312	29.130	18.564	71.265	20.087	53.212	35.248
B-carotene	0.000	0.000	0.000	0.000	150.055	107.687	141.182	154.240	34.066	12.743	7.745	15.237	186.034	202.244	176.038	67.653
canary xanthophyll ester(2)	0.000	0.000	0.000	0.000	101.412	55.828	77.466	101.246	0.000	0.000	0.000	0.000	57.002	58.428	93.435	44.772
ketocarotenoid ester	0.000	0.000	0.000	0.000	21.782	12.523	15.343	21.260	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
canary xanthophyll ester(3)	0.000	0.000	0.000	0.000	30.484	21.615	30.931	38.849	0.000	0.000	0.000	0.000	11.656	14.874	42.198	16.938
canthaxanthin ester	0.000	0.000	0.000	0.000	80.685	57.778	98.049	86.720	0.000	0.000	0.000	0.000	52.085	40.346	57.409	26.976
ketocarotenoid ester(2)	0.000	0.000	0.000	0.000	53.555	35.091	47.032	53.718	0.000	0.000	0.000	0.000	21.370	20.455	26.001	11.451
xanthophyll ester(1)	0.000	0.000	0.000	0.000	27.081	14.144	16.187	28.351	12.201	1.428	7.679	4.144	9.778	9.789	10.382	6.112
lutein ester(2)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	65.298	20.663	52.960	28.480	0.000	0.000	0.000	0.000
lutein ester(3)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	35.222	16.759	24.472	7.002	0.000	0.000	0.000	0.000
apocarotenoid ester	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	3.855	9.472	21.643	5.399	0.000	0.000	0.000	0.000
zeaxanthin ester	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	29.756	4.091	11.393	5.076	0.000	0.000	0.000	0.000
B-carotene ester	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	35.235	7.804	34.215	10.803	0.000	0.000	0.000	0.000
# individuals	5	5	5	4	5	5	5	5	5	5	5	4	4	5	5	5

Table 3-5: Results of a general linear model for total alkaloid and carotenoid concentration.

	Quantity of Carotenoids			Quantity of Alkaloids		
	df	F	Significance	df	F	Significance
Corrected Model	15	105.514	0.000	15	4.366	0.000
Intercept	1	7191.417	0.000	1	372.537	0.000
Site	3	512.235	0.000	3	17.808	0.000
Sampling Area	1	0.013	0.909	1	0.063	0.803
Sex	1	6.941	0.011	1	0.182	0.672
Site*Sampling Area	3	0.567	0.639	3	0.734	0.540
Site*Sex	3	5.803	0.001	3	0.422	0.739
Sampling Area*Sex	1	0.489	0.487	1	3.608	0.067
Site*Sampling Area*Sex	3	2.85	0.045	3	1.582	0.213
Error	61			32		
Total	77			48		
Corrected Total	76			47		

Table 3-6: Results of a generalized linear model for alkaloid and carotenoid diversity.

	Number of Carotenoids			Number of Alkaloids		
	df	**F**	**Significance**	**df**	**F**	**Significance**
Site	3	802.52	0.000	3	19.9	0.000
Sampling Area	1	0.05	0.822	1	0.39	0.532
Sex	1	0.1	0.758	1	1.17	0.280
Site*Sampling Area	3	1.05	0.370	3	2.33	0.073
Site*Sex	3	2.85	0.036	3	1.45	0.225
Site*Sampling Area*Sex	1	2.15	0.142	4	2.71	0.028

Table 3-7: Average alkaloid concentrations in frog skins (µg per frog) by sex, study area and population (continued after break).

Population	Aguacate				Almirante				Isla Colon				Isla Solarte			
Site	Disturbed		Undisturbed		Disturbed		Undisturbed		Disturbed		Undisturbed		Disturbed		Undisturbed	
Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
3,5-I 167E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.077
Pip 183A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.267	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Tricyclic 191F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.339	0.000	0.521	0.000	0.000
DHQ 195A	39.821	17.359	9.257	9.400	3.700	3.000	47.800	28.833	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3,5-I 195B	0.000	0.000	0.000	0.000	1.367	8.933	0.000	2.667	9.817	26.247	9.996	23.269	0.000	35.609	20.314	3.423
3,5-P 195F	0.000	0.000	0.000	0.000	0.000	0.700	0.667	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3,5-I 195G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	3.836	17.200	0.000	31.556	38.387
5,8-I 195I	0.000	0.000	0.000	0.000	0.467	0.700	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pyr 197B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.900	14.440	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pip 197F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	3.584	0.000	0.000	0.000	0.000	0.000
5,6,8-I 197G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.733	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,8-I 203A	2.330	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.886	0.000	0.361	0.297	0.000	0.000	0.000	0.000
5,8-I 205A	41.866	28.295	21.146	42.657	0.000	0.433	25.500	0.000	22.267	13.523	80.885	15.443	0.000	0.000	0.431	1.486
Trycylic 205B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	3.500	0.000	0.000	0.000	0.000	0.521	12.532	6.811	1.796
5,8-I 207A	4.626	16.555	1.287	2.514	0.000	0.000	9.300	1.267	33.520	4.274	73.267	10.793	0.000	0.000	0.569	2.080
Tricyclic 207GH	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	25.906	0.000	0.000	0.000	0.000
Unclass 207N	5.464	1.943	0.767	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.392	0.000	0.000	0.000	0.000
Deoxy PTX 207O	17.420	6.028	7.063	8.822	0.000	0.000	0.000	0.000	2.267	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,6,8-I 209C	0.000	0.000	0.000	0.000	0.000	1.133	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Unclassifiable 209G	0.000	0.000	0.000	0.000	0.000	0.833	0.000	0.000	15.689	3.250	0.000	0.381	0.000	0.000	0.000	0.000
5,8-I 209I	133.704	43.921	39.635	108.241	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3,5-P 209K	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.498	0.000	0.000	0.000	0.000	0.000	0.000
3,5-P 209Q	0.000	0.000	0.000	0.000	0.000	0.000	0.200	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Unclassifiable 209S	0.000	0.000	0.000	0.000	0.000	0.000	0.433	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
DHQ 211A	0.000	0.000	0.000	0.000	0.300	0.000	4.000	1.467	0.000	0.000	0.000	0.000	2.006	1.671	0.571	1.769
Izidine 211B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.314	0.000	0.000	0.000	0.000
Izidine 211F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.650	0.000	0.000	0.000	0.000	0.000	0.000
Pip 211I	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.849	0.000	0.000	0.000	0.000	0.000	0.000
Pip 211J	0.000	28.528	0.000	0.000	1.233	10.300	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pip 213A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.476	0.000	0.000	0.000	0.000	0.000
Unknown 221	2.157	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Unknown 223	12.591	2.134	2.368	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,6,8-I 223A	16.849	7.452	4.481	293.251	0.000	0.267	0.000	3.367	74.160	22.749	40.001	50.309	0.000	0.000	0.000	0.587
3,5-I 223AB	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.200	0.000	16.765	0.000	0.000	0.000	0.000
5,8-I 223D	0.984	3.085	0.578	1.345	0.000	0.000	0.200	0.000	0.302	0.000	0.624	0.000	0.000	0.000	0.000	0.000
DHQ 223F	34.394	45.376	82.806	36.308	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3,5-P 223H	38.912	50.798	34.885	38.584	0.167	0.000	2.800	16.367	13.522	0.365	19.245	8.252	0.624	0.000	13.055	15.720
5,8-I 223J	151.157	45.905	53.564	135.056	0.000	0.000	0.000	0.500	0.831	0.000	2.250	15.479	0.000	0.000	0.000	0.000
Pip 223K	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.385	0.000	0.000	0.000	0.000
Tri 223P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.331
5,6,8-I 223X	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.501	0.000
Unknown 225	2.069	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Izidine 225A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.655	0.000	0.000	0.000	0.000	0.000	0.000
Pip 225B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.363	0.000	0.000	0.000	0.000	0.000	0.000
Pip 225I	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.011	0.000	0.000	0.000	0.000
5,6,8-I 231B	7.898	0.247	0.769	7.953	0.000	3.567	5.733	15.000	14.249	2.430	0.000	3.326	0.000	0.000	0.000	0.000
5,8-I 231C	26.771	0.000	0.000	0.642	0.000	0.000	1.900	0.200	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,8-I 233NEWA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.390	6.365	3.401	2.180
5,8-I 233D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.242	1.300	3.572
3,5-I 233J	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.112	0.000	0.000	0.000	0.000	0.000
3,5-P 233H	0.000	0.000	0.000	0.000	0.000	0.000	0.667	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,8-I 235	0.000	0.000	0.000	0.000	0.000	0.733	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,8-I 235B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	31.610	17.354	14.874
5,8-I 235B'	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.233	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Unclassifiable 235S	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.328	0.000	0.559	2.092	0.000	0.000	0.000	0.000
SpiroP 236	0.000	0.000	0.000	0.000	0.567	14.267	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,8-I 237	37.879	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,8-I 237NEW	0.000	1.751	2.593	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,6,8-I 237C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500	0.000	0.000	0.000	0.528	0.000	0.000	0.586	5.782
5,8-I 237D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.355	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pip 239L	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.520	0.000	0.000	0.000	0.000
NEW 241	0.000	0.000	0.000	0.000	0.567	3.567	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pip 241D	0.000	0.000	0.000	0.000	0.000	0.000	2.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pip 241G	0.000	0.000	0.000	0.000	0.000	0.433	0.000	0.967	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
aPTX 241H	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.684	3.387	1.383	0.000	0.000	0.000	0.000
NEW 242	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,8-I 243B	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
New Pip 251	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3,5-P 251K	0.000	0.000	1.480	14.768	0.000	0.000	0.000	7.400	5.138	0.000	34.074	3.900	0.000	0.340	0.863	2.883
4,6-Q 251Y	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.378	0.000	0.000	0.000	0.000
5,6,8-I 251S	0.000	0.000	0.000	0.000	0.000	1.767	0.000	0.000	0.000	0.000	0.000	0.758	0.000	0.000	0.000	0.000
5,6,8-I 251T	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	15.320	0.000	0.000	0.000	0.000	0.000	0.000
SpiroP 252A	0.000	0.000	0.000	0.000	0.000	5.233	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.680
SpiroP 252B	0.000	0.000	0.000	0.000	0.000	0.267	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
NEW 253	33.569	7.035	7.116	25.624	0.000	0.000	0.000	0.867	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Tri 253S	0.000	0.940	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,6,8-I 253H	0.000	0.000	0.328	1.095	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.990
Pyr 253I	0.000	0.000	0.000	0.000	0.167	0.233	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pip 253J	0.000	0.000	0.000	0.000	0.000	0.633	2.467	7.500	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pip 253U	0.000	0.000	0.000	0.353	0.000	0.000	0.000	0.000	0.000	0.000	0.293	0.000	0.000	0.000	0.000	0.000
SpiroP 254	0.000	0.000	0.000	0.000	0.000	0.267	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,8-I 257C	0.000	0.000	0.000	0.000	0.000	0.000	0.233	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1,4-Q 257D	0.000	0.000	0.000	0.000	0.000	1.167	2.867	2.333	0.544	0.000	0.000	0.409	0.000	0.000	0.000	0.000
5,8-I 259B	2.168	0.000	2.130	1.337	0.000	0.000	0.000	0.000	0.880	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,8-I 261D	11.159	4.505	0.000	0.943	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Tricyclic 261F	0.000	0.000	0.000	0.000	0.800	7.733	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,6,8-I 263A	45.248	57.340	3.957	0.578	0.000	0.000	0.600	0.967	37.501	17.310	11.721	21.092	0.000	0.000	0.297	0.407
PTX 265D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.533	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3,5-P 265J	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	3.194	0.000	0.000	0.000	0.000	0.000
5,8-I 267G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.798	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pip 267K	0.000	0.000	0.000	0.000	0.000	0.000	0.433	0.967	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
DHQ 269B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.389	0.000	0.000	0.000	0.000	0.000	0.000	0.000
DHQ 269AB	60.211	37.697	24.233	57.845	0.000	0.000	0.000	0.000	7.341	0.000	0.000	1.818	0.000	0.000	0.000	0.000
Pip 269C	0.000	0.000	0.000	0.000	0.000	0.800	0.567	1.433	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Dehydro-5,8-I 269D	1.995	5.371	4.039	0.000	0.000	0.000	0.000	0.000	10.782	5.224	3.199	5.723	0.000	0.000	0.000	0.000
5,8-I 271A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	4.366	2.908	1.789	2.180	0.000	0.000	0.000	0.000
Unclassifiable 271E	7.049	0.000	8.681	3.357	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,8-I 273B	0.000	1.946	0.000	0.000	0.000	0.000	0.000	0.000	3.125	1.397	1.065	0.297	0.000	0.000	0.000	0.000
Unknown 275	0.000	0.000	11.640	1.446	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Lehm 275A	10.582	1.625	25.426	19.023	0.167	0.333	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3,5-I 275C	0.000	1.397	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.623	2.397	34.541	0.000	0.000	0.000	0.000
5,6,8-I 275E	8.667	18.528	1.623	0.000	0.000	0.000	0.000	0.167	8.036	4.919	2.800	5.124	0.000	0.000	0.000	0.000
Lehm 275G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.094	3.301	2.826	110.514	0.000	0.000	0.000	0.000
Lehm 277A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	12.069	0.000	0.000	0.000	0.000
1,4-Q 277D	0.000	0.000	0.000	0.000	0.133	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,6,8-I 277E	10.096	70.015	5.367	0.994	0.000	0.000	0.833	1.000	40.456	26.108	13.102	19.527	0.000	0.000	0.000	0.000
5,6,8-I 279F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.688	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Unclassifiable 279I	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.649
HTX 283A	3.665	2.878	0.000	0.000	0.000	0.000	0.000	0.000	1.969	0.000	0.000	0.000	0.000	0.000	0.000	0.000
HTX 285A	4.667	17.960	0.000	0.000	0.000	0.000	0.000	0.000	1.855	0.000	0.000	0.000	0.000	0.000	0.000	0.000
HTX 287A	0.000	8.567	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
HTX 291A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	12.913	1.052	0.000	0.000	0.000	0.000
Izidine 291B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.132	0.000	0.000	0.000	0.000
Lehm 291F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.596	0.000	0.000	0.000	0.000
PTX 291G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.005	0.000	14.869	0.000	0.000	0.000	0.000
DHQ 293A	0.000	0.000	0.248	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5,6,8-I 293C	0.000	13.563	0.000	0.000	0.000	0.000	0.000	0.000	20.836	12.858	8.152	12.392	0.000	0.000	0.000	0.000
PTX 293E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.685	2.351	0.454	1.116	0.000	0.000	0.000	0.000
Lehm 293I	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	4.209	0.000	0.000	4.485	0.000	0.000	0.000	0.000
aPTX 305A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.926	0.000	3.920	4.328	0.000	0.301	0.000
Unclassifiable 305H	0.000	0.000	0.000	0.000	4.600	12.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PTX 307A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	51.860	33.034	6.731	26.254
PTX 307F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	5.416	0.000	0.637	5.859
PTX 307G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	13.874	0.000	0.000	0.517
PTX 309A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.430	0.000	11.410	0.000	0.000	2.799
PTX 309C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.682	0.000	0.000
aPTX 309D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	28.663	12.609	5.538	11.607	0.000	0.000	0.000	0.000
PTX 321A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	12.368	3.021	0.925	4.287
PTX 323A	0.000	9.267	0.000	0.000	0.000	0.000	0.000	0.000	3.221	2.825	1.333	2.025	127.768	27.892	3.557	75.222
PTX 323A (artifact)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	4.537	1.491	0.000	2.945
aPTX 323B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.624	0.000	0.000	0.000	0.000
Unclassifiable 323I	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.229	0.000
aPTX 323J	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	9.388	6.071	4.812	2.355	19.005	6.633	0.000	0.000
aPTX 325A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	5.575	1.283	0.883	3.005	5.950	0.000	0.000	0.000
aPTX 339A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.415	0.000	0.000	0.000
Unknown A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.244	0.313	0.000	1.435	0.000	0.000	0.000	0.000
Unknown B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.078	0.000	0.000	0.000	0.000
Unknown C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.600	0.000	0.000	0.000	0.000
Unknown D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.499	0.000	0.000	0.000	0.000
Unknown E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.364	0.000	0.000	0.000	0.000
Unknown F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.572	0.000	0.000	0.000	0.000
Pip NEWA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.302	0.000	0.000	0.000	0.000	0.000
# individuals	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

Chapter 4

GENETIC STRUCTURE UNCOUPLED FROM ANTI-PREDATORY PHENOTYPES IN AN APOSEMATIC FROG

Abstract

Reduced gene flow and reproductive isolation can arise between adjacent populations if selection acts on phenotypic traits that serve as signals for predator avoidance and mate choice. Thus the role of selection in driving early stages of differentiation can be inferred from patterns of admixture and connectivity between phenotypically divergent populations of species like the poison frog *Oophaga pumilio*, which exhibits a geographic mosaic of aposematic color pattern variation thought to be under natural and sexual selection. Here we use cline analyses to investigate fine scale genotypic and phenotypic transitions across a region of Panama where red and blue colored populations of *O. pumilio* are divided by a polymorphic population exhibiting a continuum of red to brown to blue coloration. Our aim was to assess the extent to which selection acts to maintain color pattern variation in *O. pumilio*. We found that genotypic clines and clines of aposematic phenotypic traits are not concordant nor coincident, suggesting that natural selection does not govern population genetic structure across color

pattern transitions. Consistent with this, selection coefficients ranging from 0.003-0.26 (min/max) estimated from clinal structure suggest that natural selection is weak. Our findings complement recent predation studies which have shown no fitness costs associated with immigrant inviability, and demonstrate how weak selection can permit phenotypic divergence in the Bocas del Toro archipelago.

Introduction

Poison frogs (family Dendrobatidae) have emerged as a model system for studying the mechanisms responsible for rapid phenotypic divergence driven by natural and sexual selection. Many poison frogs are aposematic prey species that exhibit warning coloration that is associated with diet-derived alkaloid (Saporito et al. 2009) defensive traits and that can be utilized in mate assessment (Cummings and Crothers 2013). As in some other aposematic groups (e.g., *Heliconius* butterflies), the striking diversity in color and pattern polytypism in some poison frogs (e.g. *Dendrobates tinctorius*) has been shown to contribute to prezygotic isolation by means of immigrant inviability, where natural selection favors local over non-local phenotypes (Noonan and Comeault 2009). Locally adapted populations of sympatric co-mimic sister species are very common in some genera, like the genus *Ranitomeya* (Symula et al. 2001, Yeager et al. 2012, Twomey et al. 2014). Of the polytypic poison frog species, *Oophaga pumilio* exhibits an exceptionally high number of distinct phenotypes (>16), and thus has been of long-standing interest as a model for research on evolutionary and ecological mechanisms of phenotypic divergence (Daly and Myers 1967).

The impressive phenotypic diversity in *O. pumilio* appears to have evolved rapidly, and phenotypes are thought to reflect natural and sexual selection (Summers et

al. 1997, Brown et al. 2010). Divergent *Oophaga pumilio* phenotypes occur with varying levels of geographic isolation, ranging from contiguous Panamanian mainland populations to insular populations across the Bocas del Toro archipelago (Wang and Shaffer 2008). Female mate choice experiments have demonstrated that females often prefer their own phenotype (Summers et al. 1999, Reynolds and Fitzpatrick 2007, Maan and Cummings 2008, Richards-Zawacki and Cummings 2011), although captive *O. pumilio* from different islands will readily breed and produce viable offspring (Summers et al. 2004) that do not exhibit intrinsic post-zygotic incompatibilities (Dugas and Richards-Zawacki 2015). Similarly, field-scale predation experiments have increasingly demonstrated inconsistent support for natural selection acting on color pattern in *O. pumilio* (Hegna et al. 2013, Richards-Zawacki et al. 2013, Dreher et al. 2015, Yeager et al. *in review*), possibly because selection pressures are much weaker than expected (Yeager et al. *in review)*.

As experimental assays of selection do not always reflect natural conditions (Richards-Zawacki et al. 2012), assessments involving other complementary approaches could offer novel perspectives on the extent to which selection contributes to color pattern differentiation in *O. pumilio.* Other approaches could also afford opportunities to assess alternative hypotheses. For example, coupled drift has been proposed as an alternative to a selection-based theory explaining phenotypic divergence, where sexual selection initially results in phenotypic divergence between populations, which continues via drift (Tazzyman and Iwasa 2010). In this study, we investigated clinal variation between *O. pumilio* populations from the Bocas del Toro region of Panama (Figure 1) that encompass a phenotypic transition between two monomorphic red regions (Isla San

Cristobal Island and mainland Almirante), and a monomorphic blue region (Aguacate Peninsula) that are separated by a polymorphic region (Aguacate Peninsula). We used clinal patterns of naturally occurring variation in *O. pumilio* as a lens for examining genotypic and phenotypic divergence and to infer the strength of selection under conditions of gene flow.

Oophaga pumilio in the Bocas del Toro archipelago and adjacent mainland regions have diverged from an ancestral red-bodied phenotype to a diverse compliment of distinct phenotypes (Wang and Shaffer 2008) distributed across islands and the mainland (Yeager et al. *in review*). However, based on mitochondrial DNA (mtDNA) sequence variation, Hagemann and Pröhl (2007) suggested that there are only three distinct lineages of *O. pumilio*: a Northern (Costa Rican), Southern (Bocas del Toro region of Panama), and Eastern (Isla Escudo de Veraguas, Panama). Other genetic evidence suggests that multiple colonization events likely occurred into the Bocas del Toro archipelago from mainland Panama, likely originating from populations in Southeast Costa Rica (Wang and Shaffer 2008). The northern Aguacate peninsula also appears to represent a region of genetic transition or vicariance between 'Northern' and 'Southern' lineages of *O. pumilio* (Hauswaldt et al. 2011). Frogs from both the nearby Isla San Cristobal to the North East, as well as much of the populations from the mainland to the West of the polymorphic population display red bodies with varying degrees of blue on the legs. Rudh et al. (2007) found evidence of genetic clustering based on amplified fragment length polymorphisms, where Cerro Brujo (Aguacate Peninsula) and nearby Isla San Cristobal form a genetic cluster together relative to other Bocas del Toro archipelago populations. Based on coalescent analysis of the mtDNA d-loop region,

Brown et al. (2010) also showed a lack of reciprocal monophyly for Aguacate populations, with some individuals placed ancestral to a derived clade containing Almirante and San Cristobal populations and other Aguacate individuals.

The phenotypic polymorphism and lack of resolution in the ancestry of populations in the Aguacate region offers an enticing opportunity for further investigation of genotype-phenotype relationships, including whether color variation is governed by selection. By comparing multilocus genotypic (i.e., admixture frequencies) and multiple phenotypic (i.e., dorsal and ventral coloration) clines across the Aguacate region, we tested the prevailing hypothesis that concordance and coincidence should occur when multiple forms of selection are acting on phenotypic signals (Cummings and Crothers 2013) . The coincidence, concordance, stability and slope of clinal transitions (i.e., tension zones) have been widely used to infer the nature and strength of selection as well as isolating barriers (Barton and Hewitt 1985), including in aposematic species like *Heliconius* butterflies (Mallet and Barton 1989, Mallet et al. 1990, Blum 2002, Rosser et al. 2014). With reference to prior work on *O. pumilio* and hybrid zone theory, we 1) assessed the extent to which neutral genetic structure corresponds to the distribution of phenotypic variation across the Aguacate transition zone; 2) assessed the extent to which the structure of genotypic and phenotypic clinal transitions is governed by selection (i.e., clines are steep, coincident and concordant); and lastly 3) inferred the strength of selection according to the structure of the Aguacate transition zone. Doing so offered novel and independent perspectives on the origins and maintenance of phenotypic differentiation in *O. pumilio*, a species that has been become central to understanding rapid divergence and outcomes of natural and sexual selection.

Materials and Methods:

Specimen collections: Across the phenotypic transition 16-22 individuals from 14 locations were collected, spaced ~250 meters wherever suitable habitat was identified. Samples from six potential red parental populations (three Almirante, three Isla San Cristobal) were additionally collected in close proximity to the phenotypic transition, as well as six monomorphic blue locations throughout the Aguacate Peninsula, and additional three additional nearby locations that differ in color pattern (Figure 1, sites 23-25). Tissue samples (toe-clips) were collected from 491 individuals from 30 sampling locations (Figure 1, Table 1) between June 2009 and December 2012 and preserved in salt-saturated dimethyl sulfoxide (DMSO) and ethylenediaminetetraacetic acid (EDTA) solution at room temperature for up to three months prior to DNA extraction.

Phenotypic trait measurements: Background coloration within the study area included all shades along a continuum from red to brown to blue. At all sampled localities, dorsal background coloration is uniform within an individual, though individuals differ in the extent of dorsal black spotting. A few sampled populations (locations 24 and 25) were distinct, with frogs being white with large black dorsal blotches (Figure 1). For all individuals, dorsal phenotypes were quantified by eye using a scale from 0 to 5 (0 = blue, 1 = blue/brown, 2 = brown, 3 = red/brown, 4 = red). Ventrally, frogs in the study were either a single uniform color, or had a mottled venter comprised of red and blue patches of color. A separate scale (0 to 4) based on proportional composition of red and blue was used to score ventral coloration: 0 = entirely blue; 1 = 75% blue; 25% red, 2 = 50% blue, 50% red; 3 = 75% red, 25% blue; 4 = entirely red). These dorsal and ventral phenotypic scores were plotted along transects to visualize phenotypic clines.

For a subset of frogs (n=277, Table 1), which included both putative parental populations and some frogs from the phenotypic transition area, color photographs were taken on a standardized (Rite in the Rain® paper) background in the field using a Canon 7d DSLR camera with a Canon MR-14 ring flash. Images were saved in the RAW image file format. For these individuals, RGB values were extracted from photographs to further quantify phenotypes. We began by cropping a 252x252 pixel square of the dorsal and ventral (dominant, and minority colors if present) color and processing each square using the ImageJ macro Batch RGB (Abràmoff et al. 2004). Melanistic color pattern elements and white regions attributable to flash were avoided when choosing the square for RGB for measurement. We used a principal components analysis (PCA) to reduce R, G, and B values to uncorrelated composite variables, extracting each PC with eigenvalue > 1 for dorsal and ventral coloration separately, the resulting PC1 values were used for dorsal and ventral cline analyses to afford higher resolution of phenotypic variation (Table 2). We analyzed each as a separate cline because dorsal coloration is thought to be under sexual and natural selection whereas ventral coloration may be more important to sexual selection (Maan and Cummings 2008, Cummings and Crothers 2013) To ensure that phenotypic categorical scores tracked RGB values in evaluating phenotypic variation, we plotted PC values according to phenotypic categorical scores (Figure 2).

Microsatellite genotyping: We genotyped 491 *O. pumilio* individuals from 30 localities (Figure 1, Table 1) across the Aguacate region. Each individual was genotyped at 12 highly variable microsatellite loci previously developed for *O. pumilio* (Hauswaldt et al. 2009, Wang and Summers 2009). Genomic DNA was extracted from toe-clips using the Qiagen DNeasy® Blood and tissue kit (Qiagen, Valencia, CA, USA) following the

protocol for animal tissue. The following microsatellite loci were amplified for each specimen using polymerase chain reactions (PCR): the dinucleotide Oop_O1 (Hauswaldt et al. 2009), the trinucleotide Dpum92 (Wang and Summers 2009), and the tetranucleotide Dpum44, Dpum110 (Wang and Summers 2009), Oop_G5, Oop_C11, Oop_E3, Oop_F1, Oop_B8, Oop_B9, Oop_C3 and Oop_D4 (Hauswaldt et al. 2009). Amplifications were done in 10 μL reaction volumes that included 4 μL GoTaq Green Master mix® (Promega, Madison, WI, USA), and 0.5 μL of a 10uM solution with each of the forward and reverse primers. Reactions with the Hauswaldt et al. (2009) primers contained 1 μL of undiluted genomic DNA, whereas reactions using the Wang and Summers (2009) primers contained 1.5 μL undiluted template DNA plus 1 μL of 2.5 mM $MgCl_2$. Thermal cycling conditions followed Hauswaldt et al. (2009) for the Oop primers, except we used an annealing temperature of 55°C. For the Dpum primers, reaction conditions followed Wang and Summers (2009) except we used an annealing temperature of 52°C for Dpum44 and 60°C for Dpum110. PCR products were characterized on an ABI 3730xl (Applied Biosystems Inc., Forest City, CA) and scored using GeneMarker v1.90 (Softgenetics, State College, PA) against a LIZ 500 size standard (Applied Biosystems®, Waltham MA). Allele calls were confirmed and any deviations were accounted for in further analyses of genotype frequencies. We tested for Hardy-Weinberg equilibrium (HWE), calculated allele frequencies, observed (H_O) and expected (H_E) were estimated in Arlquin (Excoffier et al. 2005, Table 3).

Analysis of multilocus admixture and genetic differentiation: We used the program STRUCTURE v. 2.3.4 to assess population genetic structure and genotypic admixture according to geography and phenotype (Pritchard et al. 2000). Five independent

preliminary STRUCTURE runs were performed with the full data set, where each run consisted of 30,000 burn-in steps and 3,000,000 data collection steps with K values ranging from 1 to 15. To visualize population structure we used the main pipeline from CLUMPAK (http://clumpak.tau.ac.il/index.html). This analysis indicated that the most likely number of genetic clusters supported by the data set (K) was two, which roughly separated individuals and populations according to putative parental phenotype across the transitional zone (Figure 3). To identify the origin of the red phenotype in the transitional region we ran additional, separate analyses with a reduced data set that included the parental red population from either the mainland (Almirante) or insular (San Cristobal) region, which also excluded peripheral populations that exhibited other phenotypes (e.g. black/white and blue/green). For each of these analyses, we performed five replicate STRUCTURE analyses, each time allowing K to vary from 1 to 5, using 30,000 burn-in and 3,000,000 data collection steps. These analyses indicated that the most likely number of genetic clusters supported by the data sets (K) was four, which identified the insular San Cristobal populations as more genetically similar to populations in the polymorphic region of interest. We therefore used San Cristobal populations to anchor our cline analyses as the parental red phenotype (described below), where individuals with posterior probability assignments >90% from the average of five STRUCTURE runs were assigned to one of the four genetic clusters identified with the reduced data set and where all others were classified as admixed individuals (Figure 4).

Clinal variation in genotypic and phenotypic traits: We used the R package *HZAR* (Derryberry et al. 2013) to describe clinal transitions in the frequency of multi-locus genotype, dorsal phenotype, and ventral phenotype across the Aguacate transect anchored

with the insular San Cristobal populations. Genotypic clines were built using the average admixture frequencies from five independent STRUCTURE runs, including all populations from the San Cristobal parental red transect. Linear distances were generated by HZAR using the northernmost population on San Cristobal as the terminus of the cline. Phenotypic clines built from PC values were constructed using the same locality distances.

HZAR fits cline models to both molecular and phenotypic traits utilizing Metropolis-Hastings Monte Carlo Markov Chain (MCMC) algorithms by applying likelihood function tests for alternative cline model shapes. The Autofit feature in HZAR automates model selection between 10 quantitative trait models options for genotypic and phenotypic clines that vary in scaling (fixed, free and none) and tail options (left, right, mirror, both and none) to describe the shape of clinal transitions using Akaike's Information Criterion (AIC, Akaike 1973). Though the majority of transition within traits occurs over the width of the cline, some transition continue past the width into the tails, which can indicate a breakdown in the strength of selection approaching parental populations. "Left" or "right" tail models indicate gene flow higher in the direction of one tail than predicted by cline width, where as "mirror" tail models have identical tail shape. "Both" tail model refers to two independent tail shapes and "none" tail model dictates tails do not differ from the sigmoidal transition cline. Maximum likelihood cline profiles are used to select the cline model shape, and confidence intervals and likelihood profiles are used to assess coincidence and concordance between genotypic and multiple phenotypic clines.

Model fits for genotypic admixture transects were tested against the null model of no change across the transect. Models were accepted with AIC scores within 2 units of the lowest AIC value (AIC= -2(logLik)+2K). Cline center and width support estimates were then generated in HZAR from the set of MCMC clines within 2 log likelihood units of the maximum likelihood. Cline concordance and coincidence were compared between traits (admixture genotypes, dorsal phenotype and ventral phenotype) by comparing relative AIC values (ΔAIC to the minimum AIC) of the likelihood profile where if the cline models selected for each given value vary by ≥2 AIC values those clines are classified as non-coincident (cline centers) or discordant (cline widths) (Burnham & Anderson 2002, Anderson 2008).

Selection coefficients: We estimated the strength of selection in maintaining clinal transitions by calculating selection coefficient (*s*) where:

$$w = \sqrt{\frac{8\sigma^2}{s}}$$

Following Barton and Hewitt (1985), this estimation takes into account the width of the cline (*w*) as well as linear inter-generational dispersal distances (σ^2). In the laboratory, sexual maturity (~19 mm SVL, Donnelly 1989) in *O. pumilio* takes place in approximately 1 year (JY *pers. obvs.*), during which time chemically defended juveniles likely disperse in the wild. Because nothing is known about juvenile dispersal and no dispersal distances are available for *O. pumilio*, we used two different values from related poison frogs: *Allobates femoralis,* which has an average annual adult dispersal of 17.8 m (Ringler et al. 2009); and *R. imitator,* for which a generational dispersal of 97 m has been estimated (see Twomey et al. 2014 supplementary materials). We note that juvenile

anuran dispersal may be significantly higher than adults (Berven and Grudzien 1990) and because no juvenile poison frog dispersal estimates are available we acknowledge this may result in underestimation of selection strength.

Results

Phenotypic traits: Dorsal coloration PC 1 and 2 explained 99.65% of variation, with PC1 explaining over 90% of variation (Figure 2). Dorsal coloration PC1 was primarily negatively driven by red variation, where PC2 is positively associated with blue and green. Principal components 1 and 2 explained 99.15% of ventral color variation with PC1 alone explaining 68%. Ventral coloration PC1 is negatively driven by red and positively by blue, whereas PC2 is positively associated with red and green.

Genotypic and Phenotypic clinal variation: As already noted, Bayesian assignment analyses recovered evidence of population structure and admixture on multiple levels, with both individuals and populations corresponding to hierarchically nested groups (Figure 2). Multilocus admixed frequencies had an estimated center of 9.56 km from the transect terminus, and exhibited a steep clinal transition with a width of 0.08 km (Table 4). The cline exhibited no difference in sigmoidal shape past the estimated width of the center of the cline into the tails, and the tale shape on both sides of the transition were symmetrical, though not identical. Higher levels of population-level admixture were found in parental red San Cristobal populations where our sampling failed to fully capture the far left side of the transition as compared to populations on the side across the Aguacate Peninsula (Figures 5,6).

Dorsal and ventral phenotypic and PC clines consistently exhibited steep transitions. Clines generated from categorical phenotypic scores exhibited steep

transitions with widths of 0.59 km and 0.34 km, respectively, and had estimated centers located at 7.55 km and 7.34 km, respectively (Table 4, Figure 5). The shapes of their tails did not depart from a sigmoidal transition, similar to the genotypic cline. The dorsal and ventral phenotype clines reflecting PC1 scores exhibited a width of 2.87 km and 2.61 km, respectively, and had estimated centers located at 6.84 km and 5.02 km, respectively (Figure 6). Ventral PC1 cline retained similar model shape to all previous cline models with a sigmoidal shape and symmetric tails. Dorsal PC1 cline shape differed with a fixed model selected, and had independent tail shapes with a slower transition on the left side of the cline in the red/polymorphic regions than blue regions.

Comparison of Genotype and Phenotypic clines: Dorsal and ventral phenotypic clines estimated categorically and using PC1 scores were not coincident or concordant (Figure 4). Corresponding phenotypic clines (e.g., categorical dorsal to PC1 dorsal) consistently differed in center location and width (Figures 6, 7). Similarly, corresponding phenotypic clines estimates using each approach (e.g., PC1 ventral to PC1 dorsal values) were not coincident or concordant (Figure 6), indicating that phenotypic variation in these traits may not be tightly linked. We also found that the genotypic cline was not coincidence or concordant with any of the phenotypic clines (Table 4). The genotypic cline is offset and remarkably narrower than any of the phenotypic clines, suggesting that phenotypic transitions do not directly influence the location of genotypic transitions, and that stronger selection maintains genetic boundaries than phenotypic boundaries.

Estimation of Selection: All quantitative traits had small selection coefficients, though the range of estimates reflected the dispersal distance used (reported as low/high dispersal estimate based on values from *A. femoralis* and *R. imitator*). Coefficients for categorical

dorsal and ventral phenotype scores (0.003/0.094 dorsal and 0.004/0.124 ventral, Table 4) were lower than coefficients for genotypic scores (0.009/0.257). Coefficients for phenotypic PC1 scores were even lower (dorsal <0.001/0.043, ventral <0.001/0.045).

Discussion

Explaining the rapid phenotypic differentiation of *O. pumilio* across the Bocas del Toro archipelago and nearby mainland remains an elusive goal, with each successive study revealing more about the complexity of the region. Examining naturally occurring phenotypic variation across the Aguacate peninsula, we applied hybrid zone theory and cline analysis to gain valuable insights into relationships between genotype and phenotype and the strength of selection under conditions of gene flow. We did not find the predicted pattern of clinal concordance and coincidence expected under conditions of strong selection. Rather, our analyses indicate that the genotypic transition across the peninsula is off center from phenotypic transitions and that the genotypic transition is much sharper than phenotypic transitions, indicating that genetic boundaries among color morphs are not solely a reflection of phenotypic variation. We also found evidence indicating that selection is much weaker than has been thought. Selection coefficients estimated from the structure of phenotypic and genotypic clines are remarkably low, particularly in comparison to coefficients estimated for other aposematic species, such as *Heliconius* butterflies (Benson 1972, Mallet 1986, Mallet and Barton 1989, Mallet et al. 1990, Kapan 2001), which are known to exhibit color pattern variation shaped by strong natural selection and sexual selection.

We found that all clines exhibited steep transitions, which suggests that strong selection is shaping boundaries across the geographic mosaic of aposematic polytypism

in *O. pumilio*. Considering the low selection coefficients that were estimated, however, it is likely that limited dispersal is contributing to the sharp transitions observed in the Aguacate region. Though dispersal distances are not known for *O. pumilio*, the species is known to maintain small, well defended territories (Pröhl and Berke 2001), and other poison frogs exhibit remarkably low dispersal distances (Ringler et al. 2009; Twomey et al. 2014). We also found much narrower cline widths for *O. pumilio* (Table 4) compared to other species in Panama for which cline attributes have been estimated (e.g., manakins, Brumfield et al. 2001), including *Heliconius* butterflies that are under strong selective pressure (Mallet 1986, Blum 2002). The classic *Bombina* toad hybrid zone, which encompasses much wider clines (~6 km; Szymura 1993) than those estimates here, offers an additional point of comparison, as *Bombina* toads are much more vagile than dendrobatids (i.e., *Bombina* exhibit generational dispersal distances of ~430 m).

Cline width would be expected to be wider under a neutral diffusion model (Endler 1977). Using the same dispersal estimates as in our selection coefficient, and assuming ~1000 years since contact between Isla San Cristobal and the mainland (Anderson and Handley 2002) we would estimate neutral diffusion cline widths to range from 1.41-7.70 km. Even halving the time since last contact (500 years) would produce a minimum estimated neutral diffusion cline width of 2.00 km, where our genotypic/phenotypic clines are steeper and range from 0.08-0.59 km.

Steep clines can also be associated with habitat transitions that may inhibit gene flow between ecologically adapted populations (Jiggins et al. 1997, McMillan et al. 1997, Blum 2008). In European wood tiger moths, for example, thermal environment structures the signal divergence between aposematic coloration and melanization, which

influences thermoregulation (Hegna et al. 2013). In *O. pumilio*, differences in aggression and foraging behaviors have been found between geographically and phenotypically distinct populations (Rudh et al. 2013) that might be attributable to habitat differences. However, a predation experiment found that attack frequencies between phenotypically different frogs were not associated with differences in forest irradiance, which is a proxy measure of canopy cover (Yeager et al., *in review*). Clinal frequency transitions might also be influenced by mating behaviors that are contingent on habitats. For example, the outcome of male/male interactions and the establishment of territories could be contingent on conspicuous (versus cryptic) individuals being more prone to agonistic interactions in certain environments (Rudh et al. 2013). Further work that clarifies whether predation risk or behavior varies by microhabitat would in turn clarify whether clinal transitions track environmental or ecological gradients.

The low selection coefficient values estimated for *O. pumilio* reinforce inferences from field predation studies that selection is weak. Estimates of selection from predation studies are often considered with suspicion because overall attack frequencies are typically very low (e.g., 7.9-14.8%, Yeager et al. *in review*). Low selection coefficients are consistent, however, with evidence that attack frequencies by wild predators do not differ between frog phenotypes (Yeager et al. *in review*) and that divergent frog phenotypes do not affect the rate of naïve predator education or predict predatory behavior towards novel phenotypes (Yeager et al. *in review*). Nonetheless, our estimates require further confirmation because we calculated coefficients partly based on dispersal distances derived from other species. Accordingly, further work on adult and juvenile dispersal distances in *O. pumilio* would likely yield more precise estimates of selection.

Evidence of weak selection and displacement suggests that genotypic and phenotypic clines at color pattern boundaries in *O. pumilio* may be unstable and prone to movement. Though all of the observed cline transitions were steep, suggesting sharp genotypic and phenotypic segregation occur over a small geographic range, we found that the center of the genotypic cline is displaced into the region dominated by frogs exhibiting a blue phenotype indicating that 'red frog' genotypes have introgressed into monomorphic blue populations. Displacement and introgression can be due to greater permeability of hybrid zones to neutral traits (e.g., microsatellite loci), but the observed asymmetry across the Aguacate zone suggests that other factors are structuring gene flow among neighboring populations. It is possible, for example, that the observed pattern is a reflection of genetic asymmetries, such as dominance drive (Mallet 1986, Blum 2002), or that 'red frog" genomic attributes confer some sort of selective advantage. If so, then the genotypic cline could be highly mobile (e.g., Blum 2002), where elements of the 'red frog' genome would introgress deeper into the Aguacate peninsula, resulting in greater genotype/phenotype disjunction.

Though strong selection often serves as the most parsimonious explanation of phenotypic divergence, our findings bolster other evidence from recent studies that does not provide support for strong selection acting on *O. pumilio* (e.g. sexual selection: Richards-Zawacki et al. 2012, natural selection: Dreher et al. 2015). Though it remains possible that the geographic mosaic of color variation in *O. pumilio* is governed by selection (i.e., selection could be acting in a manner that is not readily detected by clinal or experimental analysis), our findings open the door to considering possible alternative

mechanisms, such as drift, that could structure genetic variation and differentiation among populations.

Our estimates of weak selection may only reflect one form of selection, and may not fully capture the full consensus of natural and sexual selection, or all the potential interactions between natural selection and sexual selection. It is also possible that as an artifact of the regions we sampled, natural and sexual selection could oppose, and cancel one another out, leading us to estimate low selection. Alternatively, we may have identified an example of stochastic selection tied to extrinsic factors where environmental components could serve as importance independent driver of divergence.

To more comprehensively assess sexual selection contemporary pedigree analyses coupled with mate choice trials from the region could provide an estimate of the strength of mating preferences, and most importantly mate choice decisions (Richards-Zawacki et al. 2012). Weak preferences or a lack of assortative mating, coupled with our findings, would support coupled drift (Tazzyman and Iwasa 2010) as an alternative hypothesis explaining phenotypic divergence rather than a strong selection hypothesis.

Figure 4-1: Locations sampled. Pie charts represent the frequency of frog coloration for each locality. Left inset shows study area within Bocas del Toro archipelago and adjacent mainland; right inset shows sampling along the phenotypic transition in the area of Dolphin Bay. Scale bar is 5KM

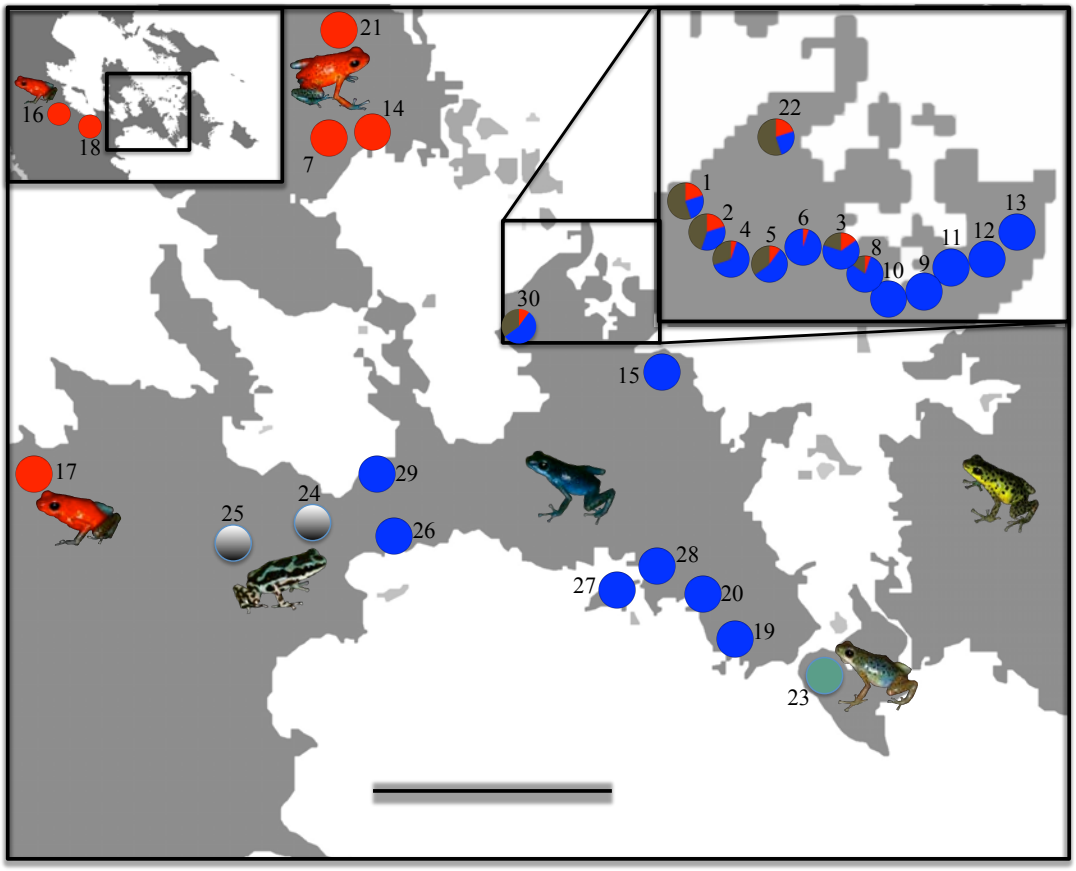


***Figure 4-2**:* Plot of RGB Principal Components (PC1 x PC2) sorted by Phenotype Category scores (0-4, see methods).

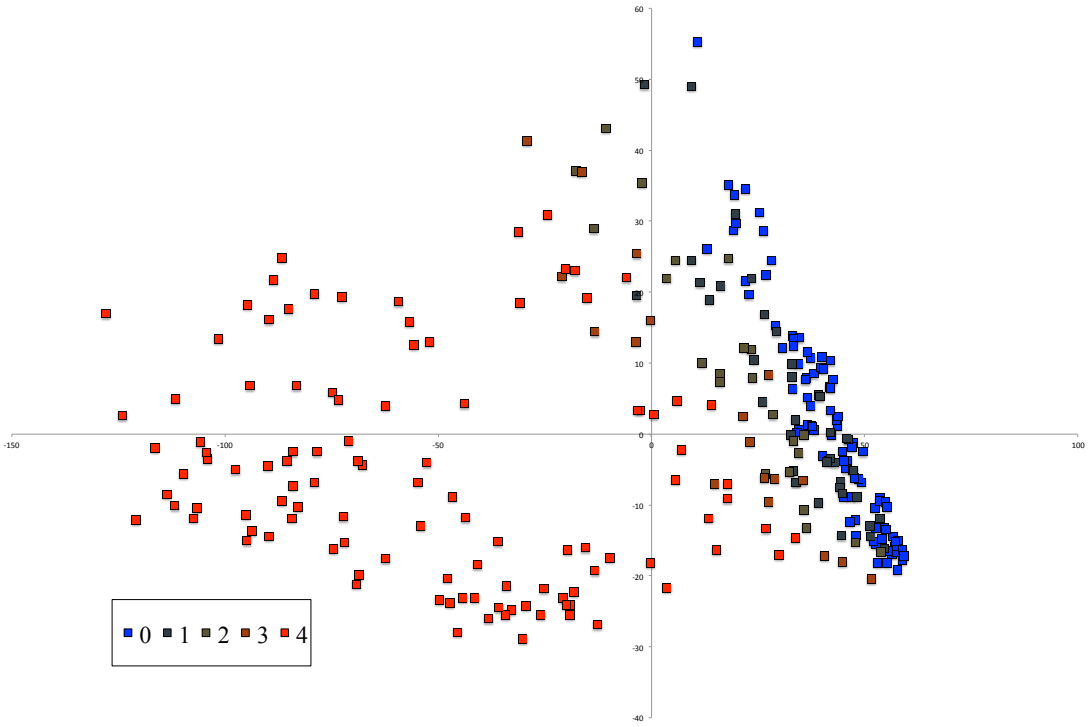


Figure 4-3: Structure results averaged from five independent runs. Probability for each K1-15 run (top) and genetic clustering across the San Cristobal reduced data set with rough phenotypic groupings including San Cristobal (parental red), samples across the phenotypic transition (brown), monomorphic blue populations within the transect (transitional blue) and Aguacate Peninsula populations (parental blue).

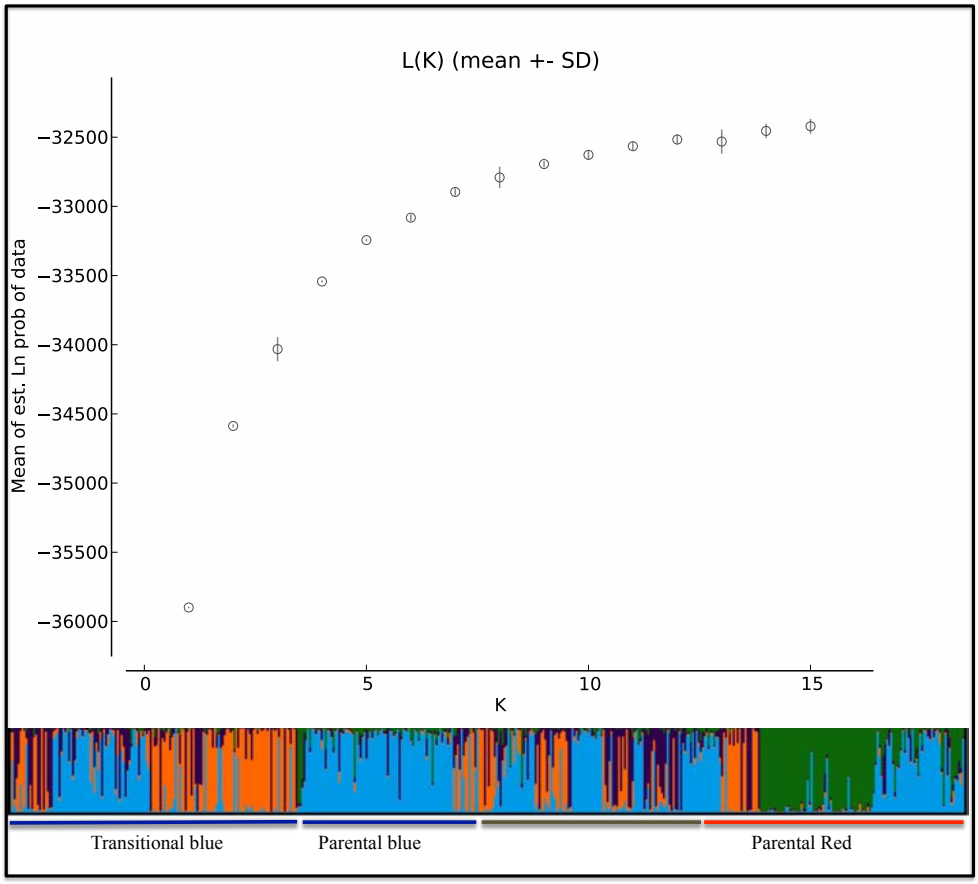


Figure 4-4: Frequency of dominant genotypes at each locality based on STRUCTURE assignments (K=4). Brown represents admixed individuals and blue, purple, orange, and green represent individuals assigned to one of four genetic clusters from STRUCTURE. Scale bar is 5KM.

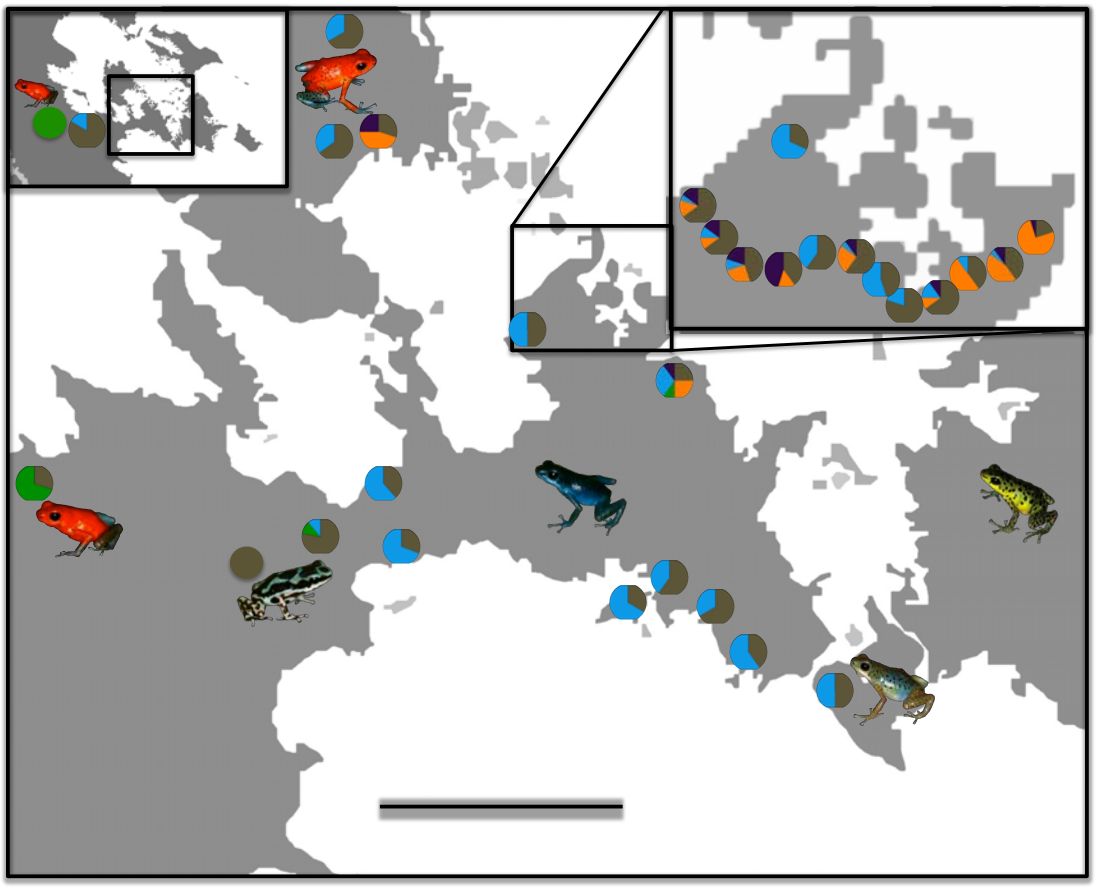


Figure 4-5: Clinal transition for admixed genotype and phenotypic categorical scores for dorsal and ventral phenotypic clines. The far left side of the cline refers to populations from Isla San Cristobal (populations 7, 14, 21), the far right side of the cline shows populations on the far side of the Aguacate Peninsula (populations 19, 20).

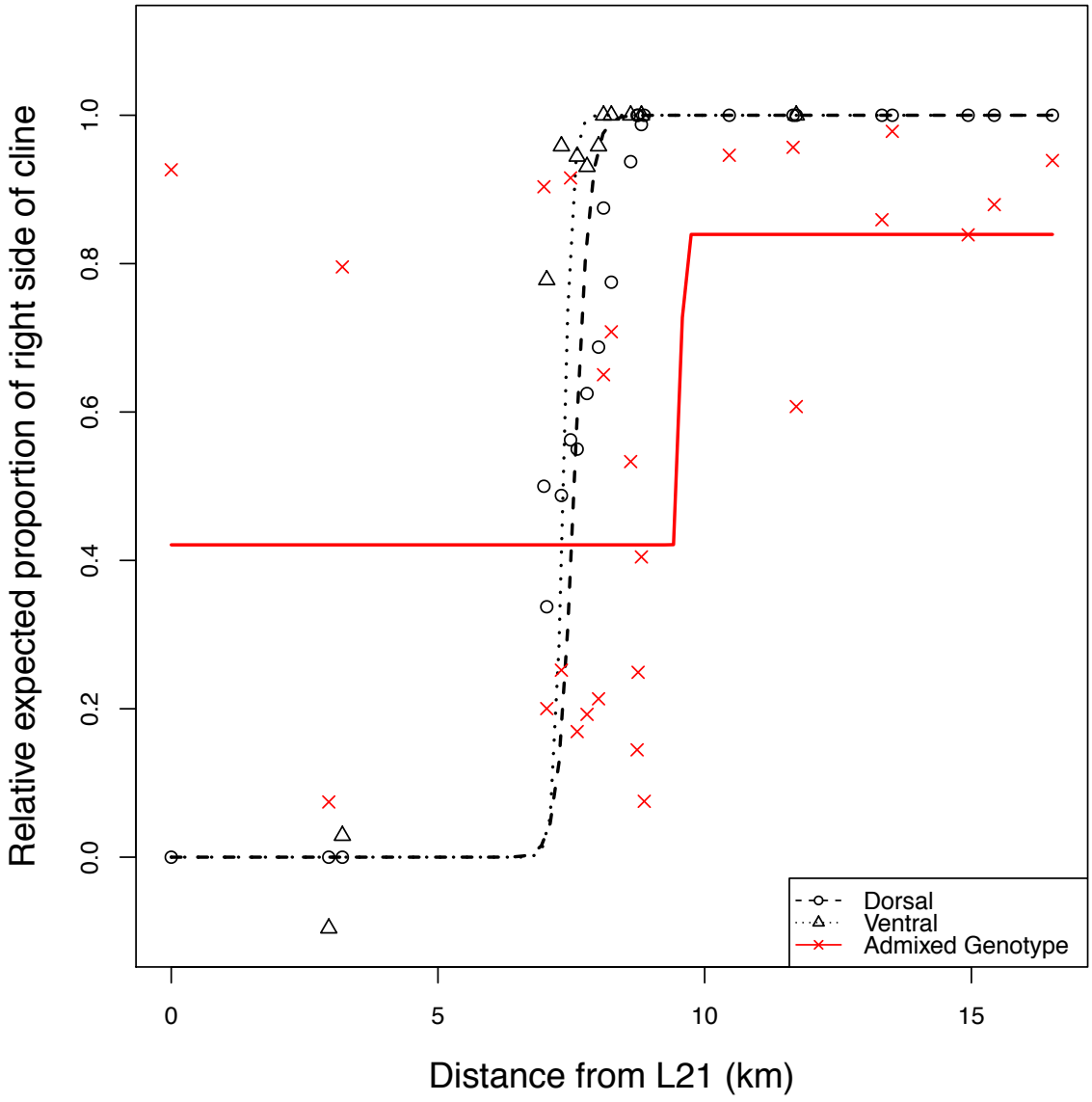


Figure 4-6: Clinal transition for admixed genotype and phenotypic RGB PC1 values dorsal and ventral clines. The far left side of the cline refers to populations from Isla San Cristobal (populations 7, 14, 21), the far right side of the cline shows populations on the far side of the Aguacate Peninsula (populations 19, 20).

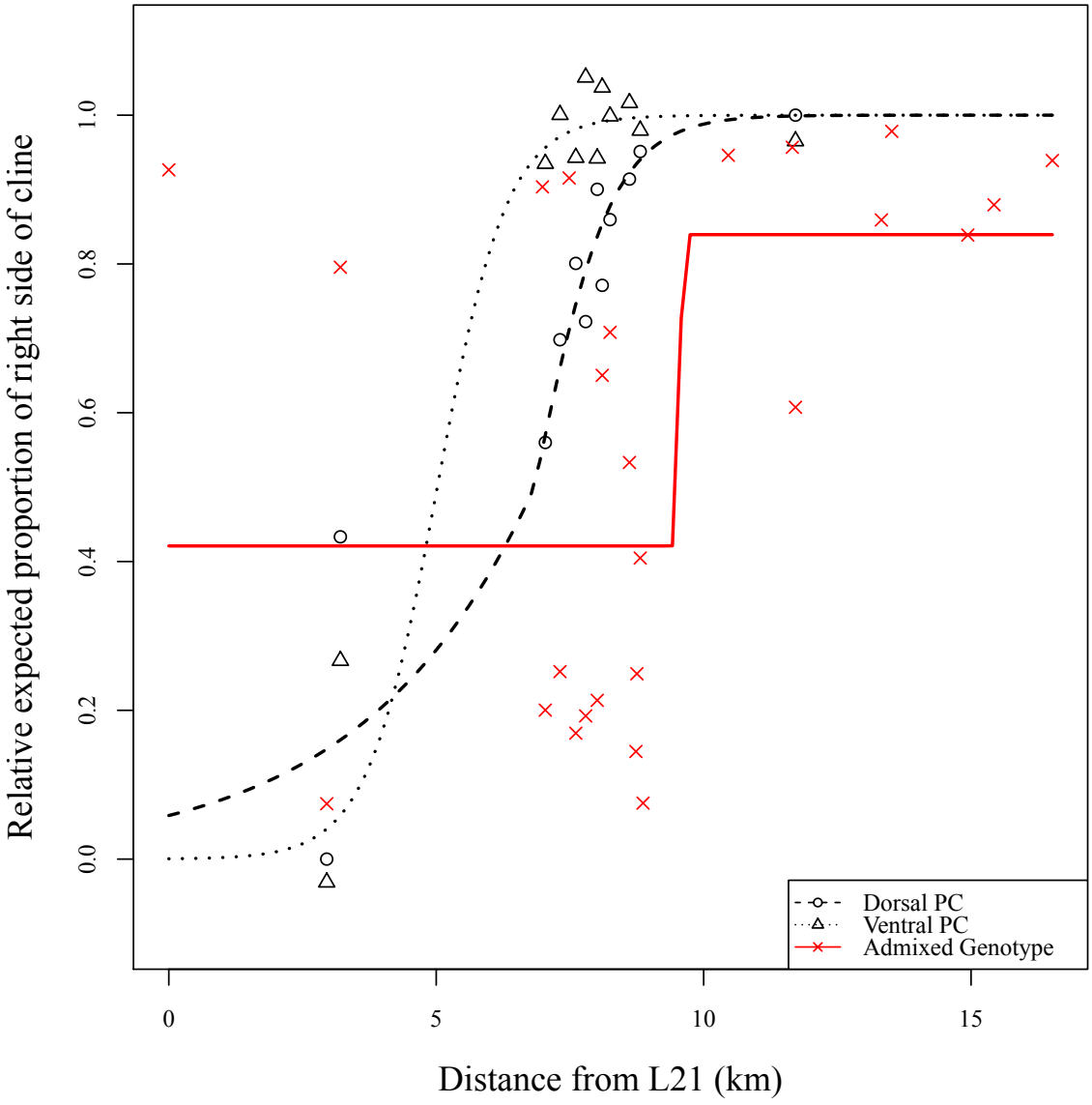


Figure 4-7: Estimated clinal transition between dorsal and ventral phenotype and multilocus admixture frequency genotypes. Scale bar is 5KM.

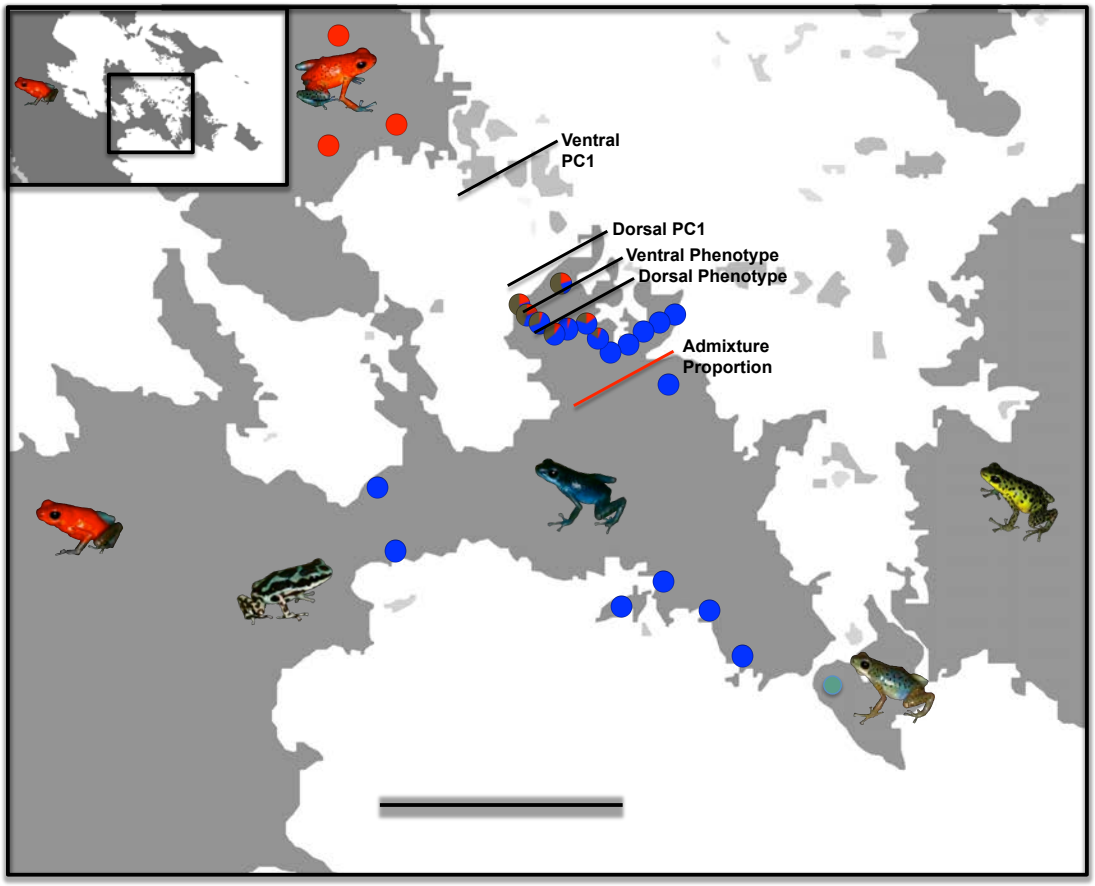


Table 4-1: Summary data for 30 localities of *O. pumilio* sampled from parental and transitional populations. Almirante populations were omitted from cline analyses (see methods). All individuals had dorsal and ventral phenotypic scores, and Dors/Vent RGB columns show the number available for RGB values extracted from standardized color photographs. Distance column refers to the linear pairwise distance from the Northernmost Isla San Cristobal population and were used in cline analyses (location 21).

Locality	Latitude (N)	Longitude (W)	Individuals	MSAT	Used in Cline	Dors RGB	Vent RGB	Distance
1	9°12'59.2"	82°13'31.8"	20	20	Yes	20	20	7.040
2	9°12'53.2"	82°13'23.8"	20	20	Yes	20	20	7.314
3	9°12'44.6"	82°12'53.2"	20	20	Yes	20	20	8.011
4	9°12'46.2"	82°13'16.4"	20	20	Yes	20	20	7.610
5	9°12'43.5"	82°13'08.8"	20	20	Yes	20	20	7.794
6	9°12'37.7"	82°12'58.6"	20	20	Yes	20	20	8.104
7	9°14'42.9"	82°15'38.9"	20	20	Yes	10	10	3.208
8	9°12'39.6"	82°12'46.5"	20	20	Yes	19	19	8.250
9	9°12'35.1"	82°12'32.0"	20	20	Yes	20	20	8.613
10	9°12'33.6"	82°12'22.8"	20	20	Yes	20	20	8.814
11	9°12'40.3"	82°12'18.3"	20	20	Yes	0	0	8.734
12	9°12'41.1"	82°12'16.3"	20	20	Yes	0	0	8.752
13	9°12'44.3"	82°12'06.4"	20	20	Yes	0	0	8.867
14	9°14'51.9"	82°15'40.6"	20	20	Yes	20	20	2.953
15	9°11'16.5"	82°11'27.6"	20	20	Yes	9	9	11.718
16	9°12'25.0"	82°22'01.5"	20	20	No	20	20	-
17	9°11'56.3"	82°20'41.4"	40	40	No	39	39	-
18	9°12'16.21"	82°21'43.55"	12	12	No	0	0	-
19	9°9'6.30"	82°11'6.70"	5	5	Yes	0	0	15.429
20	9°9'15.40"	82°11'23.40"	3	3	Yes	0	0	14.939
21	9°16'24.6"	82°15'14.6"	12	12	Yes	0	0	0.000
22	9°13'15.70"	82°13'6.70"	22	22	Yes	0	0	6.987
23	9°8'58.70"	82°10'11.07"	6	6	Yes	0	0	16.520
24	9°10'27.7"	82°18'05.4"	9	9	No	0	0	-
25	9°9'18.50"	82°19'22.90"	3	3	No	0	0	-
26	9°10'05.2"	82°15'33.6"	13	13	Yes	0	0	11.658
27	9°09'38.9"	82°12'39.0"	12	12	Yes	0	0	13.324
28	9°9'46.90"	82°12'4.02"	5	5	Yes	0	0	13.518
29	9°10'44.8"	82°15'42.0"	13	13	Yes	0	0	10.461
30	9°12'38.6"	82°13'42.2"	16	16	Yes	0	0	7.486

Table 4-2*:* Principal components analysis of dorsal RGB color scores from photographs

Trait	***PC1***	***PC2***	***PC3***
Dorsal Red	-0.987	-0.087	0.137
Dorsal Green	-0.160	0.665	-0.729
Dorsal Blue	0.027	0.741	0.670
Ventral Red	-0.664	0.687	-0.297
Ventral Green	0.298	0.606	0.737
Ventral Blue	0.686	0.401	-0.607

Table 4-3: Microsatellite genetic and genotypic diversity of *O. pumilio* populations.

Locality	Num. Samples	Num. Alleles	Obs. Het.	Exp. Het	P-val.
1	20	17	0.846	0.908	0.288
2	20	17	0.829	0.907	0.241
3	20	17	0.818	0.922	0.282
4	20	18	0.806	0.913	0.261
5	20	19	0.828	0.920	0.333
6	20	16	0.714	0.869	0.334
7	20	13	0.770	0.862	0.498
8	20	14	0.795	0.871	0.338
9	20	15	0.781	0.853	0.380
10	20	18	0.788	0.896	0.225
11	20	19	0.846	0.927	0.221
12	20	16	0.754	0.901	0.172
13	20	16	0.854	0.913	0.452
14	20	18	0.833	0.922	0.224
15	20	16	0.801	0.901	0.227
16	20	8	0.888	0.752	0.223
17	40	14	0.864	0.828	0.072
18	12	10	0.749	0.847	0.403
19	5	6	0.875	0.876	0.684
20	3	5	0.861	0.906	0.756
21	12	11	0.824	0.874	0.453
22	22	14	0.779	0.846	0.374
23	6	6	0.778	0.838	0.480
24	9	10	0.828	0.892	0.398
25	3	4	0.806	0.839	0.788
26	13	11	0.808	0.894	0.359
27	12	10	0.771	0.873	0.298
28	5	6	0.833	0.844	0.755
29	13	10	0.833	0.868	0.504
30	16	11	0.719	0.834	0.325

Table 4-4*:* Summary of phenotypic and genotypic cline models including their estimated centers, widths, Akaike's Information Criterion (AIC) and likelihood values. Cline scaling and tails refer to elements of the shape of clines (see text for explanation), all distance measures are presented in kilometers starting from furthest location on Isla San Cristobal (location 21, Figure 1).

Cline	Cline Scaling Model Selected	Cline Tails Model Selected	Estimated Cline Center (in Km)	Estimated Cline Width (in Km)	Loglikelihood	AIC Score	Selection Coefficient (*Low*)	Selection Coefficient (*High*)
Dorsal Score	**free**	**none**	**7.552647**	**0.59006765**	**657.7280**	**-1301.4560**	**0.003297882**	**0.093991005**
Ventral Score	**free**	**none**	**7.346839**	**0.33802777**	**170.5295**	**-327.0590**	**0.004357223**	**0.124182674**
Admixture	**free**	**none**	**9.561755**	**0.07904223**	**-152.1863**	**318.3726**	**0.009010655**	**0.256807442**
Ventral PC1	free	none	5.020250	2.61089688	-921.2349	1856.4698	0.001567802	0.044683001
Dorsal PC1	fixed	both	6.837210	2.86656093	-891.2771	1796.5541	0.001496254	0.04264387

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Biography:

Justin grew up in rural Amish country with an acute interest in leaving farmland for exploring the rainforest. This dream was facilitated in large part to highly accommodating parents, and a permissive university, and was the spark that propelled him to study evolutionary biology. His work to date has been focused in behavioral ecology. This research primarily focused in studying the evolution of warning coloration, particularly in the context of polymorphism in prey species, as well as investigating the processes that can lead to reproductive isolation between populations.