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THE ANTIPREDATOR BEHAVIORS OF CRYPTIC AND APOSEMATIC ANURANS AND VARIABILITY IN ALKALOIDS, CONSPICUOUSNESS, AND PATTERN WITHIN DENDROBATES AURATUS (DENDROBATIDAE)

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THE ANTIPREDATOR BEHAVIORS OF CRYPTIC AND APOSEMATIC ANURANS AND VARIABILITY IN ALKALOIDS, CONSPICUOUSNESS, AND PATTERN WITHIN *DENDROBATES AURATUS* (DENDROBATIDAE)

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By
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Table of Contents

Methods

Results

Discussion

Tables and Figures

ABSTRACT

Crypsis and aposematism are common antipredator strategies that have evolved as defensive mechanisms to prevent predation. Prey that employ these defensive strategies also exhibit antipredator behaviors meant to avoid or deter predation. These behaviors include: (1) escape or immobility in the presence of an immediate predator or (2) exhibiting bold behavior by accepting the risk of potential predation in a novel environment in exchange for the benefits of foraging and mating opportunities. In this study, the escape and bold behaviors of cryptic members of *Craugastor* and the aposematic dendrobatid *Dendrobates auratus* were tested to compare these alternative antipredator strategies. *Craugastor* behaved more cryptically and was less bold than *D. auratus* in response to simulated predators and when emerging from a cover object. Further, a human and bird model were used as simulated predators to compare the escape behaviors exhibited by *Craugastor* and *D. auratus* in response to each. The results of this study support previous findings that cryptic anurans commonly rely on immobility to maximize camouflage, whereas aposematic anurans exhibit movement that enhance their warning signals. Also, movements exhibited by *D. auratus* were distinctive based on the identity of the approaching predator, suggesting that predator type is important when studying the escape behavior of an aposematic species. Furthermore, although crypsis and aposematism are thought to be alternative strategies, a continuum ranging from cryptic to aposematic may exist within aposematic species. The color/pattern and alkaloid chemical defense of individual *D. auratus* were measured and compared to their antipredator behavior to establish a potential spectrum of antipredator strategies. Two populations of *D. auratus* were found to exhibit alternative antipredator strategies – the Atlantic population was more bold, conspicuous, and chemically defended than the Pacific population. The two populations of *D. auratus* support the possibility that crypsis and aposematism are not mutually exclusive. Instead, populations of *D. auratus*, in response to unique selective pressures, can utilize a combination of antipredator strategies including morphology and behavior.

INTRODUCTION

Over the course of evolutionary time predation has been a strong selective force on prey species, resulting in the evolution of a diversity of complex strategies for avoiding predation (Lima and Dill, 1990; Brodie *et al.*, 1991). Many of these defensive adaptions are morphological, which are intended to deter, prevent, or warn predators from attacking – these include spines, bristles, hairs, and quills (Speed and Ruxton, 2005); poison glands containing noxious or unpalatable chemical defenses (Saporito *et al.*, 2010, 2012); the use of cryptic coloration as a camouflage (Caro, 2005; Buresch *et al.*, 2011); and the use of conspicuous coloration or patterning as a warning signal (Sherratt, and Beatty, 2003). There are also numerous behavioral responses that act to reduce the probability of detection or attack by predators (Lima and Dill, 1990; Ruxton *et al.*, 2004), such as: immobility or spatial/temporal shifts in microhabitat use (Brodie *et al.*, 1974, Werner *et al.*, 1983; Wilbur, 1987; Sheriff *et al.*, 2009); the unken reflex (Toledo and Haddad, 2009); and body raising (Blanchette and Saporito, 2016). Most prey species use a combination of traits, which act together to provide defense (David *et al.*, 2014). Although prey defensive adaptations have been well characterized, our understanding of how these adaptations mediate the ecological and evolutionary nature of predator–prey interactions remains surprisingly incomplete.

Crypsis and conspicuousness represent the two extremes of a continuum of color-based defensive adaptations that have evolved in many invertebrate and vertebrate prey species (Ruxton *et al.*, 2004; Caro, 2005). Cryptic coloration enables a prey species to camouflage itself by background matching, countershading, or disruptive coloration, all of which are strategies to conceal them from predators (Ruxton *et al.*, 2004; Caro, 2005). Alternatively, conspicuous coloration facilitates prey detection, typically by the expression of bold colors or patterns that are easily visible to predators (Ruxton *et al.*, 2004). The use of conspicuous colors or patterns can function as an advertisement (or warning) of secondary defenses, an adaptation known as aposematism (Cott, 1940; Ruxton *et al.*, 2004). Secondary defenses can include morphological structures (Dyrynda, 1986; Speed and Ruxton, 2005; Sugiura and Yamazaki, 2014) or defensive chemicals such as amines, peptides, proteins, terpenes, steroids, and alkaloids (Mebs *et al.*, 2010; Savitzky *et al.*, 2012). Although some cryptic prey species also utilize chemical defenses, conspicuous species advertise the presence of their defensive chemicals. In many cases, the degree of prey conspicuousness (e.g., brightness of several gastropod genera; Cortesi and Cheney, 2010) is positively correlated with levels of toxicity, and therefore, unprofitability to a predator (Summers and Clough, 2001; Franks *et al.,* 2008); however, there are also examples in which conspicuousness and defense are not positively correlated (Blount *et al.,* 2009; Summers *et al.,* 2015). Crypsis and aposematism have evolved concurrently with a suite of specific behavioral attributes that increase the effectiveness of these predator defenses (Willink *et al.,* 2013); thus, studies comparing cryptic and aposematic organisms can provide important information on the effectiveness of signaling or camouflage as a predator defense.

In general, cryptic and aposematic prey will use one of two behavioral strategies when perceiving and responding to potential predators: immobilization or escape (Miyatake *et al.,* 2007). The immobility reaction is considered a type of fear paralysis, and is thought be an innate or learned reflex response (Sargeant and Eberhardt, 1975; Cooper *et al.,* 2008a). Although cryptic and aposematic organisms can remain immobile in the presence of a predator (Cooper *et al.,* 2009a, b; Ozel and Stynoski, 2011), this behavioral response appears to be a result of different defensive strategies. Cryptic animals tend to remain immobile and use their camouflage to reduce the risk of being detected by a predator, whereas aposematic prey remain immobile in the presence of a predator, relying largely on the fact that they are displaying a warning signal that is detectable by a predator (Cooper *et al.,* 2009b). In some cases, cryptic animals will flee, and research suggests that their initial movement is quick and far (Cooper *et al.,* 2008b). For example, frogs in the genus *Craugastor* rely on crypsis for protection and, when approached by a predator, generally remain immobile; however, in instances in which they do move, it is usually one large jump ($\geq$ 0.45m) before they conceal themselves in the leaf litter (Cooper *et al.,* 2008a). Similarly, the dwarf chameleon (*Bradypodion transvaalense*) exhibits body flattening and immobility, flipping on a branch, or quickly dropping from a branch to escape a predator (Stuart-Fox *et al.,* 2006). Alternatively, when aposematic organisms flee from a predator they typically move more slowly and not as far as do cryptic prey (Wiklund and Sillén–Tullberg, 1985; Ruxton *et al.,* 2004; Cooper *et al.,* 2009a). For example, in a study of aposematic monarch butterflies (*Danaus* *plexippus*), Wiklund and Sillén–Tullberg (1985) found that adults approached by an avian predator either fluttered their wings or flew a short distance away, possibly increasing the exposure of their conspicuous coloration, which was sufficient to deter predation.

The threat of predation may have an effect on the behavior of cryptic and aposematic prey, even in the absence of predators. For example, exhibiting exploratory behavior of a novel environment may be adaptive if it allows individuals increased access to foraging or mating opportunities. However, the risk of predation may be greater in a novel environment. Therefore, individuals that exhibit exploratory behavior regardless of the risks are classified as bolder than those that do not (Canestrelli *et al.,* 2016). These behaviors (escape and boldness) may combine in species to help create personality differences among individuals, a concept known as a behavioral syndrome (Sih *et al.,* 2004). Individuals that delay escape or exhibit immobility in the presence of a predator may also be more likely to quickly leave a shelter and enter a novel environment in which a predator may or may not be present. These individuals would be considered bolder than those that immediately flee from an approaching predator or never leave a shelter (Sih *et al.,* 2004; Canestrelli *et al.,* 2016). Bolder individuals, however, may be at a higher risk of predation or injury when compared to individuals that flee from a predator quickly (Ahlgren *et al.,* 2015). However, escape from a predator may interrupt courtship, whereas avoidance of a novel environment may prevent access to plentiful resources in the form of food or mates. Theoretically, aposematic individuals with greater chemical defense and more conspicuous coloration should behave more boldly in the presence of a predator, and exhibit increased exploration of a novel environment than less chemically-defended and conspicuous individuals (Pröhl and Ostrowski 2011; Willink *et al.,* 2013; Dugas *et al.,* 2015; Summers *et al.,* 2015). Although crypsis and aposematism are common and well-documented defensive strategies (Cott, 1940; Ruxton *et al.,* 2004), relatively little is known about how cryptic and aposematic animals at risk of natural predation behave across varied situations (Ruxton *et al.,* 2004; Ozel and Stynoski, 2011).

Members of Craugastoridae are cryptic, non-chemically defended frogs that bury themselves in leaf litter or remain immobile on the leaves of small trees to avoid predator detection (Hedges *et al.,* 2008). Many members of *Craugastor* have been studied for their immobility and escape behaviors. In a study comparing the escape behavior of three species within *Craugastor* (*C. fitzingeri, C. noblei,* and *C. mimus*), Cooper *et al.* (2008a) found that 30 out of 32 individuals approached directly by a human did not attempt escape and instead relied on immobility and camouflage. Similarly, *C. bransfordii* has been found to remain immobile longer than the aposematic frog *Oophaga pumilio* when approached by a human (Ozel and Stynoski, 2011). Studies of escape behavior often use a cryptic anuran species as a control for an aposematic species, however no study has compared the strategies of crypsis and aposematism across multiple behavioral contexts. If cryptic anurans rely on immobility as a method of camouflage in response to a potential predator, they may be less willing to leave a shelter to explore a novel environment due to decreased cryptic efficacy. Conversely, if aposematic anurans behave more boldly in the presence of a potential predator, then they may be more willing to explore novel environments, possibly increasing their foraging and mating opportunities (Dingemanse and Réale, 2005; Pröhl and Ostrowski, 2011; Ahlgren *et al.,* 2015).

Dendrobatid frogs are a well-studied group of aposematic organisms that vary in their conspicuousness and alkaloid-based chemical defenses (Savage, 2002; Saporito *et al.,* 2007a, 2012). The alkaloid defenses of dendrobatids are sequestered largely from a diet of ants and mites (Saporito *et al.,* 2007a, 2012), and are known to vary based on life-stage, age, sex, and geographic and temporal scales (Saporito *et al.,* 2006, 2007a, 2010; Jeckel *et al.,* 2015; Murray *et al.,* 2016). As a group, dendrobatids are well known for their complex social behaviors that include elaborate courtship, territoriality, and both reproductive and defensive advertisements via visual and acoustic displays (Donnelly, 1989; Savage, 2002; Pröhl, 2005), making them a model group to study how aposematic traits may influence behavior. Among dendrobatids, some studies have found frog coloration (brightness, hue, and chroma) and alkaloid defense (based on alkaloid diversity, lethality, and quantity) to be correlated (Summers and Clough, 2001; Santos *et al.,* 2003), whereas other studies have found an inverse relationship between color and alkaloid defense (Blount *et al.,* 2009; Wang, 2011). These studies on poison frogs are beginning to provide evidence that crypsis and aposematism lay on a continuum of defensive strategies, rather than be discrete defensive strategies (Mappes *et al.,* 2005). Behavior may also lie on a continuum within aposematic species, wherein more conspicuous, chemically-defended individuals exhibit unhurried movement or immobility to possibly relay a warning signal to a potential predator that an individual is unprofitable (Summers *et al.,* 2015; Rojas, 2016). Conversely, less conspicuous or chemically-defended individuals may exhibit immobility more frequently to reduce the probability of detection. However, no studies have simultaneously examined the relationship between coloration, defense, and behavior for the same species.

Cooper *et al*. (2009b) suggested that conspicuous dendrobatids exhibit characteristic behavior of aposematic prey with their unhurried movements through the leaf litter, or in some cases, immobility, which may be related to certain behavioral tendencies and fitness strategies in this group of frogs. Immobility may be an adaptive defensive behavior, because the highest concentration of unprofitable chemicals and strongest color signals are located on the frog's dorsum (Siddiqi *et al.,* 2004; Saporito *et al.,* 2010). Therefore, if an immobile frog directs this region towards a predator, the likelihood of exposing alkaloid defenses may be increased without the frog moving away from a valuable resource they have acquired (e.g., space, calling site, oviposition site; Ozel and Stynoski, 2011; Dugas *et al.,* 2015). Unhurried movements may allow a potential predator to assess the warning signal and deter attack which results in the individual expending little energy in escape (Cooper *et al.,* 2009a; Cooper and Blumstein, 2015). Unhurried movement may also be important to aposematic dendrobatids, as aerial predators may perceive immobile individuals as fruit on the forest floor (Paluh *et al.,* 2015). Visually-oriented predators, such as birds, represent a predation threat to aposematic dendrobatids (Hegna *et al.,* 2012; Paluh *et al.,* 2015), but frogs that move may be attacked less frequently, possibly because of enhanced warning signal efficacy (Paluh *et al.,* 2014). Most studies of frog escape behavior have used humans as a simulated predator (Cooper *et al*. 2009a, b; Ozel and Stynoski 2011; Pröhl and Ostrowski 2011; Dugas *et al*. 2015; Cooper and Blumstein 2015), with a few recent studies having used a simulated bird predator (Cooper *et al.,* 2008b; Willink *et al.,* 2013; Blanchette *et al.,* 2017). Different colored morphs of *O. granulifera* exhibit varied escape responses to a bird model, with red morph individuals initiating movement more quickly, potentially to enhance their warning signal, than do the green, less conspicuous morph that may have relied more on immobility and low detectability (Willink *et al.,* 2013). *Oophaga pumilio* has been tested for its escape behavior in response to a human and a model Rufous

Motmot, wherein frogs were found to exhibit fleeing behavior directly away from the human, but sporadic movement when responding to the bird model (Blanchette *et al.,* 2017). A study that directly compares the chemical defense, color, and bold behavior of aposematic individuals within and across populations may help explain the observed variation across dendrobatids with respect to their morphology and antipredator behavioral tendencies.

The Green and Black Poison Frog, *Dendrobates auratus*, is a conspicuously colored dendrobatid that exhibits variation in color and alkaloid defenses across its geographic range (Daly *et al.,* 1994a, b, 2000; Savage, 2002; Patrick and Sasa, 2009). *Dendrobates auratus* has variable black and green patterning over its entire body (Cove and Spínola, 2013), ranging from leaf green to blue and dark black to bronze (Savage, 2002; Patrick and Sasa, 2009). Both color and blotched patterns may be important for *D. auratus* in avoiding predation, because the contrasting light and dark pigmentation may create false edges that distort the body outline, potentially hindering a predator's detection ability (Köhler, 2012; Honma *et al.,* 2015). Further, the combination of color and pattern may provide an appearance that is conspicuous up close but turns cryptic with increasing distance (Tullberg *et al.,* 2005). Alternatively, predators may be able to more easily remember the relationship between color and chemical defense if the color is associated with a pattern because together color and pattern are honest indicators of a secondary defense (Rojas, 2016). Within Costa Rica, populations of *D. auratus* located in the Caribbean lowlands exhibit lighter coloration and different alkaloid defenses compared to individuals found in the Pacific lowlands (Daly *et al.,* 1987; Patrick and Sasa, 2009); however, no studies have directly compared the chemical defense and color pattern of *D. auratus.* Based on data from other dendrobatids (e.g., *O. granulifera;* Wang, 2011; *O. pumilio;* Saporito *et al.,* 2007a, b, 2010; Paluh *et al.,* 2014; Murray *et al.,* 2016; *Phyllobates lugubris;* Mebs *et al.,* 2014; *P. vittatus*; Mebs *et al.,* 2014), it is likely the alkaloids of *D. auratus* are also variable, but a study that directly characterizes the alkaloid composition (number, type, and quantity of alkaloid) of *D. auratus* is required.

The behavior of *D. auratus* has been studied in an attempt to understand if the species behaves in a manner characterized as aposematic, represented by unhurried movement or immobility in response to the risk of predation. Cooper *et al*. (2009b) compared escape behaviors between *D. auratus* and *O. pumilio* in northeastern Costa Rica, and found that *D. auratus* fled at a slightly further distance in response to a simulated predator (a stick with painted eyes), whereas *O. pumilio* allowed the predator to approach closer before fleeing; however, both species only fled once the simulated predator was considered to be within a potential attack range (< 0.5 m), which was interpreted as both frogs relying on aposematism to deter predation. In a similar study (Cooper *et al.,* 2009a), prior to fleeing, *D. auratus* remained exposed and hopped leisurely (i.e., slowly) in the leaf–litter when responding to an approaching simulated predator (walking human), but was more likely to flee when approached quickly or directly. These studies suggest that *D. auratus* assesses the level of risk associated with their bold and exploratory behavior, but comparative studies focused on understanding the relationships between coloration, chemical defense, and behavior are lacking in this species.

The purpose of my study was to determine how the cryptic and aposematic antipredator strategies of anurans are reflected in their escape and bold behaviors, within and among populations, using the aposematic dendrobatid *D. auratus* and cryptic members of *Craugastor*. I also compared the escape behavior of *D. auratus* and *Craugastor* between simulated human and bird predators to compare frog defensive behavior in the presence of different potential threats. Further, I tested for the relationship between color, pattern, alkaloids, and behavior in *D. auratus* to determine if this species exhibits a spectrum of antipredator strategies ranging from characteristically cryptic to aposematic. To empirically test these ideas, I (1) measured the escape behavior of *D. auratus* and *Craugastor* in escape assays using a human and bird model as the simulated predators; (2) measured the bold behavior of *D. auratus* and *Craugastor* by way of simulating exploration of a novel environment; (3) measured the color and pattern of *D. auratus;* and (4) quantified and characterized the alkaloid profiles of *D. auratus.*

METHODS

Sex determination in *Dendrobates auratus*

Morphological measurements. Behaviorally, males and females of *Dendrobates auratus* differ in that females are more active and tactile during courtship, and respond to calling males, but males provide all of the parental care (Summers, 2014). Currently, however, there are no external secondary sexual characteristics that allow for easy sex determination of *D. auratus* in the field. In some anuran species, external morphological measurements (e.g., snout-to-vent length (SVL), girth, mass, toe pad width, etc.) have been used to reliably determine sex (Monnet and Cherry, 2002; Chang, 2008; Kraus, 2008). Therefore, I first conducted a study to determine if morphological measurements could be used to reliably determine sex in *D. auratus* by examining specimens that are part of museum collections.

After examining the availability of *D. auratus* samples from the Natural History Museum of Los Angeles, The Field Museum, and the University of Michigan Museum of Natural History, two loans of 50 frogs each were requested from the American Museum of Natural History, New York. All of the *D. auratus* collected were from Panama, and an attempt was made to ensure that they were collected from a similar locality (Cocle, Colon, and Panama provinces). From these 100 *D. auratus*, 30 adult male (SVL > 25.0 mm) and 30 adult female (SVL > 25.0 mm) specimens were selected for inclusion in the study. Juveniles were not included in the analysis. For each individual frog, SVL, mass, toe pad width of the third phalange on the left front manus, and width at the center of the abdomen was measured. Frog SVL was measured to the nearest 0.01 mm using digital calipers, and mass was measured using a Pesola PPS200 digital pocket scale to the nearest 0.01 g. Each measurement was taken in triplicate to calculate an average for each morphological character for each individual frog. Following morphological measurements, all frogs were dissected via a small lateral incision on the abdomen and sex was determined by the presence of testes, eggs, or oviducts (Figure 1).

Statistical analyses. A Discriminant Function Analysis was used to determine which morphological character (or combination of characters) are the best predictors of sex in *D. auratus.* Analyses were conducted with SPSS v. 14 for Windows.

Behavioral assays with *Dendrobates auratus* and *Craugastor.*

Species and study sites. Three distinct populations of *D. auratus* were observed in the field under natural conditions. La Selva Biological Station (10°26' N, 83°59' W) and Isais (10°27' N, 84°03' W) were located in the Atlantic lowlands of Costa Rica, and the

Firestone Center for Restoration Ecology (9°16' N, 83°51' W) was located in the Pacific lowlands of Costa Rica (Figure 2). Twenty adult *D. auratus* (SVL males 25–40 mm; females 27–42 mm) were collected at each location. The discriminant function analysis enabled the identification of male and female (see below), and therefore an equal number of adult male and adult female *D. auratus* were collected. There are no external secondary sexual characteristics that allow for the identification of male and female *Craugastor.* Twenty adult *C. fitzingeri* were collected at La Selva Biological Station and twenty *C. stejnegerianus* were collected at the Firestone Center for Restoration Ecology because *C. fitzingeri* is absent at this site.

The collection of *D. auratus* occurred during daylight hours from 0600–1000, when the frogs were most active (Dunn, 1941; Summers, 1990; Cove and Spínola, 2013). The collection of *C. fitzingeri* and *C. stejnegerianus* occurred during nighttime hours, from 1900–2200, when the frogs were most easily captured. Upon collection, all *D. auratus* were measured for snout-to-vent length, toe pad width of the third phalange on the left manus, width at the center of the abdomen to the nearest 0.01 mm using Traceable® Digital Calipers. Frog mass was measured using a Pesola PPS200 digital pocket scale to the nearest 0.01 g. All *D. auratus* were sexed, using the discriminant function equation (see below). Upon collection of *C. fitzingeri* and *C. stejnegerianus,* the snout-to-vent length and mass were measured. All frogs were collected in individual Ziploc bags that were dampened with water and contained leaf litter. Frogs were then transported to a field laboratory and stored in individual plastic terraria with moist leaf-litter for up to 48 hours prior to conducting behavioral assays (Cooper *et al.*, 2009a). All frogs were returned to their original site of collection after completing the behavioral assays. To avoid retesting the same individuals, frogs were not collected from the same place after they were released.

The same individual frog was used in three behavioral assays to measure: (1) boldness in escape response to human approach; (2) boldness in escape response to simulated avian predator approach; and (3) boldness exhibited by exploration of a novel environment. After completion of one behavioral assay, each frog was returned to its terrarium for at least 24 hours prior to use in the next behavioral assay. The order of behavioral assays that each frog was used in was randomized using a random number generator. Reuse was necessary to draw comparisons between escape and exploratory behaviors of the frog and its color, pattern, and alkaloid composition (methods below). Reuse also allowed for comparisons in escape behavior in response to human and avian predator approach, which may provide insight into how frogs perceive differently-sized potential threats. Prior to the start of each behavioral assay trial, the temperature and humidity at the center point under the cover object and at a point 5 cm outside the cover object was measured using a Traceable® Humidity/Temperature Pen.

Escape behavior assay. To quantify escape behavior as a measure of boldness, the flight initiation distance (FID) was measured for each individual frog. The FID is the distance between a predator and prey at the point in which the prey initiates fleeing (Cooper *et al.,* 2012). The FID attempts to measure risk perception and how close a potential predator can approach before prey attempts to escape, if at all. The shorter the FID, the closer a predator is able to approach before the prey attempts to flee (Berger, 2006). The FID was measured as the distance in meters between the original position of the frog when it first moved in response to approach and the position of the approaching predator (bird vs. human; see below for details) (Rodríguez-Prieto and Fernández-Juricic, 2005; Cooper *et al.,* 2009a, b; Ozel and Stynoski, 2011). In addition to FID, the latency (seconds), angle of escape (degrees), and distance fled (cm) were also recorded for each individual (following the methods of Bulbert *et al.*, 2015). Each experiment took place on a black plastic (30.5 × 30.5 cm) experimental arena in a forest clearing (Blanchette *et al.*, 2017).

To begin each behavioral assay, an individual frog was placed in the center of the experimental arena that was level and flush against the ground. Every frog was approached from the same starting point, regardless of the treatment. FID was measured as the distance between the predator and the frog when the frog first moved in response to approach. Latency was recorded with a stopwatch as the time from the beginning of the predator approach to the point at which the frog moved. If the frog attempted to escape, the angle of escape was measured in degrees based on the simulated predator approaching from $0^o/360^o$ (Figure 3). The distance fled was measured as the distance between the original start point of the frog and its location after it did not move for 10 seconds. Flags were placed at 90^o, 180^o, and 270^o with respect to the approaching predator as a perimeter around the experimental arena at a distance of 1.5 m to mark the maximum distance fled so that *D. auratus, C. fitzingeri,* and *C. stejnegerianus* could be recaptured if they fled from the approaching predator. Dark green cotton blinds were constructed and placed at 90° and 270° at a distance of 2.5 m so that the cover object could be lifted and the researcher could remain hidden from the frog (Figure 3). The researcher alternated which side the cover object was lifted from to prevent bias in frog behavior. Between trials, the experimental arena and cover object were cleaned with a 1:3 solution of distilled water and ethanol and dried to remove any potential confounding effects of frog odor.

Human predator. Although humans are not natural predators of most anurans, an approaching human presents a potential risk to prey, and therefore it is expected that animals will flee in a predictable manner consistent with their response to natural predators (see Cooper, 2009a; Cooper *et al.*, 2009a; Camp *et al.*, 2012). Prior to beginning Human Predator trials, each frog was placed under a dark container and allowed to acclimatize for 5 minutes (Cooper *et al.*, 2009b). Upon acclimatization, the container was lifted, and the frog was given 10 seconds to adjust to its surroundings before it was approached from 9 meters away at approximately 1.8 m/s (Cooper, 1997; Cooper, 2003; Cooper, 2009a; Cooper *et al.*, 2009a). Once approach was initiated, a stopwatch was started to measure latency. If the frog moved within the 10 second adjustment stage, the trial was discarded and the individual was recaptured and tested approximately 24 hours later. Once the frog moved, the researcher stopped their stopwatch and stopped walking to mark their position with a small flag (Ozel and Stynoski, 2011). The facing angle and escape angle of the frog were marked with flags and measured with a protractor. The FID and distance fled were measured in meters using a measuring tape. If the frog did not respond to the treatment, the latency, distance fled, and FID were recorded as zero. Upon completion of the FID measurement, the frog was recaptured and placed in its holding bag for use in other experiments. Behavior was characterized as one of four responses: escape; pivoting; body raising; or no movement. Escape behavior was identified as an individual exhibiting movement that included hopping away from the experimental arena; pivoting was identified as an individual changing orientation, but not moving more than 1cm; body raising was identified as an individual vertically raising the legs and pointing its snout towards the ground, resulting in a raised and arched dorsum (Blanchette and Saporito, 2016); and no movement was recorded if an individual did not exhibit any of the former movements.

Simulated bird approach. A model avian predator was constructed using a 3-D printed bird model (body length: 28 cm; wingspan: 33 cm; body depth: 6.35 cm). The model was printed on white, hard plastic and painted cream with gray tipped wings to represent a general bird form and not a specific species found in Costa Rica. Similar to the human approach treatment, the frog was allowed to acclimate under a dark cover object for five minutes. Once acclimated, the cover object was lifted and the frog given 10 seconds to adjust to its surroundings. If the frog moved during the adjustment phase, it was recaptured and retested approximately 24 hours later. After adjustment, the bird was released from its position on a rig made out of PVC pipe at a height of 2 m, 9 m from the frog (Blanchette *et al.*, 2017). The bird model glided towards the frogs on a nylon line and came to a rest approximately 6.5 meters behind the frog. The bird model travelled at a speed of 1.8 m/s and was at a height of 50 cm when directly overhead (Blanchette *et al.*, 2017). Timing the latency began when the bird was released and ended when the frog moved in response to the bird. A flag was placed at the position of the bird when the frog moved, to measure FID. The facing angle and escape angle of the frog were marked with flags and measured with a protractor. The FID and distance fled were measured in meters using a measuring tape. If the frog did not respond the latency, distance fled, and FID were recorded as zero. The flags were placed after the trial concluded and the frog was recaptured so the frog's fleeing was not interrupted or influenced by the researcher.

To examine whether or not frogs considered the bird model as a "bird predator", each frog was also tested for a response to a similarly colored approaching circular disk. The disk was approximately 33 cm in diameter, constructed with a 3-D printer, and painted to match the bird coloration. The behavioral assay was conducted in the same manner as the bird model and variables measured as previously stated.

Bold behavior assay. Similar to previous studies (Hedrick, 2000; González-Bernal *et al.*, 2014), bold behavior was assessed by measuring the time it took for a frog to emerge from a cover object. Each individual was placed under a cover object consisting of a black plastic box (15 × 15 × 7 cm) with an opening (4 × 4 cm) cut out. A weighted door (12 × 5.5 cm) rested flush against the box and ground to prevent the frog from escaping during a five minute acclimatization period (González–Bernal *et al.*, 2014; Hovey and Saporito, unpublished data). Upon acclimatization, the door was removed via an attached string (2.5-3 m) to minimally disturb the frog while standing 2.5-3 m away perpendicular to the cover object opening (Hovey and Saporito, unpublished data). The appearance, emergence, and waiting times of the individual were measured for each trial. Based on the methods of Martin *et al.* (2003), the appearance time was recorded as the time at which an individual's head appeared at the opening, and the emergence time was recorded as the time at which the individual's entire body left the cover object. An animal may be able to assess the presence or absence of a predator in between appearance at and emergence from the opening. The waiting time is the difference between appearance and emergence, and if an individual perceives danger, the waiting time may be longer than an individual that does not. These times are not independent of each other but represent behavioral decisions of the individual (Martin *et al.*, 2003). The assay concluded upon emergence of the frog or once 30 minutes elapsed (Hovey and Saporito, unpublished data). Between assays, the cover object and door were cleaned with a 1:3 solution of distilled water and ethanol and allowed to dry to remove chemical odors.

Alkaloid Extraction and Characterization

Alkaloid extraction. To draw comparisons between frog behavior and alkaloid defenses, alkaloids were extracted from each of the individual *D. auratus* prior to use in the behavioral assays. The alkaloids were collected from each frog by use of a Transcutaneous Amphibian Stimulator (TAS), which resulted in no harm or death to frogs (Grant and Land, 2002). To extract alkaloids, a weak electric current (Frequency 50 Hz; Pulse width 2 ms; Amplitude 9V) was applied to the skin on the dorsum of each frog for three minutes. This induced the secretion of contents from the frog's granular glands (Hantak *et al.*, 2013; Bolton *et al.*, 2017). A 6 mm circle of bibulous paper, created using a paper hole-punch, was used to wipe off the alkaloid secretions every minute during the three minute TAS process and then placed in 2 mL of 100% methanol in covered glass vials with Teflon-lined lids (Bolton *et al.,* 2017).

Alkaloid fractionation. To separate the alkaloids from the ethanol solution, an acid-base extraction was performed on 1 mL of each alkaloid sample collected from *D. auratus.* One mL of the EtOH/alkaloid solution, 50 µL of 1N HCl, and 100 µL of nicotine (internal standard) were added to a conical glass vial. The solution was slowly blown down with nitrogen to ca. 100 µL, after which ca. 200 µL of distilled water was added. Extraction occurred with 300 µL of hexane 4 times and the hexane layer was discarded. The remaining layer was basified with saturated $NaHCO_3$ (ca. 6 drops). Anhydrous Na_2SO_4 was added to a separate conical vial and extracted with 300 µL of ethyl acetate 3 times. The ethyl acetate layer was added to the vial with anhydrous Na_2SO_4 and the waste layer discarded. The solution was carefully blown down with nitrogen to dryness. Methanol equivalent in volume to frog weight was added (ca. 100 µL).

Alkaloid characterization. The alkaloid extracts were characterized by Gas Chromatography-Mass Spectrometry, using a Varian 3900 GC coupled with a Varian Saturn 2100 T ion trap MS fitted with a 30 m x 25 mm i.d. Varian Factor Four VF–5 ms fused silica column. The GC–MS ran a temperature program from 100–280^{o}C, at a rate of 10^{o}C per minute, using helium as a carrier gas (1 ml/min). Electron impact–mass spectrometry (EI–MS) and chemical ionization–mass spectrometry (CI–MS) were used to analyze all samples. Identification of alkaloids was based on comparison of retention times and mass spectral data to known dendrobatid alkaloids (e.g., Daly *et al.,* 2005; Saporito *et al.,* 2006). Alkaloid quantity and number were calculated for Isais and Firestone populations. Alkaloid diversity (not quantity) was calculated for La Selva, because of stress experienced by *D. auratus* during handling that resulted in the loss of alkaloids. Only the alkaloids present in quantities greater than 0.05 µg were included in the study.

Color Assessment

A portable Ocean Optics (Dunedin, FL) USB 4000 UV-VIS spectrophotometer with a PX-2 pulsed xenon light source and a R400-7-SR reflectance probe with a 400 µm core diameter was used to quantify conspicuousness of each individual *D. auratus* after use in the behavioral assays. The spectrophotometer was held approximately 6 mm from the frog's dorsum at a 45^o position. Three random locations along the frog's dorsum were selected and three readings measured at each location per green and black color. The average of the three green locations was used in analyses. White standard measurements were taken using a Labsphere certified reflectance standard in between each frog. The Java-based program CLR, version 1.05 (Montgomerie, 2008), following equations detailed by Endler (1990) was used to calculate brightness and hue. The 20 individuals used in behavioral assays at Isais and Firestone were measured for their color values whereas 15 individuals from La Selva were measured. To quantify pattern, the dorsum of all 60 *D. auratus* was photographed with a digital camera attached to a tripod 15 cm above the frog, and the images were analyzed using Image-J (Patrick and Sasa, 2009). To calculate the percent of the dorsum covered with pattern (blotches), the total dorsum area was divided by the sum of the area of each blotch on the dorsum (Köhler, 2012).

Statistics

Prior to analyses, all behavioral data were checked for normality (Shapiro-Wilk's test) and homogeneity of variance (Levene's test). The behavioral data were not normally distributed and attempts to transform using log-, natural log-, exponential-, square root-, and squared transformations did not result in the data meeting the assumptions of normality. Therefore, the analogous non-parametric statistical analyses were performed. The behavioral data did not have equal variances and therefore all analyses were run using an unequal variances model.

Escape behavior. The same individual frogs (experimental and control) were used to compare escape behavior between bird and human predator treatments; therefore, paired difference, two-tailed Wilcoxon tests were used to assess differences in FID, latency time, and distance fled. Comparisons were made between predator treatments, between frog species, within male and female *D. auratus,* and among geographic locations, and temperature and humidity were used as a covariate in all analyses.

Bold behaviors. Unpaired, two-tailed Mann-Whitney U tests were used to detect differences in appearance, emergence, and waiting times between frog species and male and female *D. auratus* at each geographic location. One-way ANOVA's were used to compare the appearance, emergence, and waiting times between geographic locations of *Dendrobates auratus.* Unpaired, two-tailed Mann-Whitney U tests were used to compare the appearance, emergence, and waiting times of *Craugastor* at each location. Temperature and humidity were used as a covariate in all analyses.

Behavioral syndrome. Linear regression was used to test for a relationship between escape and bold behaviors of *D. auratus* within geographic locations. The flight initiation distance of in individual in response to the human or bird predator was tested against the appearance, emergence, and waiting time of the same individual.

Alkaloid characterization. Statistical differences in alkaloid profiles with respect to the number, quantity, and type of alkaloids were examined with a one-way analysis of similarity (ANOSIM). To graphically represent differences in alkaloid profiles between populations, non-metric multidimensional scaling (nMDS) was used. Two-tailed Wilcoxin tests were used to compare the quantity of alkaloids between the Isais and Firestone populations. The number of alkaloids were compared between La Selva, Isais, and Firestone using one-way ANOVA.

Color and pattern. Differences in brightness, hue, chrome, and proportion of black patterning between La Selva, Isais, and Firestone were examined using one-way ANOVA.

Relationships between frog defensive/bold behaviors and frog coloration/pattern/ alkaloid defenses. A number of linear regressions were used to assess relationships between defensive behaviors, bold behaviors, coloration (brightness, hue, and chroma), pattern (percent of dorsum covered in pattern), and alkaloid defenses (number of alkaloids and quantity of alkaloids). Coloration, pattern, and alkaloid defenses were only examined in *D. auratus*. Linear regression was used to: (1) determine the relationship between defensive and bold behaviors within and between each location, and for both frog species; (2) examine the relationship between defensive behaviors and coloration, pattern, and alkaloid defense for *D. auratus*; and (3) examine the relationship between exploratory behaviors and coloration, pattern, and alkaloid defenses. Logistic regression was used to compare the defensive behavior of frog species within geographic locations.

RESULTS

Sex determination in *Dendrobates auratus*

On the basis of the discriminant function analysis, snout-to-vent length, toe pad width, and width at the abdomen center are accurate predictors of sex in *D. auratus* (Canonical Correlation = 0.734; $\chi^2 = 39.05$; df = 3; $p < 0.05$). The discriminant function equation to determine sex was: discriminant function = -4.008 + 0.213(SVL) + -3.905(TPW) + 0.387 (width at abdomen center). Individuals were classified as female if their discriminant function was approximately 0.948 and as male if their discriminant function was approximately -1.185. An intermediate value of -0.1185 was calculated to create a cutoff value for individuals whose discriminant function fell between the two identified sex groups. An individual was classified as female if its discriminant function ranked higher and male if its discriminant function ranked lower than the intermediate value.

Escape and bold behaviors of male and female D. auratus. There were no significant differences in the escape and bold behaviors of male and female *D. auratus* within geographic locations.

Antipredator behavior of aposematic and cryptic anurans

Escape behavior. During escape behavior assays *D. auratus* exhibited one of four behaviors: no movement; pivoting; body raising; or escape. Further, *Craugastor* were more likely to remain immobile (Table 1).

Aposematic *D. auratus* were significantly more likely than cryptic *Craugastor* to exhibit movement in response to the bird model at La Selva (Wald = 9.05; p = 0.003; Exp(B) = 10.52), Isais (Wald = 3.96; p = 0.047; Exp(B) = 4.64), and Firestone (Wald = 8.90; p = 0.003; Exp(B) = 28.50). *Dendrobates auratus* were significantly more likely than *Craugastor* to exhibit movement in response to the human at La Selva (Wald = 12.18; $p < 0.05$; Exp(B) = 17.0), Isais (Wald = 13.82; $p < 0.05$; Exp(B) = 22.67), and Firestone (Wald = 14.79; $p < 0.05$; Exp(B) = 36.0).

The FID of *D. auratus* was significantly greater than *Craugastor* in response to the bird model at La Selva ($p = 0.0006$), Isais ($p = 0.015$), and Firestone ($p = 0.0012$) and the FID of *D. auratus* was significantly greater than *Craugastor* in response to the human at La Selva ($p = 0.0002$), Isais ($p < 0.001$), and Firestone ($p = 0.022$; Table 2).

The distance moved by *D. auratus* was significantly greater than *C. fitzingeri* at La Selva ($p = 0.003$) and Isais ($p = 0.05$); however, there was no significant difference in distance moved between *D. auratus* and *C. stejnegerianus* at Firestone ($p = 0.23$). The distance moved in response to the human was significantly greater for *D. auratus* at La Selva ($p = 0.004$), Isais ($p = 0.002$), and Firestone ($p = 0.006$; Table 2).

Dendrobates auratus did not exhibit movement as quickly as *Craugastor* in response to the bird model, which was reflected in their increased latency at La Selva ($p = 0.005$). However, there was no significant difference in the latency to movement between *D. auratus* and *Craugastor* in response to the bird model at Isais ($p = 0.07$) or Firestone ($p = 0.23$). *Dendrobates auratus* did not exhibit movement as quickly as *Craugastor* in response to the human, which was reflected in their increased latencies at La Selva ($p = 0.001$), Isais ($p = 0.0002$), and Firestone ($p = 0.009$; Table 2).

Bold behavior. In the boldness assay, *D. auratus* exhibited bolder behavior than *Craugastor,* which was reflected in their appearance, emergence, and waiting times. *Dendrobates auratus:* appeared at the door significantly more quickly than *Craugastor* at La Selva ($p < 0.0001$), Isais ($p < 0.0001$), and Firestone ($p < 0.0001$; Figure 4a); emerged from the cover object significantly more quickly at La Selva ($p < 0.0001$), Isais ($p < 0.0001$), and Firestone ($p < 0.0001$; Figure 4b); and had significantly shorter waiting times at La Selva ($p < 0.0001$), Isais ($p < 0.0001$), and Firestone ($p < 0.0001$; Figure 4c).

Antipredator behavior of an aposematic anuran species

Escape behavior of Dendrobates auratus *within populations.* The flight initiation distance of *D. auratus* was not significantly different in response to the bird model and human at La Selva ($p = 0.25$), Isais ($p = 0.54$), or Firestone ($p = 0.08$). The distance moved in response to the bird model and human was not significantly different at La Selva ($p = 0.86$), but *D. auratus* fled significantly farther, and typically directly away, from the human at Isais ($p = 0.04$) and Firestone ($p = 0.008$). The latency to movement was not significantly different in response to the bird model and human at La Selva ($p = 0.52$); however *D. auratus* exhibited movement more quickly in response to the bird, as represented by a decreased latency to movement at Isais ($p = 0.04$) and Firestone ($p = 0.01$; Table 2; Figures 5-7).

The flight initiation distance of *D. auratus* was not significantly different in response to the bird model and disk at La Selva ($p = 0.15$) or Firestone ($p = 0.53$), but *D. auratus* from Isais responded more quickly to the bird than the disk, which was reflected in their increased flight initiation distances ($p = 0.01$). The distance moved in response to the bird and disk was not significantly different at La Selva ($p = 0.15$) or Isais ($p = 0.81$), but was significantly greater in response to the disk at Firestone ($p = 0.002$). The latency to movement was not significantly different in response to the bird and disk models at La Selva ($p = 0.24$), Isais ($p = 0.86$), and Firestone ($p = 0.16$; Table 2).

Escape behavior of Dendrobates auratus *among populations*. The flight initiation distance in response to the bird model was not significantly different between *D. auratus* populations from La Selva and Isais ($p > 0.05$) or Isais and Firestone ($p > 0.05$). However, *D. auratus* from La Selva responded significantly more quickly to the bird and exhibited greater flight initiation distance than Firestone ($p < 0.05$). Further, the distance moved in response to the bird model was not significantly different between *D. auratus* populations from La Selva and Isais or Isais and Firestone, but the La Selva population moved significantly farther away from the human than the Firestone population ($p < 0.001$). The latency to movement was not significantly different between *D. auratus* from La Selva and Isais or Isais and Firestone; however, *D. auratus* at Firestone responded more quickly, reflected in their decreased latency, than La Selva ($p < 0.05$).

The flight initiation distance in response to the human was not significantly different between *D. auratus* populations from La Selva and Isais ($p > 0.05$) or Isais and Firestone ($p > 0.05$). The *D. auratus* population from La Selva had significantly greater flight initiation distance than the Firestone population ($p < 0.05$). The distance moved in response to the human was not significantly different between *D. auratus* populations from La Selva and Isais ($p > 0.05$), Isais and Firestone ($p > 0.05$), or La Selva and

Firestone ($p > 0.05$). The latency to movement was not significantly different in response to the human among populations (Table 2).

Bold behavior of Dendrobates auratus *among populations.* The appearance and emergence times of *D. auratus* were not significantly different across populations. The La Selva and Isais *D. auratus* populations were not significantly different in waiting time ($p = 0.39$), but Firestone had significantly greater waiting times than La Selva ($p < 0.001$) and Isais ($p < 0.001$; Figure 4).

Behavioral syndrome within and among Dendrobates auratus *populations.* Flight initiation distance in response to the bird model and human was not a predictor of boldness within or among populations of *D. auratus* (Table 3).

Alkaloid Analysis

GC–MS analysis of 60 *Dendrobates auratus* from La Selva, Isais, and Firestone resulted in the identification of 332 unique alkaloids (including isomers) from 20 different structural classes (Table 4). In total, 10 new alkaloids from four different structural classes were identified (Appendix 1).

The data on quantity of alkaloids was analyzed using only Isais and Firestone locations, but data on number of alkaloids was analyzed using all three geographic locations. Isais had a significantly greater quantity of alkaloids than Firestone (Global R = 0.99; $p < 0.0001$; Figure 8). With respect to diversity, the alkaloid profiles were significantly different among the La Selva, Isais, and Firestone populations (Global R = 0.99; $p < 0.0001$; Figure 9).

Color and Pattern

Brightness among populations. Dorsal green brightness of *D. auratus* populations did not differ significantly between La Selva and Isais ($p > 0.05$) or La Selva and Firestone ($p > 0.05$). The dorsal green of Firestone *D. auratus* was significantly brighter than that of Isais *D. auratus* ($p < 0.001$; Table 5; Appendix 2).

Chroma among populations. Dorsal green chroma did not differ significantly between La Selva and Isais or La Selva and Firestone populations of *D. auratus*. Firestone *D. auratus* had significantly greater dorsal green chroma than Isais *D. auratus* ($p < 0.001$; Table 5; Appendix 2).

Hue among populations. Dorsal green hue did not differ significantly between La Selva and Isais or La Selva and Firestone populations of *D. auratus*. Firestone *D. auratus* had significantly greater dorsal green hue than Isais *D. auratus* ($p < 0.001$; Table 5; Appendix 2).

Pattern among populations. Firestone *D. auratus* had a significantly greater proportion of black pattern than La Selva ($p < 0.001$) and Isais ($p < 0.001$). La Selva and Isais were not significantly different in their proportion of black pattern (Table 5).

Relationships between Behavior, Alkaloids, Color, and Pattern

Color and alkaloid quantity. There was no significant relationship between the dorsal brightness, chroma, or hue and alkaloid quantity of *D. auratus* within the Isais and Firestone populations (Table 6). After the removal of three Isais outliers, there is a weak, significant, inverse trend between both dorsal brightness and chroma with alkaloid quantity between the Isais and Firestone populations; however, there is no relationship between dorsal hue and alkaloid quantity between populations (Table 6, Figures 10-12).

Color and alkaloid diversity. There was no significant relationship between the dorsal brightness, chroma, or hue and alkaloid diversity of *D. auratus* with the La Selva, Isais, and Firestone populations (Table 7). Among populations, there was a weak, significant, inverse relationship between the dorsal brightness, chroma, and hue and alkaloid diversity of *D. auratus* (Table 7).

Color and behavior. There was no significant relationship between dorsal brightness, chroma, or hue and the flight initiation distance, distance moved, or latency to movement in response to the bird model or human at La Selva and Firestone (Table 8). Within Isais, as dorsal hue increased, the latency to movement in response to the human increased (Table 8). Among populations, there was an inverse relationship between dorsal brightness and both distance moved and latency to move in response to the bird model. There was no significant relationship among populations between dorsal chroma or hue and escape behavior (Table 8). There was no significant relationship between dorsal brightness, chroma, or hue and boldness within or among the La Selva, Isais, or Firestone populations (Table 9).

Pattern and alkaloid quantity. There was no significant relationship between the proportion of dorsal black pattern and alkaloid quantity of *D. auratus* from Isais or Firestone. There was an inverse relationship between dorsal black pattern and alkaloid quantity between populations (Table 6).

Pattern and alkaloid diversity. There was no significant relationship between the proportion of dorsal black pattern and alkaloid quantity of *D. auratus* within La Selva, Isais, and Firestone. There was an inverse relationship between dorsal black pattern and alkaloid quantity among populations (Table 7).

Pattern and behavior. There was no significant relationship between the proportion of dorsal black pattern and flight initiation distance, distance moved, or latency to movement in response to the bird model or human by *D. auratus* at La Selva and Firestone. There was a weak, significant trend between the proportion of dorsal black pattern and latency to movement in response to the bird by *D. auratus* at Isais. There was no significant relationship between the proportion of dorsal black pattern and escape behavior in response to the bird model and human among populations, with the exception of a weak, significant, inverse relationship between pattern and latency to move in response to the bird model (Table 8).

There was no significant relationship between the proportion of dorsal black pattern and boldness of *D. auratus* at La Selva and Isais. There was a weak, positive relationship between pattern and waiting time at Firestone. There were weak, positive relationships between the proportion of dorsal black pattern and boldness among populations (Table 9).

Alkaloid quantity and behavior. There was no significant relationship between alkaloid quantity and escape behavior in response to the bird model or human within and between Isais and Firestone (Table 10).

There was no significant relationship between alkaloid quantity and boldness within Isais (Table 11; Figure 13). There was a weak, positive relationship between alkaloid quantity and both appearance and emergence times within Firestone (Table 11;

Figure 14). There was no significant relationship between alkaloid quantity and boldness between Isais and Firestone (Table 11; Figure 15).

Alkaloid diversity and behavior. There was no significant relationship between alkaloid diversity and escape behavior in response to the bird model or human within La Selva, Isais, and Firestone (Table 12).

There was no significant relationship between alkaloid diversity and boldness within La Selva, Isais, and Firestone with the exception of a weak positive relationship between alkaloid diversity and waiting time at Isais. There was no significant relationship among populations between alkaloid diversity and appearance or emergence times; however, a weak inverse relationship existed between alkaloid diversity and waiting time (Table 13).

DISCUSSION

Differences in behavior between a cryptic and aposematic frog

Coloration among animals ranges from highly cryptic to conspicuous, endpoints of a continuum which can represent effective and alternative defensive strategies in avoiding predator attack (Ruxton *et al.*, 2004; Tullberg *et al.*, 2005; Rudh and Qvarnström, 2013). Cryptic prey rely on camouflage to reduce the probability of detection by predators (Cooper *et al.*, 2008a), whereas aposematic organisms rely on an innate or memorable association between their conspicuous coloration or pattern and secondary defense to deter predation (Willink *et al.*, 2014). The present study tested these alternative strategies by comparing the escape and bold behaviors of cryptic frogs in the genus *Craugastor* and the aposematic frog *Dendrobates auratus.*

Overall, *Craugastor* behaved in a more cryptic manner when compared to *D. auratus*, and did not typically exhibit movement in response to the simulated predators, whereas *D. auratus* exhibited different degrees and types of movement. The immobility by *Craugastor* suggests that they rely largely on camouflage to avoid being detected by predators. Prey should only initiate escape from an approaching predator when the risk of remaining in place outweighs the benefit (Cooper and Blumstein, 2015), therefore, cryptic prey, such as members of *Craugastor*, delay escape and rely on camouflage to reduce the chance of detection by a predator (Ruxton *et al.*, 2004). Furthermore, premature movement by a camouflaged prey could result in detection and capture (Broom and Ruxton, 2005). *Craugastor* have been found to rely on immobility in previous studies of escape behavior (Cooper *et al.*, 2008a; Ozel and Stynoski, 2011), supporting the findings of the current study.

Alternatively, *D. auratus* typically exhibited some type of movement in response to the simulated predators, either by pivoting, body raising, or escape. Pivoting has been documented in the dendrobatid frog *O. pumilio* and may be a simple behavior that, when associated with a conspicuous color, effectively sends a warning to potential predators (Blanchette *et al.*, 2017). Similarly, body raising (i.e., lifting its body off the ground, and stretching its legs vertically) may be a behavior that enhances the warning signal of *D. auratus,* while exposing the dorsum and its alkaloid-containing glands to a predator (Blanchette and Saporito, 2016, 2017). Some of the *D. auratus* in the current study exhibited fleeing (escape behavior) when approached by the simulated predators. Previous studies of escape behavior with *D. auratus* suggest that they flee when the risk of predation is perceived as high (Cooper *et al.*, 2009a, b). The cost of remaining immobile may have been higher than the benefit, thus *D. auratus* fled. Taken together, the current study supports previous findings that individual *Craugastor* rely on immobility to avoid predator detection (Cooper *et al.*, 2008a; Ozel and Stynoski, 2011), whereas *D. auratus* exhibits movement to enhance its warning signal to potential predators.

Consistency in defensive behavior may be correlated across different contexts such as escape from a simulated predator and measures of boldness when emerging from a cover object. *Craugastor* were also found to behave less boldly than *D. auratus,* as they remained under the cover object significantly longer than *D. auratus. Craugastor* relied on their crypsis and immobility when threatened by the simulated predator, a behavior that was reflected in the boldness assay. The cover object may have been perceived as a threat by *Craugastor* when they were placed underneath it, thereby remaining immobile to maintain crypsis. Further, cryptic frogs are preyed upon more frequently than aposematic frogs (Poulin *et al.*, 2001), which may result in cryptic frogs behaving less boldly than aposematic frogs. Emergence from a cover object is potentially a costly behavior, due to the risk of predation associated with exposure to the outside world

(Cooper and Blumstein, 2015). The additional time required for *Craugastor* to emerge from the cover object further supports the hypothesis that cryptic anurans will behave less boldly than aposematic anurans. The findings that *D. auratus* left the cover objects more quickly suggests that they behave more boldly and further supports the notion that they rely on their conspicuous warning signal to avoid predation (Cooper *et al.,* 2009b; Cooper and Blumstein, 2015). *Dendrobates auratus* and *Craugastor* behaved consistently across the escape and bold behavior assays, which was reflected in immobility by *Craugastor* and movement by *D. auratus.*

Anuran defensive behavior in response to different types of simulated predators

The escape behaviors of anurans are often studied using humans as a simulated predator (e.g., Cooper *et al.,* 2009a, b; Ozel and Stynoski, 2011; Pröhl and Ostrowski 2011; Dugas *et al*. 2015), and the use of more biologically relevant predators, such as birds, is much less common (Cooper *et al.,* 2008b; Willink *et al.,* 2013; Blanchette *et al.,* 2017). How potential threats are perceived is likely important to frog defensive behavior, requiring an understanding of how frogs respond to different simulated predators. To test for differences in escape behavior, the current study compared the escape behavior of *D. auratus* and *Craugastor* in response to two simulated predators: a human and a bird model.

There were no differences in the flight initiation distances of *D. auratus* in response to the human and bird predator; however, *D. auratus* moved significantly farther from the human and significantly more quickly in response to the bird. Further, *D. auratus* moved directly away or perpendicular from the human, but exhibited random movement in response to the bird. In the context of the study, the bird model was considered a biologically-relevant predator, but in order to determine whether or not frogs perceived the bird as a predator, a similarly colored and sized disk was used as a control. Unlike the bird model, *D. auratus* exhibited an escape response to the disk, mostly when the disk was overhead. When the disk was directly overhead of the frog, it casted a large shadow, and previous studies have found that predator shadow is an important cue when prey assess predation risk (Cooper, 2009b). It is therefore possible that the shadow cast by the disk explains the difference in movement of *D. auratus* between the disk and bird. Although the diameter of the disk was equivalent to the wingspan of the bird (33 cm), the shadow produced by the disk when directly overhead was larger than the bird's shadow and may have indicated to individuals that there was a higher immediate risk of predation. Conversely, the bird model had a more realistic silhouette and may have elicited more natural responses such as pivoting, body raising, or escape behavior while the bird was at increased distances, whereas the disk was not considered realistic at distance, but was instead perceived as a threat when a shadow was cast.

The behavior exhibited by *D. auratus* in the present study suggests that the type of predator presented to these frogs is important, depending on the behavioral variables being measured. *Dendrobates auratus* may have fled further from the human, as well as in an opposite direction, to avoid trampling by a large approaching threat. Previous studies of escape behavior have suggested that dendrobatids respond to an approaching human similarly and move to avoid trampling rather than predation (Cooper *et al.*, 2009a; Blanchette *et al.*, 2017). Conversely, the decreased latency to movement in response to the bird may be a reflection of *D. auratus* enhancing their aposematic coloration to warn the potential predator and deter attack. Using movement to enhance visibility is a well-documented strategy of dendrobatids under the threat of potential predation by simulated predators (e.g., Cooper *et al.*, 2009a, b; Ozel and Stynoski, 2011; Pröhl and Ostrowski 2011; Willink *et al.*, 2013; Blanchette *et al.*, 2017). Further, the current study suggests that the type and directionality of movement exhibited by aposematic frogs is dependent on the simulated predator approaching the frogs. Future studies should be aware of the potential difference in the escape response of an aposematic frog to different approaching predators when choosing simulated predator types.

Cryptic *Craugastor* typically remained immobile when approached by the human and bird predators. The immobility exhibited by *Craugastor* reflects the success of their cryptic behavior as an antipredator strategy, regardless of the simulated predator approaching. A small number of *Craugastor*, however, responded to the disk model, mostly when it was overhead, suggesting that cryptic anurans may also use shadows as a cue for the risk of potential predation. Overall, the immobility response by *Craugastor* to both the human and bird predator suggests that predator type may not be as important to consider when studying the escape behavior of cryptic anurans.

A continuum of color, chemical defense, and behavior in an aposematic frog

Crypsis and aposematism are generally considered as alternative antipredator strategies at the extreme ends of a color-, chemical defense-, and behavior-continuum (Ruxton *et al.,* 2004; Mappes *et al.,* 2005). At these extremes, cryptic organisms avoid predator detection through camouflage, are non-toxic, and behave less boldly, whereas aposematic organisms are conspicuous, toxic, and behave more boldly (Ruxton *et al.,* 2004; Caro, 2005). Although numerous studies have compared these extremes in cryptic and aposematic organisms (Cooper *et al.,* 2008a; Cooper *et al.,* 2009a; Pröhl and Ostrowski 2011), recent studies suggest that a similar continuum may exist within aposematic lineages (e.g., Wang, 2011; Willink *et al.,* 2013). In particular, among aposematic dendrobatid frogs, variation in warning coloration and chemical defenses suggests that a continuum may exist, wherein some populations are more conspicuous and chemically defended when compared to others (Mappes *et al.,* 2005). Although not yet fully examined, the antipredator behavior of dendrobatid frogs may also lie on a continuum, wherein highly conspicuous, chemically defended populations behave more boldly when compared to less conspicuous and less chemically defended populations.

The current study compared the chemical defense, color/pattern, and antipredator behavior between two populations of *D. auratus* — one on the Atlantic and one on the Pacific side of Costa Rica. The Atlantic population had a higher amount of alkaloid defenses, were more conspicuous, and behaved more boldly than the Pacific population. The average quantity of alkaloids in the Atlantic frogs was nearly 7.5 times greater (519.9 µg) than that of the Pacific frogs (68.7 µg). The Atlantic population also contained a greater diversity of alkaloids (58) than the Pacific population (20). The greater amount of chemical defenses in the Atlantic population was correlated with more green coloration and less black patterning, making these frogs appear more conspicuous, whereas the Pacific population had significantly more black patterning and appeared more cryptic. Further, the Atlantic population of *D. auratus* behaved more boldly than the Pacific population. Finally, body raising, a behavior thought to increase the visibility of the aposematic signal, was a behavior unique to frogs in the more cryptic Pacific population.

Differences in defense, appearance, and behavior between Atlantic and Pacific populations of *D. auratus* illustrates the continuum of defensive strategies employed by an aposematic frog. Although the Atlantic and Pacific populations of *D. auratus* utilize different strategies to avoid predation, these two populations are likely adapted to the specific selective pressures unique to each location. The combination of more conspicuous green and bolder behavior may indicate that Atlantic *D. auratus* are characteristically aposematic in their appearance and behavior. Previous studies have found that bolder organisms emerge from shelters more quickly than less-bold individuals (González–Bernal *et al.*, 2014); further, dendrobatids are characterized as bold due to their visibility and leisurely movements even in the presence of potential predators (Cooper *et al.*, 2009a, b; Pröhl and Ostrowski, 2011). Conversely, the more cryptic Pacific population was less bold when emerging from the cover object and exhibited body raising in response to the simulated predators (Blanchette and Saporito, 2017). Body raising is a behavior intended to startle a potential predator and is thought to enhance coloration or expose the dorsum where the majority of alkaloids are present, thereby deterring predation (Ruxton *et al.*, 2004; Blanchette and Saporito, 2016). Although the Pacific population had increased black patterning (making them appear more cryptic), body raising may increase the efficacy of their warning signal by eliciting a stronger startle response by potential predators. Body raising has also been documented in *Ameerega flavopicta*, an aposematic dendrobatid that is mostly black with minimal conspicuous yellow or white coloration (Toledo *et al.*, 2004). Body raising may startle a potential predator enough to allow for escape or deter the predator completely, preventing the loss of an acquired resource (e.g. food, mates, reproductive resources) or energy expended by fleeing (Ruxton *et al.*, 2004), which may explain why some individuals within the Pacific population exhibit this behavior. Avian predators primarily rely on color cues when making foraging decisions; however, the Pacific population of *D. auratus* may combine their cryptic appearance with less bold behavior to avoid predator detection, given that they are less chemical defended. Visually-oriented predators such as birds may not necessarily avoid conspicuous prey, but make decisions to forage depending on the nutritional value of the aposematic prey, nutritional value of alternative prey, and their own condition (e.g., energetic needs or toxin burden; Richards-Zawacki, 2013; Skelhorn *et al.*, 2016). Previous studies have found that pattern decreases attack rates on models when compared to non-patterned models in insects (Schaefer and Stobbe, 2006), frogs (Rojas *et al.*, 2014; Hämäläinen *et al.*, 2015), and snakes (Brodie, 1993); thus, the Pacific population of *D. auratus* may experience the benefit of crypsis and less predation by employing a more cryptic strategy through increased patterning. Conversely, the Atlantic population of *D. auratus* may be adequately defended against potential chemosensory oriented arthropod predators as well as avian predators and can therefore be more conspicuous in appearance and behavior.

Although aposematism is typically thought to be an alternative strategy to crypsis, the current study supports the presence of a continuum from cryptic to aposematic strategies, even within an aposematic species. Crypsis and aposematism may not be mutually exclusive strategies (Mappes *et al.*, 2005), which explains why the Atlantic population of *D. auratus* was more characteristically aposematic in chemical defense, conspicuousness, pattern, and behavior than the Pacific population. The combination of pattern and color may represent a single strategy along the continuum of morphological defense. Pattern may prevent detection at a distance, but conspicuous color provides an effective aposematic signal at close distance (Barnett *et al.*, 2016), which may explain the increased black pattern of the Pacific population. The gradient of antipredator strategies that includes morphology and behavior may be the result of shifting solutions to predator encounters that increases phenotypic and behavioral diversification over time (Willink *et al.*, 2013). The explanation for variation in display and defense across dendrobatids has been attributed to many factors, including predator variation (Endler and Mappes, 2004; Bolton *et al.*, 2017), diet availability (Summers and Clough, 2001; Saporito *et al.*, 2007a), sexual selection (Maan and Cummings, 2008; Gade *et al.*, 2016), and genetic differences (Daly *et al.*, 1995); however, the current study supports the role of diet availability and predator variation and their impact on alkaloid chemical defense, color/pattern, and antipredator behavior. The current study demonstrates that there is variation within and among populations of *D. auratus* with respect to antipredator defense, and provides a foundation for future work to continue and determine how or why this variation exists.

Further, a better understanding of the morphological adaptations of *D. auratus* may give more insight into its antipredator behavior and explain the behavioral differences that are observed among populations.

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Table 1. The number of *Dendrobates auratus* and *Craugastor* that exhibited no movement, pivoting, only body raising, body raising (BR) and escape, or only escape behavior in response to the simulated bird, human, and disk predators at La Selva, Isais, and Firestone.

		Bird					**Human**					**Disk**				
		No Movement	**Pivot**	**Body Raising**	**BR + Escape**	**Escape**	**No Movement**	**Pivot**	**Body Raising**	**BR + Escape**	**Escape**	**No Movement**	**Pivot**	**Body Raising**	**BR + Escape**	**Escape**
Dendrobatesa uratus	**La Selva**	7	2	0	0	11	5	2	0	0	13	7	1	0	0	12
	Isais	11	5	0	0	4	4	3	0	0	13	11	1	0	0	8
	Firestone	10	0	10	0	0	5	1	7	2	5	6	1	3	6	4
Craugastor	**La Selva**	17	2	0	0	1	15	1	0	0	4	12	1	0	0	7
	Firestone	20	0	0	0	0	18	1	0	0	1	16	0	0	0	4

Table 2. The average flight initiation distance (FID), distance moved (DM), and latency (LAT) to movement of *Dendrobates auratus* and *Craugastor* in response to the bird, human, and disk predators at La Selva, Isais, and Firestone. Standard error values (±1 S.E.) are reported.

		Bird			**Human**			**Disk**		
		FID (cm)	**DM (cm)**	**LAT (s)**	**FID (cm)**	**DM (cm)**	**LAT (s)**	**FID (cm)**	**DM (cm)**	**LAT (s)**
Dendrobates auratus	**La Selva**	97.8±36.9	54.2±13.9	3.0±0.5	158.5±43.7	52.4±13.6	3.4±0.5	66.1±40.4	32.6±9.8	2.6±0.5
	Isais	122.3±45.5	18.6±10.4	1.9±0.5	137.2±40.1	57.4±13.6	3.7±0.5	0.04±0.01	17.3±6.5	1.9±0.5
	Firestone	23.0±14.2	0.02±0.01	0.7±0.4	35.27±16.2	27.36±10.7	2.3±0.6	0.06±0.01	35.91±10.9	2.2±0.5
***Craugastor* sp.**	**La Selva**	2.5±2.5	0.8±0.7	0.7±0.4	13.3±6.2	26.1±12.1	1.0±0.4	0.04±0.01	42.8±14.1	1.4±0.4
	Firestone	0±0	0±0	0±0	24.5±24.5	1.0±1.0	0.4±0.3	0.02±0.01	11±5.3	0.8±0.4

Table 3. The relationship between the flight initiation distance of *Dendrobates auratus* in response to the bird model or human and boldness (as measured by appearance, emergence, and waiting time) within and among the populations at La Selva, Isais, and Firestone.

		Bird		**Human**	
		FID		**FID**	
		R^2	**p**	**R^2**	**p**
Appearance	**La Selva**	0.01	0.67	0.04	0.40
	Isais	0.03	0.46	0.06	0.30
	Firestone	0.04	0.42	0.02	0.57
	Among	0.03	0.18	0.05	0.09
Emergence	**La Selva**	0.02	0.60	0.05	0.37
	Isais	0.02	0.38	0.09	0.20
	Firestone	0.03	0.45	0.02	0.57
	Among	0.03	0.19	0.05	0.08
Waiting	**La Selva**	0.04	0.75	0.05	0.34
	Isais	0.002	0.86	0.16	0.08
	Firestone	0.04	0.42	0.06	0.28
	Among	0.02	0.25	0.04	0.12

Table 4. Alkaloids detected in *Dendrobates auratus* arranged by structural class.

	1,4-Q	3,5-I	3,5-P	5,6,8-I	5,8-I	aPTX	Dehydro-5,8-I	Deoxy-hPTX	Deoxy-PTX	DHQ	hPTX	HTX	Izidine	Lehm	Pip	PTX	Pyr	SpiroP	Tri	Unclass	
	257D	**211E**	**211O**	**251M**	**193New**	**223E**	**201A**	**193F**	**265X**	**195A**(2)	**223G**	**235A**	**195K**	**275A**(3)	**183A**	**209F**(4)	**211T**	**222**	**205H**	**195E**	
	279E(2)	**223AB**(6)	**223B**(7)	**221P**	**195I**(4)	**225E**	**205L**(6)	**207O**	**281B**	**219A**(4)		**259A**(3)	**207T**	**277A**(2)	**225B**	**277B**		**234**	**207GH**(6)	**197D**(2)	
		247C	**223H**(2)	**221Q**(3)	**203A**	**267A**(2)	**207E**			**219C**		**261A**	**221N**		**225I**(2)			**236**(3)	**207J**	**209G**	
		275C	**249I**	**223A**(7)	**205A**(4)	**341A**	**207W**			**221D**		**283A**			**239I**(2)			**252B**(3)	**221G**(3)	**227**	
			251K(7)	**225K**	**207A**(7)		**265F**			**243A**(3)		**285A**(9)			**239L**(2)				**235I**(2)	**231F**	
			265J	**225L**	**207Q**					**245E**		**287A**(3)							**261F**	**235BB**	
				231B(2)	**209B**(2)					**245Q**		**287D**(5)							**263M**	**235K**	
				235E(2)	**209I**(2)					**251A**		**287L**								**235S**(2)	
				237C	**209S**					**267L**(3)		**291A**(2)								**267DD**	
				237L	**217B**					**269AB**(6)										**267G**	
				237S	**219F**					**269A**(4)										**267I**(12)	
				249U	**219J**					**269B**(7)										**267M**(3)	
				251T	**221A**					**271D**(8)										**269E**	
				253H	**221H**					**275B**(3)										**281C**	
				273A	**223D**(3)															**305H**	
				277C	**223V**																
					231C																
					233D(3)																
					235B(2)																
					237D																
					245D(2)																
					247E																
					249O																
					251O(3)																
					271A(2)																
					273B(2)																
					273C																
					273D																
					275F																
Total	2	4	6	16	29	4	5	2	2	14	1	9	3	2	5	2	1	4	7	15	**133**

Alkaloids present in quantities greater than 0.05µg in at least one frog are listed, except in the case of new alkaloids (see **Appendix 1**). Abbreviations for alkaloid classes are as follows with full names within parentheses: 1,4-Q (1,4-disubstituted quinolizidine); 3,5-I (3,5-disubstituted indolizidine); 3,5-P (3,5-disubstituted pyrrolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine); 5,8-I (5,8-disubstituted indolizidine); aPTX (allopumiliotoxin); Dehydro-5,8-I (dehydro-5,8-disubstituted indolizidine); Deoxy-hPTX (deoxy-homopumiliotoxin); Deoxy-PTX (deoxy-pumiliotoxin); DHQ (decahydroquinoline); hPTX (homopumiliotoxin); HTX (histrionicotoxin); Lehm (lehmizidine); Pip (piperidine); PTX (pumiliotoxin); Pyr (pyrrolidine); SpiroP (spiropyrrolizidine); Tri (tricyclic); Unclass (unclassified).

Table 5. The average brightness, chroma, hue, and proportion of black pattern of *Dendrobates auratus* at La Selva, Isais, and Firestone. Standard error values (±1 S.E.) are reported.

	Brightness	**Chroma**	**Hue**	**Proportion of Black**
La Selva	68.3±2.3	32.7±1.2	1.1±0.2	57.0±1.0
Isais	59.6±2.8	28.2±1.3	0.5±0.2	53.2±1.2
Firestone	73.6±3.0	35.3±1.7	1.3±0.02	71.5±0.7

Table 6. The relationship between color (dorsal brightness, chroma, and hue), pattern, and alkaloid quantity within and between the Isais and Firestone populations of *Dendrobates auratus.* Significant values are bolded.

	Isais		**Firestone**		**Between**	
	R^2	**p**	**R^2**	**p**	**R^2**	**p**
Brightness	0.06	0.29	0.09	0.18	**0.20**	**0.005**
Chroma	0.04	0.41	0.10	0.17	**0.17**	**0.01**
Hue	0.003	0.82	0.10	0.17	0.04	0.23
Pattern	0.006	0.92	0.019	0.56	**0.36**	**<0.001**

Table 7. The relationship between color (dorsal brightness, chroma, and hue), pattern, and alkaloid diversity within and among the La Selva, Isais, and Firestone populations of *Dendrobates auratus.* Significant values are bolded.

	La Selva		**Isais**		**Firestone**		**Among**	
	R^2	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**
Brightness	0.02	0.58	0.02	0.59	0.09	0.20	**0.16**	**0.003**
Chroma	0.03	0.52	0.03	0.47	0.06	0.32	**0.15**	**0.003**
Hue	<0.001	0.93	<0.001	0.98	0.01	0.61	**0.13**	**0.007**
Pattern	0.04	0.42	<0.001	0.97	0.02	0.55	**0.42**	**<0.001**

Table 8. The relationship between dorsal brightness, chroma, hue, and proportion of black pattern of *Dendrobates auratus* and the flight initiation distance (FID), distance moved (DM), and latency (LAT) in response to the bird model or human at La Selva, Isais, and Firestone. Significant values are bolded.

		Bird						**Human**					
		FID		**DM**		**LAT (s)**		**FID**		**DM**		**LAT (s)**	
		R^2	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**
Brightness	**La Selva**	0.06	0.38	0.05	0.41	0.04	0.45	0.005	0.81	0.004	0.83	0.10	0.24
	Isais	0.04	0.39	0.06	0.30	0.03	0.46	0.07	0.25	0.14	0.11	0.08	0.21
	Firestone	0.03	0.46	0.04	0.37	0.04	0.37	0.005	0.77	0.003	0.94	0.002	0.87
	Among	0.02	0.29	**0.16**	**0.003**	**0.15**	**0.003**	0.01	0.39	0.06	0.06	0.05	0.11
Chroma	**La Selva**	0.07	0.33	0.06	0.38	0.04	0.45	0.02	0.58	0.005	0.81	0.01	0.23
	Isais	0.04	0.42	0.11	0.15	0.03	0.46	0.05	0.35	0.11	0.15	0.09	0.19
	Firestone	0.01	0.63	0.04	0.42	0.03	0.43	0.0004	0.93	0.004	0.79	0.01	0.62
	Among	0.01	0.42	<0.01	0.96	0.01	0.39	0.0001	0.90	0.03	0.21	0.009	0.49
Hue	**La Selva**	0.05	0.44	0.001	0.92	0.03	0.51	0.25	0.06	0.02	0.64	0.03	0.54
	Isais	0.03	0.47	0.15	0.10	0.06	0.29	<0.001	0.99	0.007	0.73	**0.22**	**0.04**
	Firestone	0.02	0.59	0.01	0.67	0.01	0.70	0.10	0.18	0.09	0.20	0.15	0.09
	Among	0.002	0.73	0.03	0.19	0.05	0.77	0.06	0.08	0.03	0.78	0.01	0.38
Proportion of Pattern	**La Selva**	0.004	0.78	0.01	0.64	0.03	0.48	0.15	0.09	0.1	0.19	0.04	0.37
	Isais	0.06	0.29	<0.01	0.98	0.05	0.35	0.07	0.26	0.003	0.81	**0.22**	**0.04**
	Firestone	0.04	0.39	0.10	0.18	0.09	0.19	0.03	0.46	0.02	0.53	0.03	0.46
	Among	0.03	0.18	0.05	0.08	**0.07**	**0.03**	0.06	0.07	0.02	0.27	0.007	0.53

Table 9. The relationship between the dorsal brightness, hue, chroma, and proportion of black pattern of *Dendrobates auratus* and appearance, emergence, and waiting times at La Selva, Isais, and Firestone. Significant values are bolded.

		Appearance		**Emergence**		**Waiting**	
		R^2	**p**	**R^2**	**p**	**R^2**	**p**
Brightness	**La Selva**	0.02	0.66	0.02	0.65	0.02	0.64
	Isais	0.05	0.33	0.05	0.35	0.01	0.66
	Firestone	0.05	0.33	0.03	0.44	0.004	0.80
	Among	0.009	0.48	0.02	0.27	0.05	0.12
Chroma	**La Selva**	0.004	0.82	0.006	0.79	0.01	0.67
	Isais	0.05	0.33	0.04	0.38	0.001	0.87
	Firestone	0.03	0.44	0.03	0.45	0.008	0.71
	Among	0.004	0.66	0.004	0.65	0.006	0.60
Hue	**La Selva**	0.10	0.26	0.09	0.27	0.05	0.44
	Isais	0.001	0.88	<0.001	0.99	0.02	0.51
	Firestone	0.02	0.56	0.009	0.69	0.002	0.84
	Among	0.002	0.77	0.003	0.67	0.006	0.58
Proportion of Pattern	**La Selva**	0.02	0.59	0.01	0.62	0.002	0.85
	Isais	0.05	0.33	0.06	0.28	0.06	0.28
	Firestone	0.05	0.34	0.04	0.40	**0.21**	**0.04**
	Among	**0.07**	**0.04**	**0.10**	**0.01**	**0.07**	**0.04**

Table 10. The relationship between alkaloid quantity and escape behavior (flight initiation distance, FID; distance moved, DM; and latency to movement, LAT) of *Dendrobates auratus* at Isais and Firestone.

	Bird						**Human**					
	FID		**DM**		**LAT**		**FID**		**DM**		**LAT**	
	R^2	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**
Isais	0.03	0.45	0.08	0.22	0.15	0.10	0.08	0.24	0.02	0.55	0.05	0.41
Firestone	0.04	0.39	0.06	0.31	0.05	0.32	0.01	0.62	0.001	0.90	0.03	0.45
Between	0.002	0.79	0.002	0.81	0.01	0.53	0.002	0.81	0.02	0.42	0.06	0.14

Table 11. The relationship between alkaloid quantity and boldness (as measured by appearance, emergence, and waiting times) within and between populations of *Dendrobates auratus* at Isais and Firestone. Significant values are bolded.

	Appearance		**Emergence**		**Waiting**	
	R^2	**p**	**R^2**	**p**	**R^2**	**p**
Isais	0.006	0.74	0.005	0.78	<0.01	0.97
Firestone	**0.20**	**0.05**	**0.35**	**<0.01**	0.05	0.37
Between	<0.01	0.98	<0.01	0.92	0.02	0.45

Table 12. The relationship between alkaloid diversity and escape behavior (flight initiation distance, FID; distance moved, DM; and latency to movement, LAT) within and among populations of *Dendrobates auratus* at La Selva, Isais, and Firestone.

	Bird						**Human**					
	FID		**DM**		**LAT**		**FID**		**DM**		**LAT**	
	R^2	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**
La Selva	0.12	0.13	0.13	0.13	0.30	0.59	0.03	0.50	0.53	0.48	0.05	0.35
Isais	0.005	0.76	0.06	0.30	0.004	0.79	0.13	0.11	0.006	0.75	0.001	0.92
Firestone	0.13	0.11	0.06	0.31	0.06	0.28	0.13	0.12	0.02	0.59	0.004	0.78
Among	0.02	0.29	0.003	0.66	0.01	0.45	0.02	0.23	0.01	0.41	0.02	0.30

Table 13. The relationship between alkaloid diversity and boldness (as measured by appearance, emergence, and waiting times) within and among populations of *Dendrobates auratus* at La Selva, Isais, and Firestone. Significant values are bolded.

	Appearance		**Emergence**		**Waiting**	
	R^2	**p**	**R^2**	**p**	**R^2**	**p**
La Selva	0.005	0.76	0.004	0.80	0.004	0.94
Isais	0.19	0.054	0.08	0.24	**0.23**	**0.03**
Firestone	0.009	0.69	0.006	0.75	0.007	.072
Among	0.019	0.30	0.041	0.12	**0.08**	**0.03**

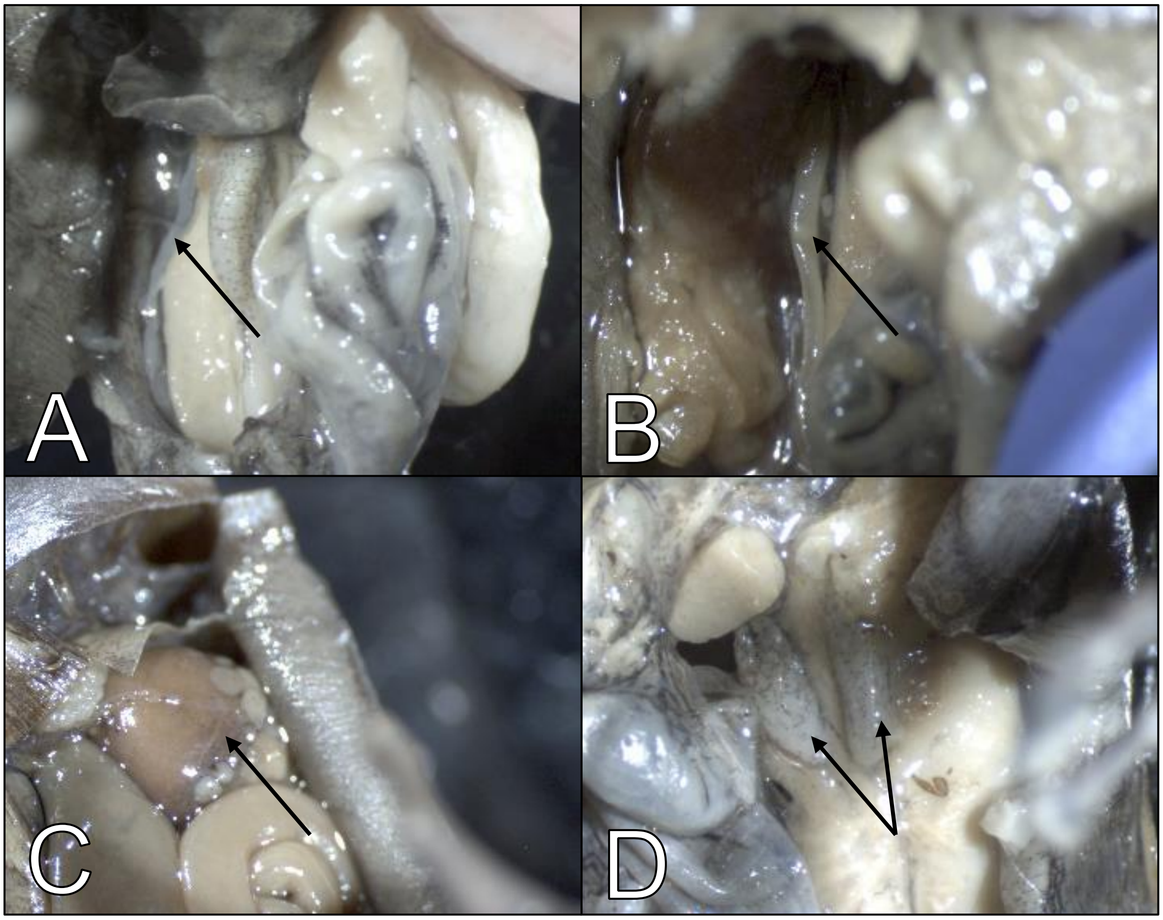


Figure 1. Key features of males and females to aid in sex determination. Incisions were made laterally on the ventral side of each individual. The arrow is pointing to (A) oviduct; (B) oviduct with egg; (C) eggs; (D) testes.

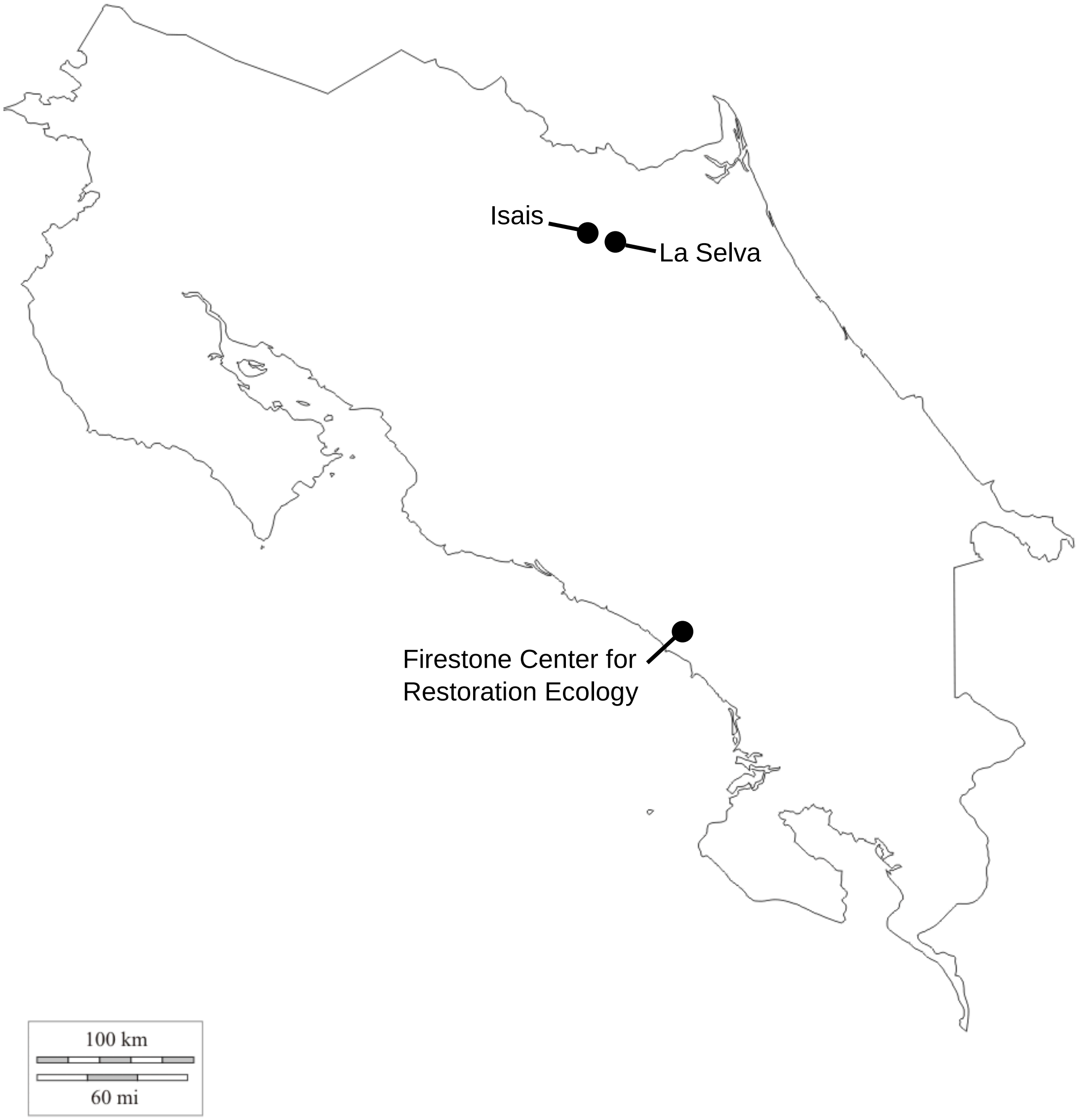


Figure 2. Map of Costa Rica showing the three populations of *Dendrobates auratus* used in the current study.

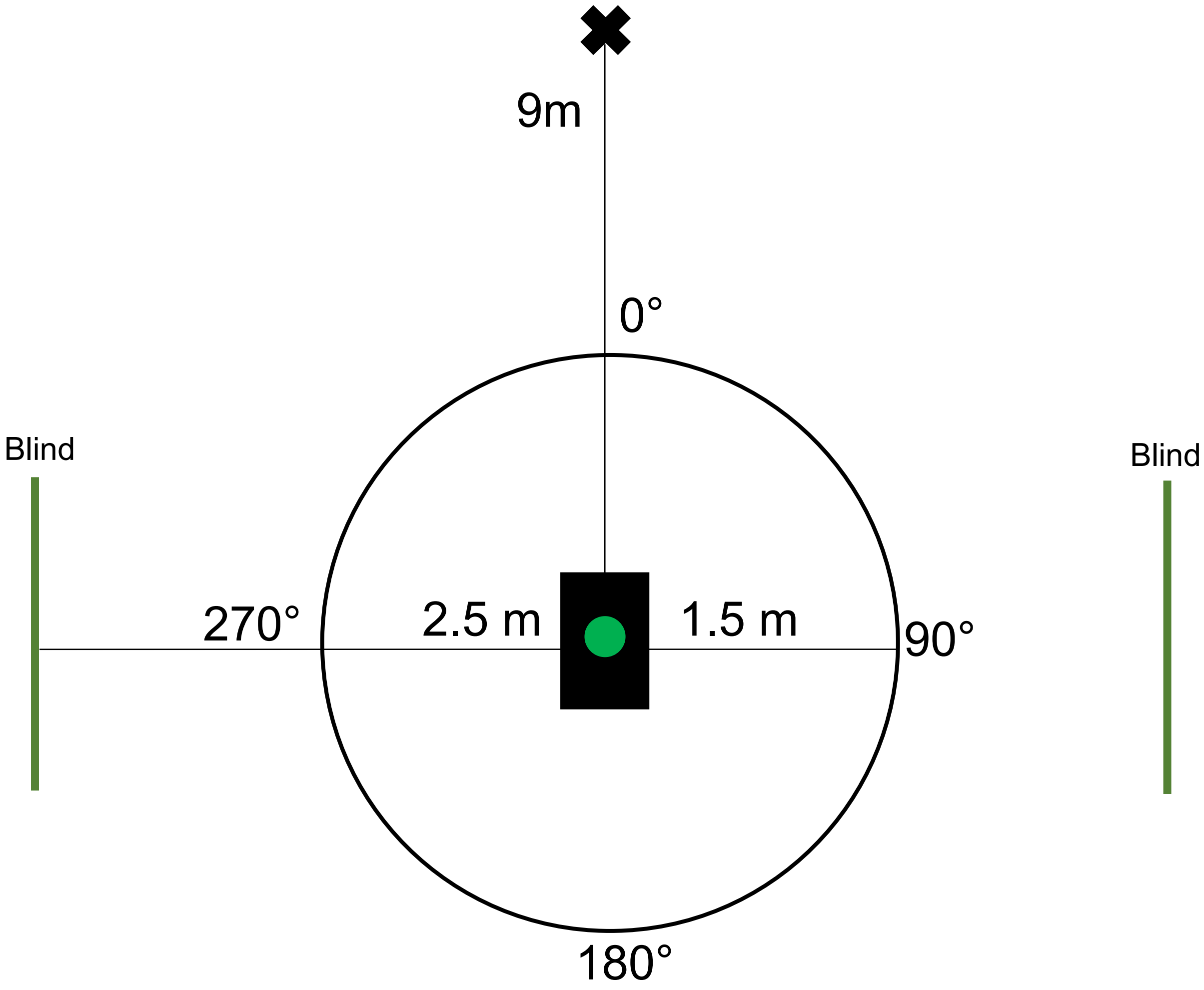


Figure 3. A schematic of the experimental design for the behavioral assays measuring flight initiation distance (FID), latency, angle of escape, and distance fled for human and bird simulated predator approach (including control shape). The start position (✖) was 9 meters from the frog (●). There was a 1.5m boundary for the distance fled and dark green blinds placed at 2.5m prevented view of the researcher by the frog.

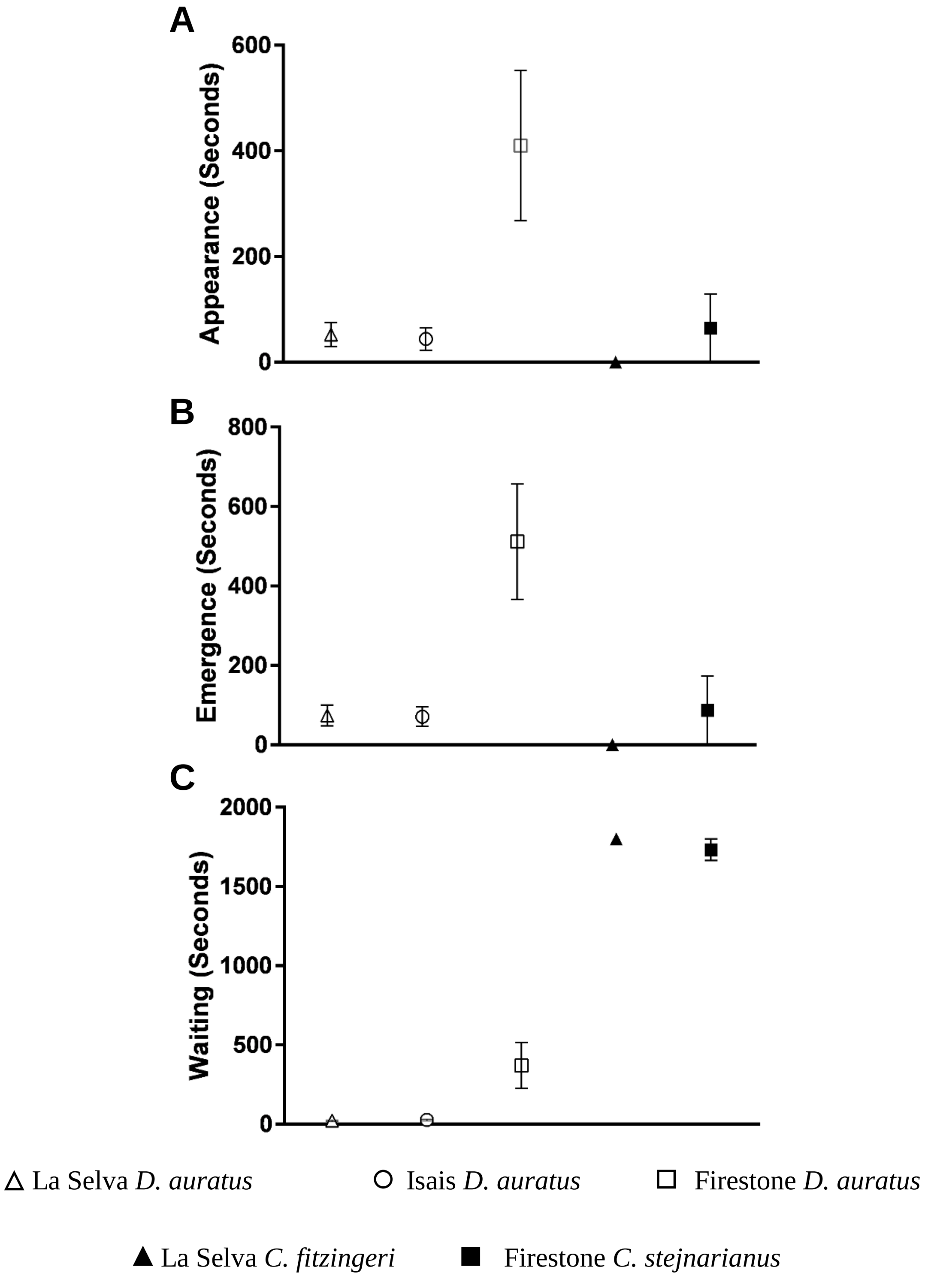


Figure 4. The average (**A**) appearance, (**B**) emergence, and (**C**) waiting times for *Dendrobates auratus* and *Craugastor* at La Selva, Isais, and Firestone. Standard error (± 1 S.E.) bars are reported.

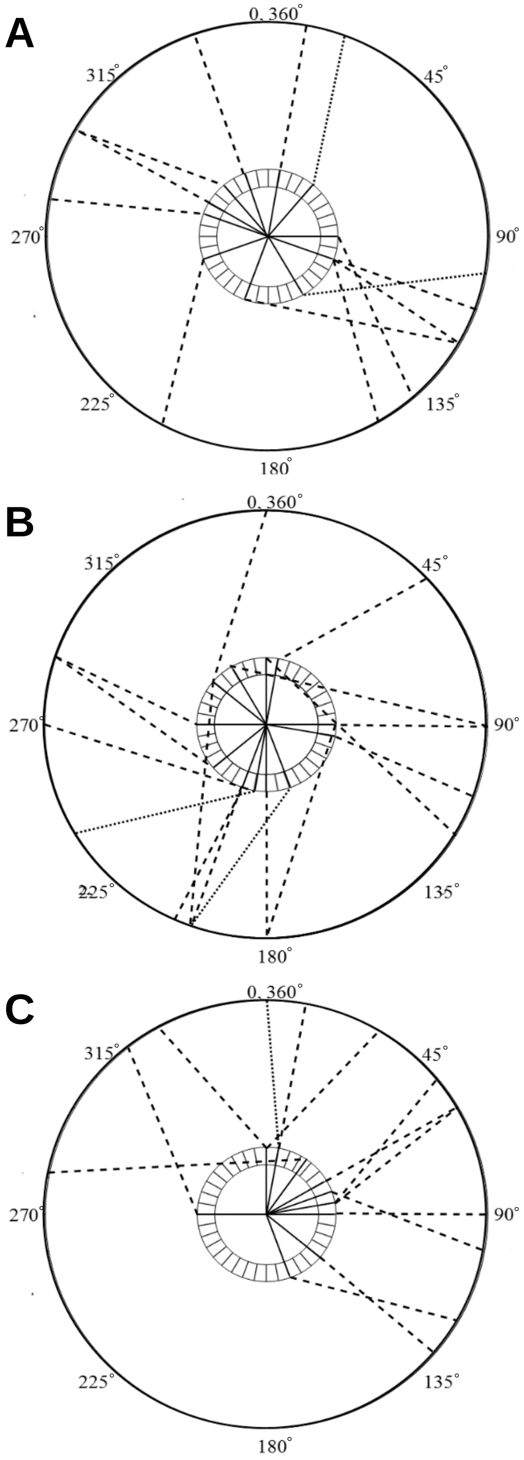


Figure 5. The initial angles and response angles of *Dendrobates auratus* during the (**A**) bird, (**B**) human, and (**C**) disk escape behavior assays at La Selva. The initial facing angles are represented by solid lines; escape angles are represented by dashed lines; and pivot angles are represented by dotted lines.

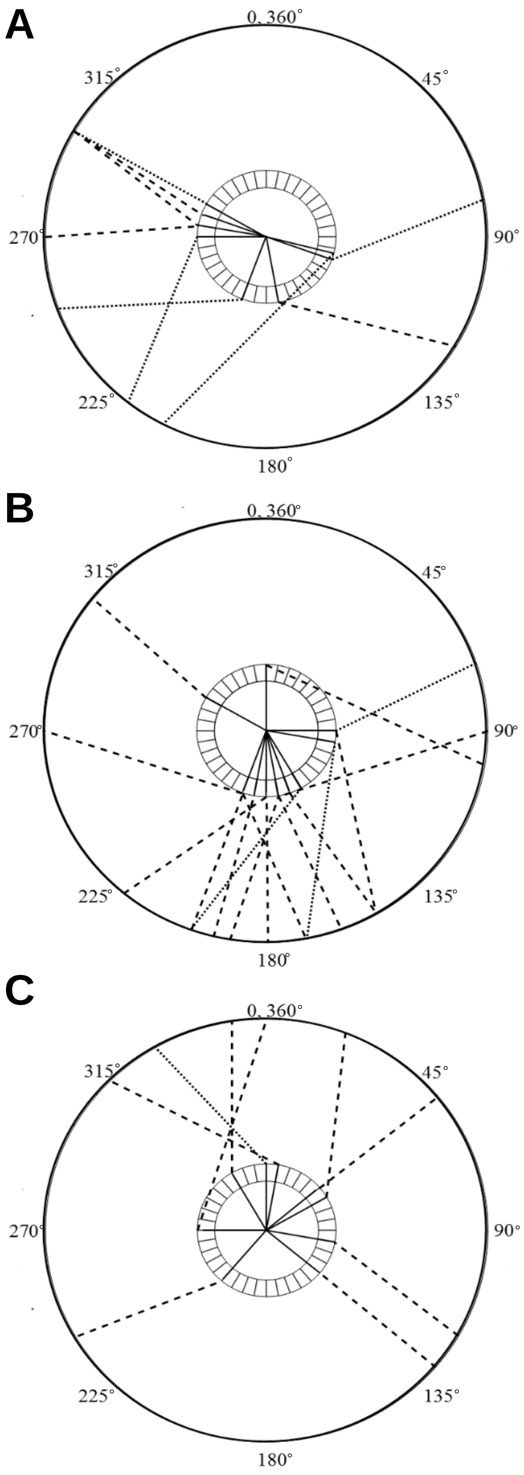


Figure 6. The initial angles and response angles of *Dendrobates auratus* during the (**A**) bird, (**B**) human, and (**C**) disk escape behavior assays at Isais. The initial facing angles are represented by solid lines; escape angles are represented by dashed lines; and pivot angles are represented by dotted lines.

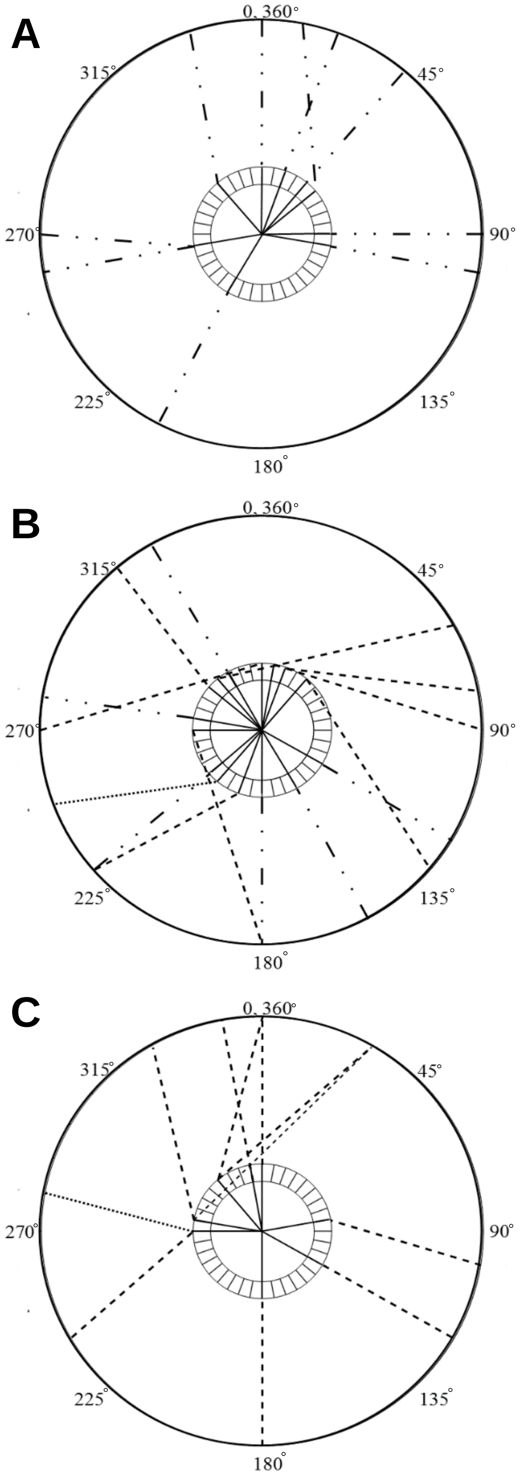


Figure 7. The initial angles and response angles of *Dendrobates auratus* during the (**A**) bird, (**B**) human, and (**C**) disk escape behavior assays at Firestone. The initial facing angles are represented by solid lines; escape angles are represented by dashed lines; pivot angles are represented by dotted lines; and body raising angles are represented by dashed/dotted lines.

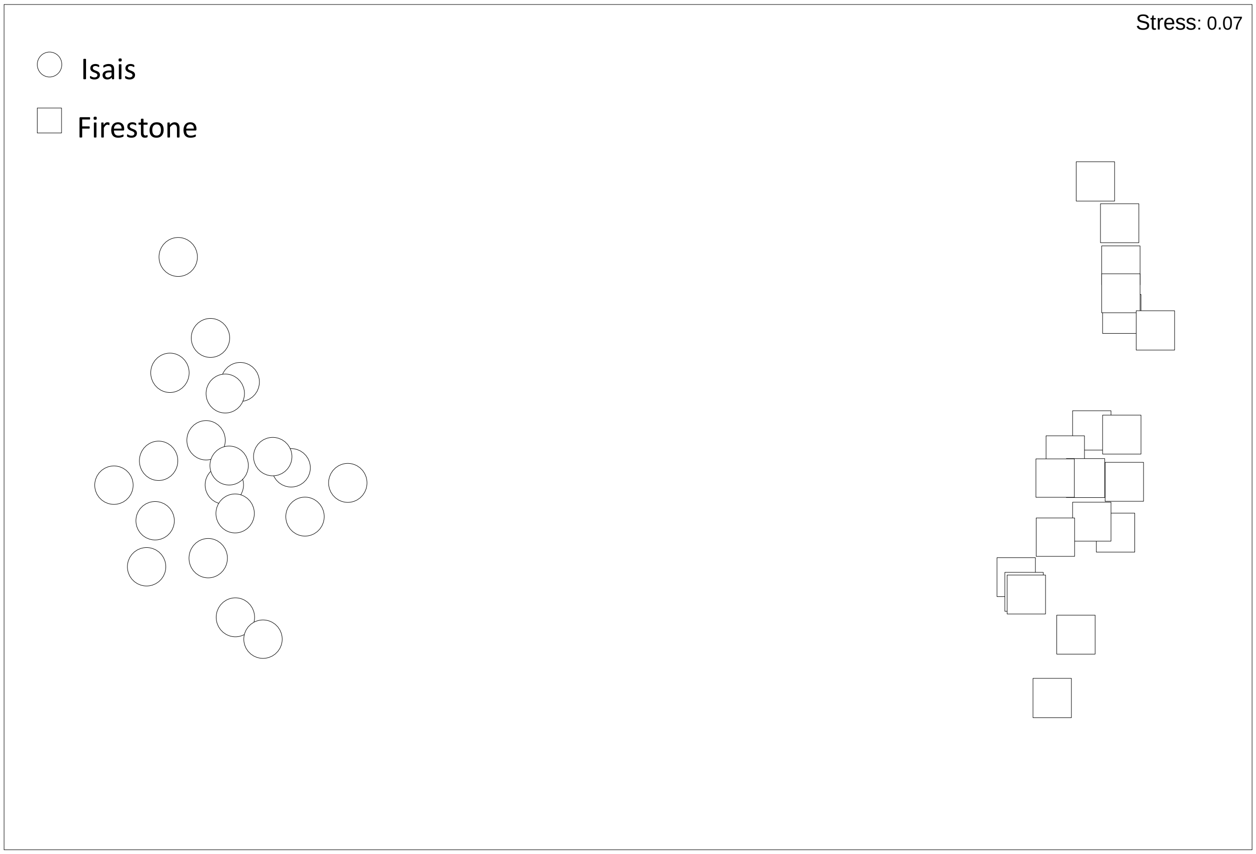


Figure 8. nMDS plot of variation in alkaloid quantity of *Dendrobates auratus* at Isais and Firestone, Costa Rica. Each symbol represents an individual frog. The distance between any two symbols represents the proportional difference in alkaloid quantity between those two individual frogs.

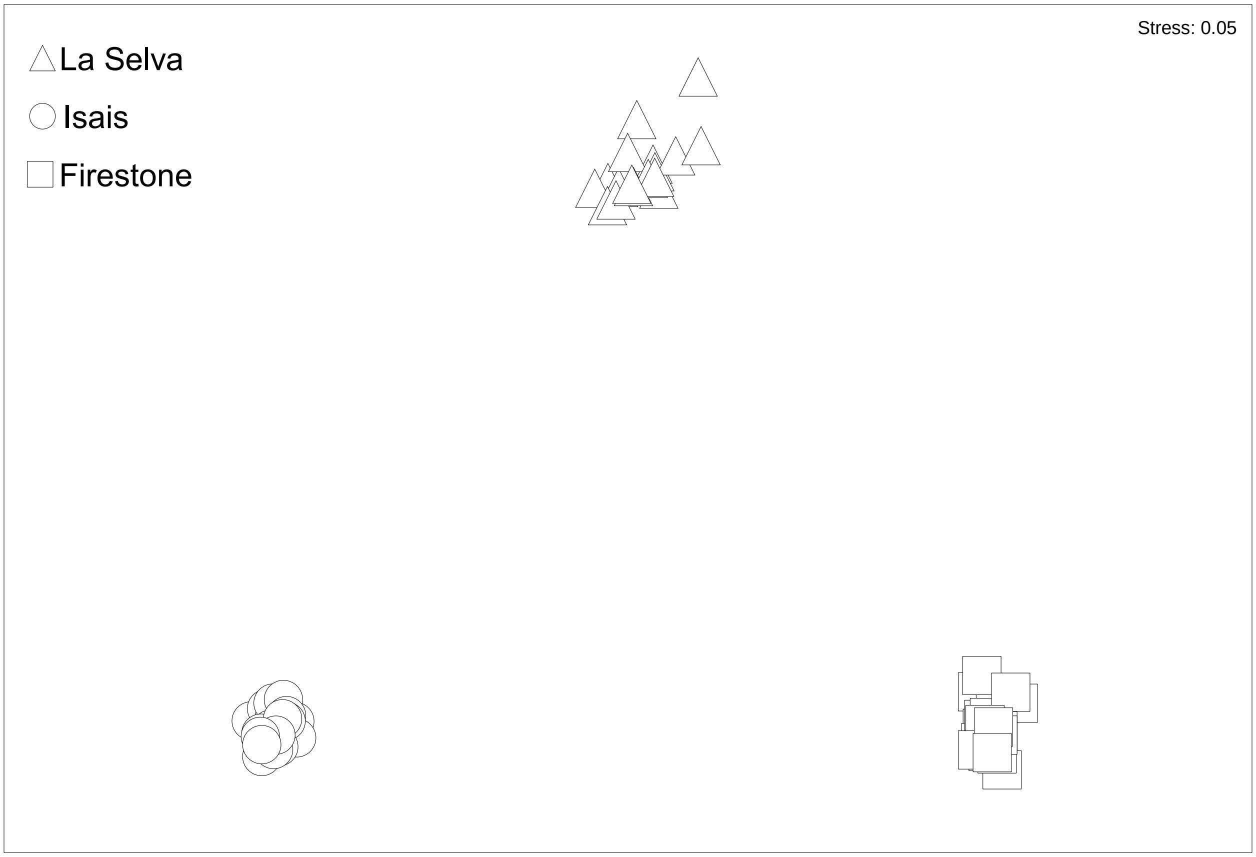


Figure 9. nMDS plot of variation in alkaloid diversity of *Dendrobates auratus* at La Selva, Isais and Firestone, Costa Rica. Each symbol represents an individual frog. The distance between any two symbols represents the proportional difference in alkaloid diversity between those two individual frogs.

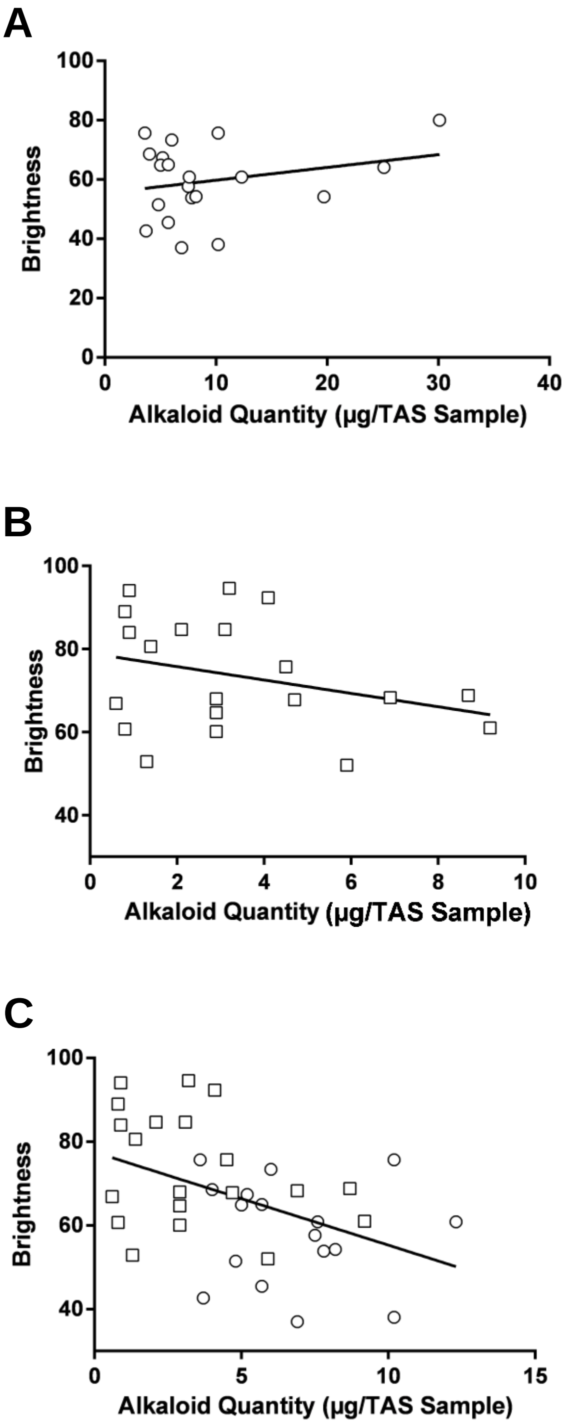


Figure 10. The relationship between alkaloid quantity and green dorsal brightness of *Dendrobates auratus* (**A**) within Isais, (**B**) within Firestone, and (**C**) between populations.

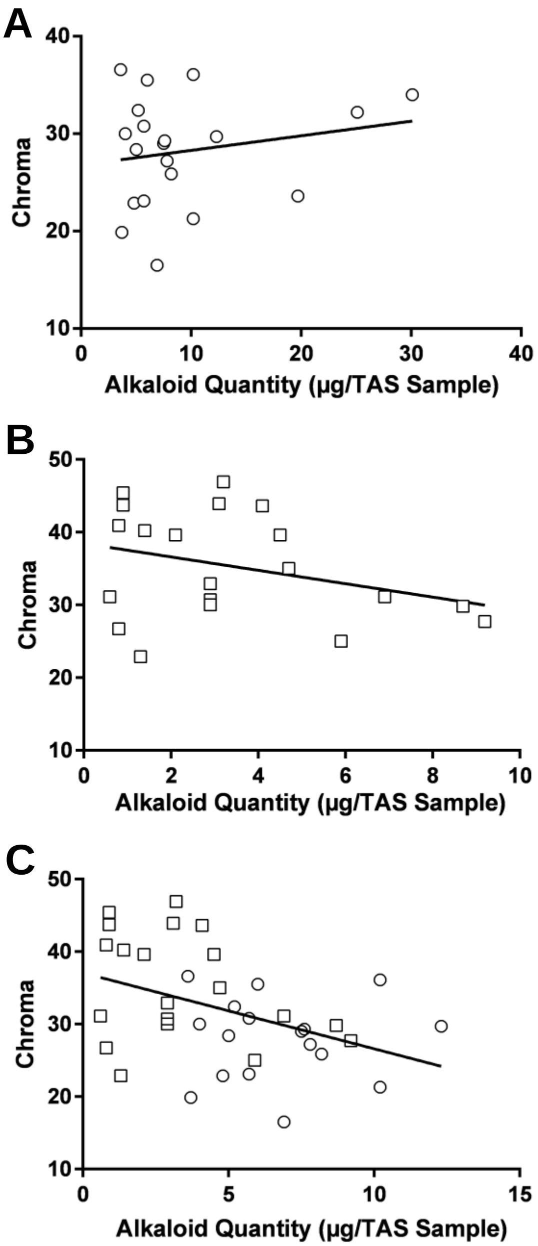


Figure 11. The relationship between alkaloid quantity and green dorsal chroma of *Dendrobates auratus* (**A**) within Isais, (**B**) within Firestone, and (**C**) between populations.

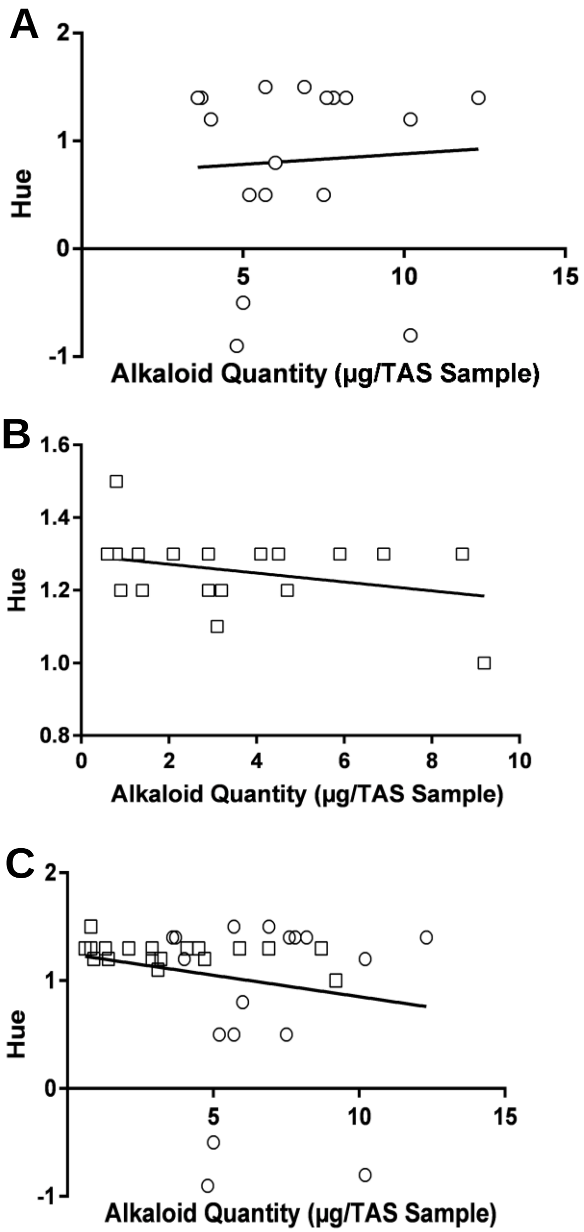


Figure 12. The relationship between alkaloid quantity and green dorsal hue of *Dendrobates auratus* (**A**) within Isais, (**B**) within Firestone, and (**C**) between populations.

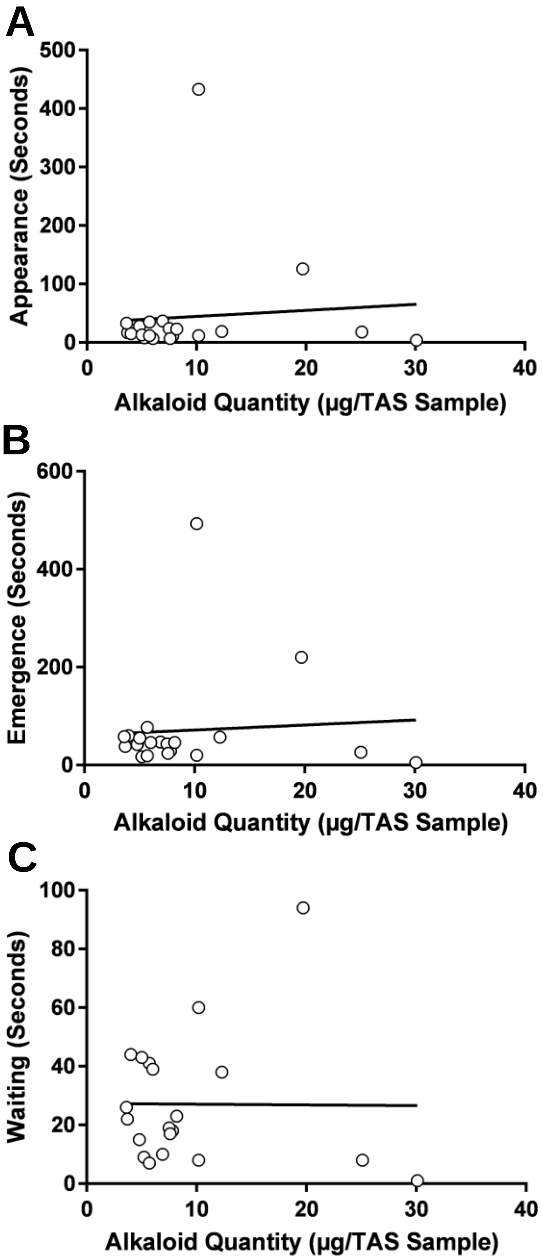


Figure 13. The relationship between alkaloid quantity and (**A**) appearance, (**B**) emergence and (**C**) waiting times of *Dendrobates auratus* within Isais.

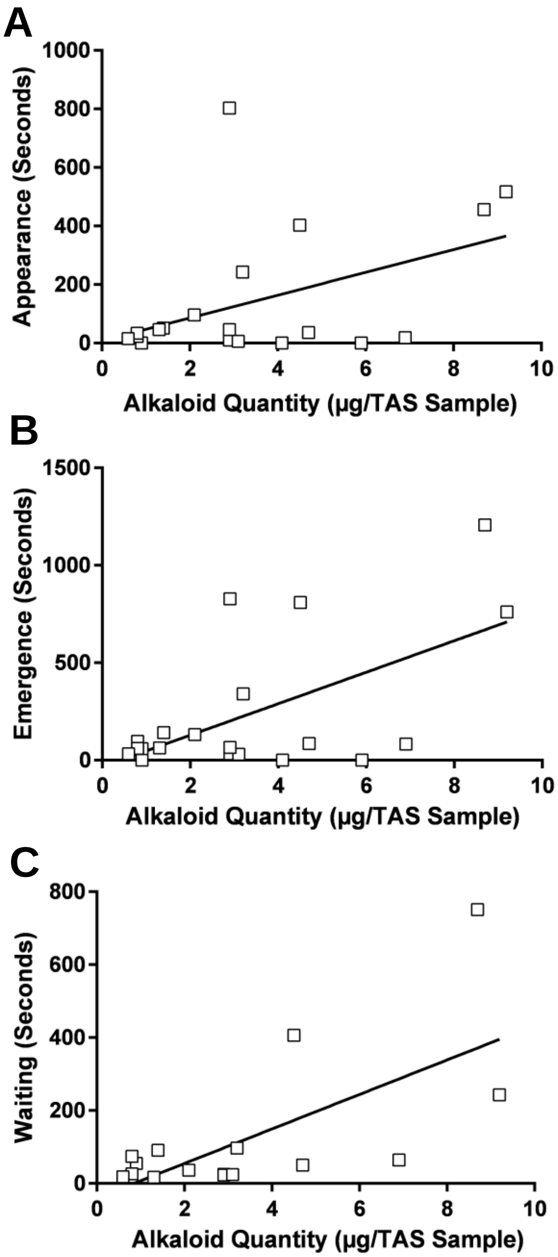


Figure 14. The relationship between alkaloid quantity and (**A**) appearance, (**B**) emergence and (**C**) waiting times of *Dendrobates auratus* within Firestone.

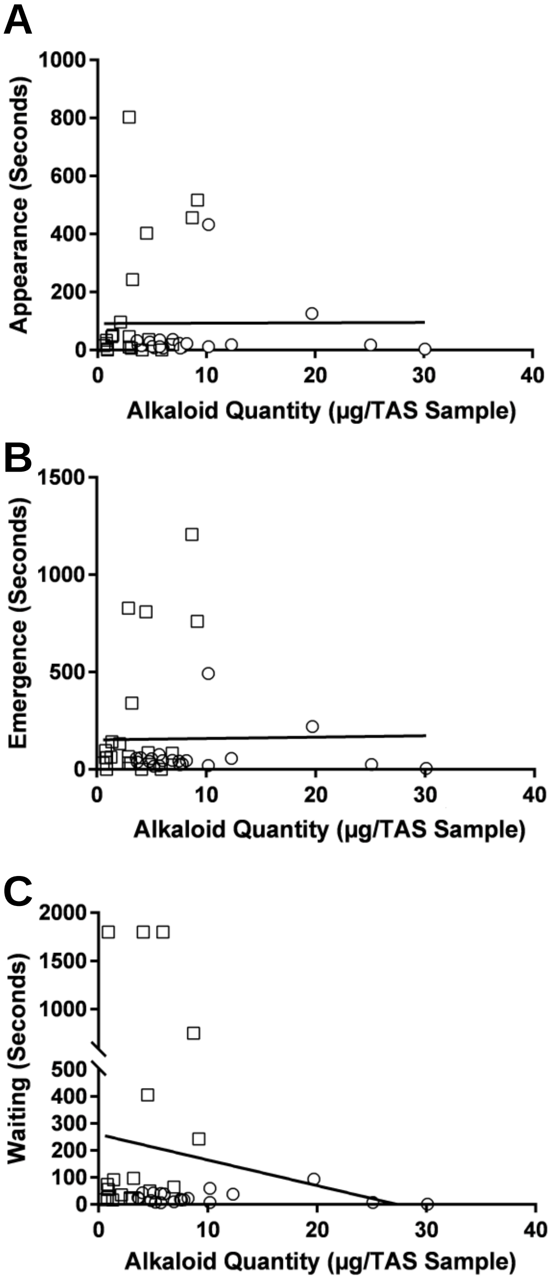
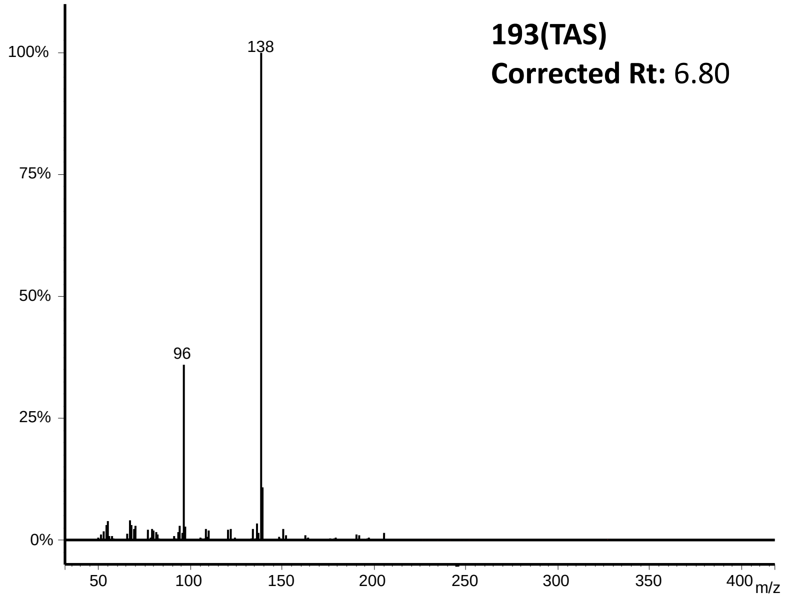


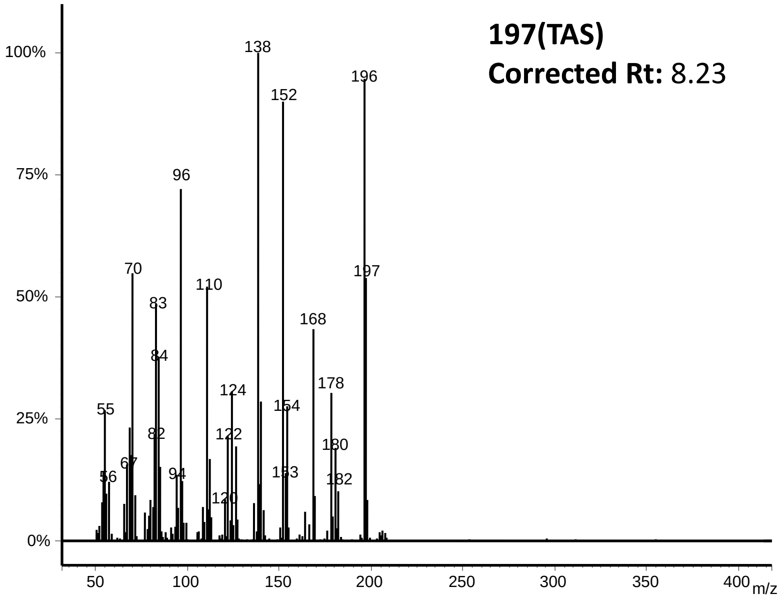
Figure 15. The relationship between alkaloid quantity and (**A**) appearance, (**B**) emergence and (**C**) waiting times of *Dendrobates auratus* among Isais (open circles) Firestone (open squares).

Appendix 1. Mass spectral data for the 12 tentatively new alkaloids detected in TAS samples of *Dendrobates auratus* from the La Selva Biological Station, Isais, and the Firestone Center for Restoration Ecology in Costa Rica. Following the methods of Jeckel et al. (2015), the retention time (Rt) reported for each alkaloid is the Corrected Rt to account for differences in elution time (approximately 0.87 seconds slower) for alkaloids in the present study compared to the alkaloid library of Daly et al. (2005). The alkaloids reported here were given code names that correspond to their molecular mass and also include "TAS" to indicate they were identified from samples collected using a Transcutaneous Skin Stimulator (TAS) and not from whole skin samples. All tentatively new alkaloids were present in three or more frogs with at least one frog containing ≥0.1µg of alkaloid per TAS sample.



193(TAS)
Corrected Rt: 6.80

Unclass



197(TAS)
Corrected Rt: 8.23

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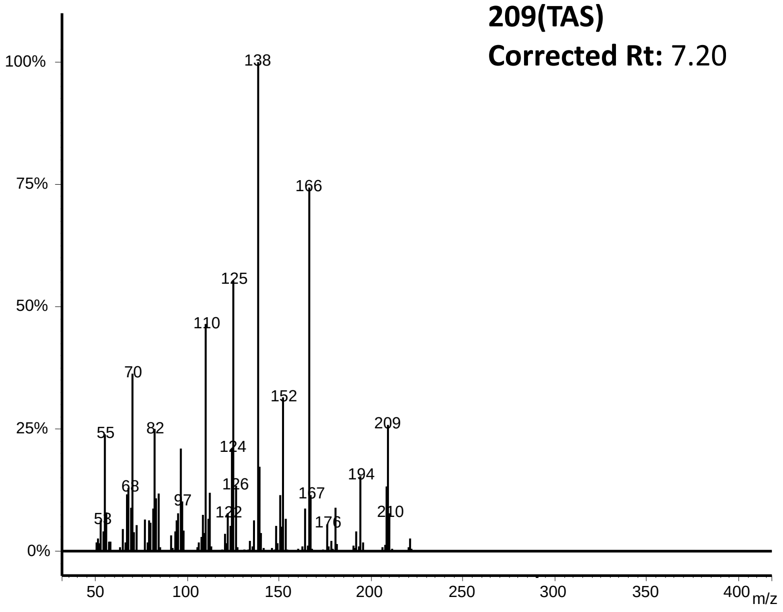
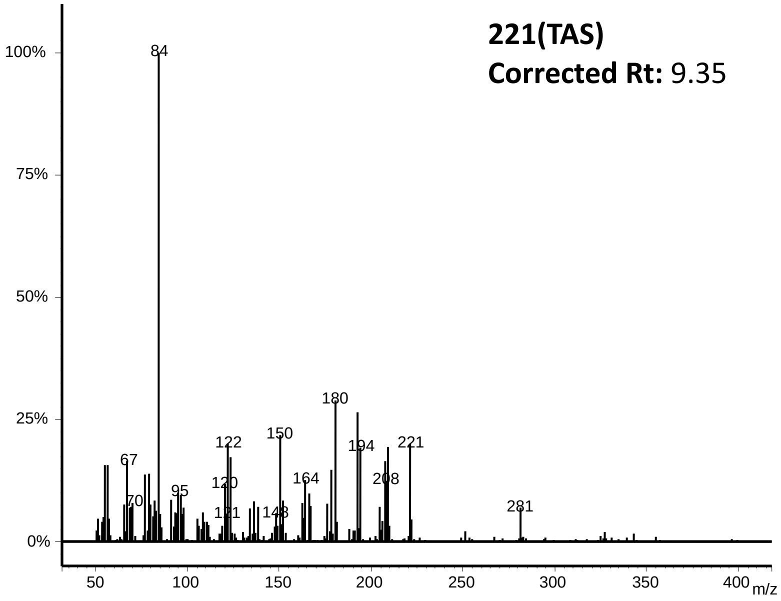


209(TAS)
Corrected Rt: 7.20

Tri


221(TAS)
Corrected Rt: 9.35

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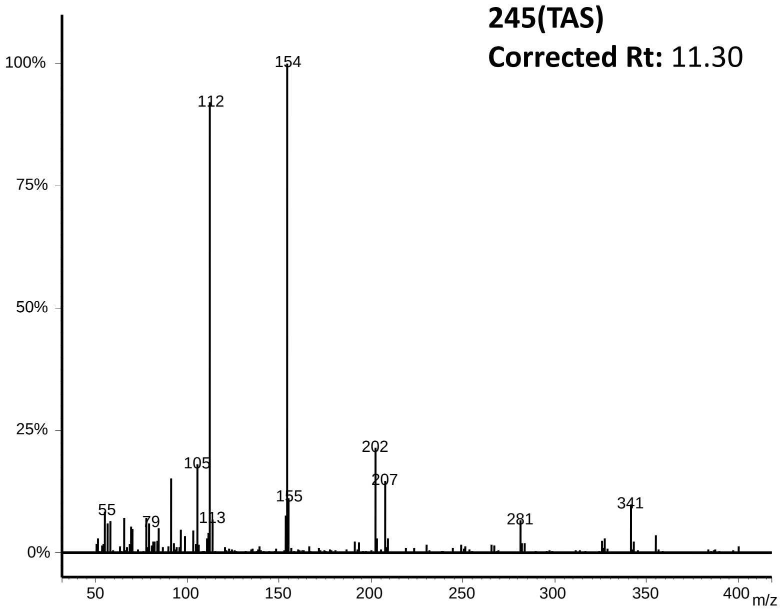
245(TAS)
Corrected Rt: 11.30

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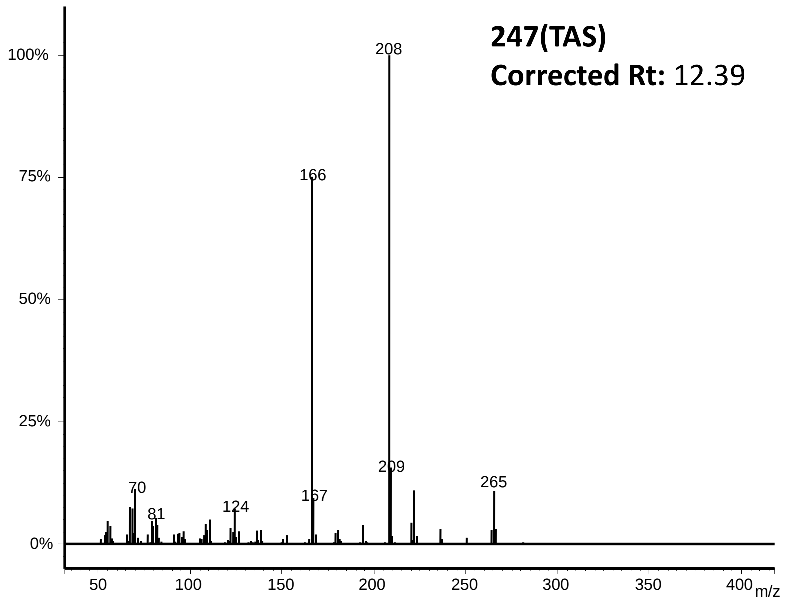
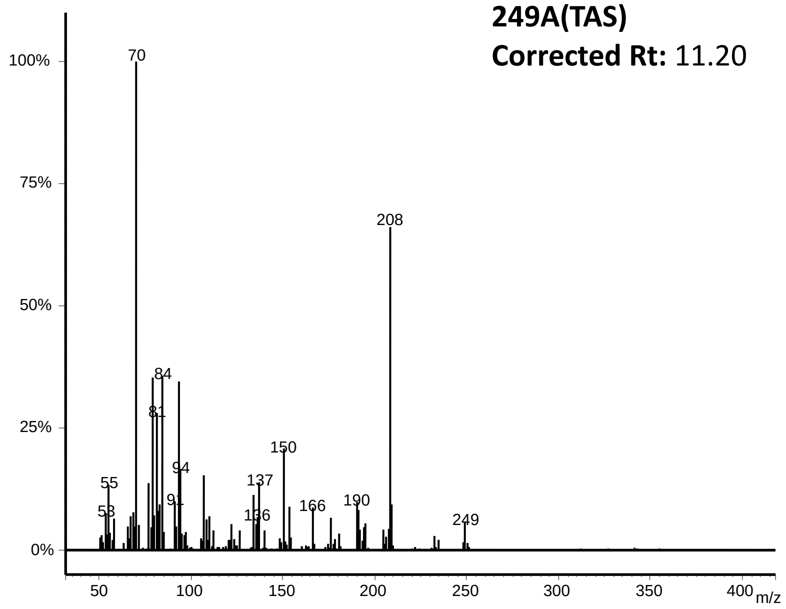


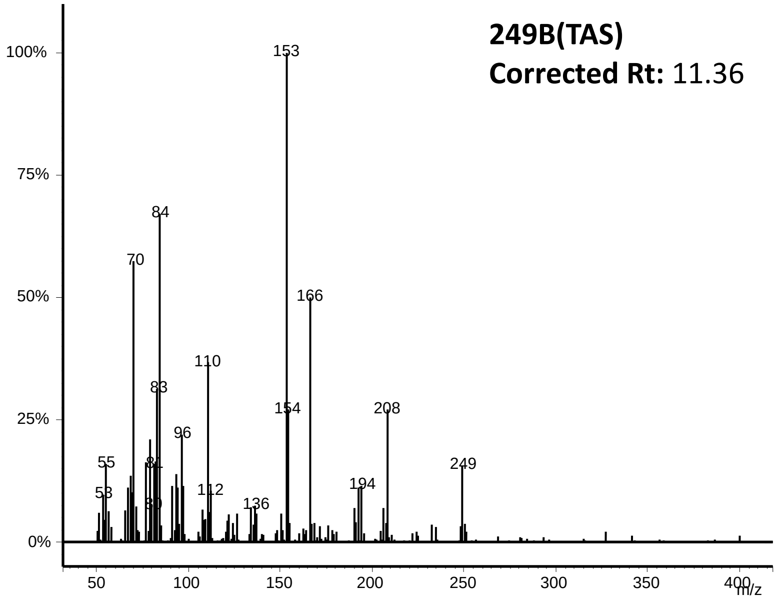
247(TAS)
Corrected Rt: 12.39

Unclass



249A(TAS)
Corrected Rt: 11.20

Unclass



249B(TAS)
Corrected Rt: 11.36

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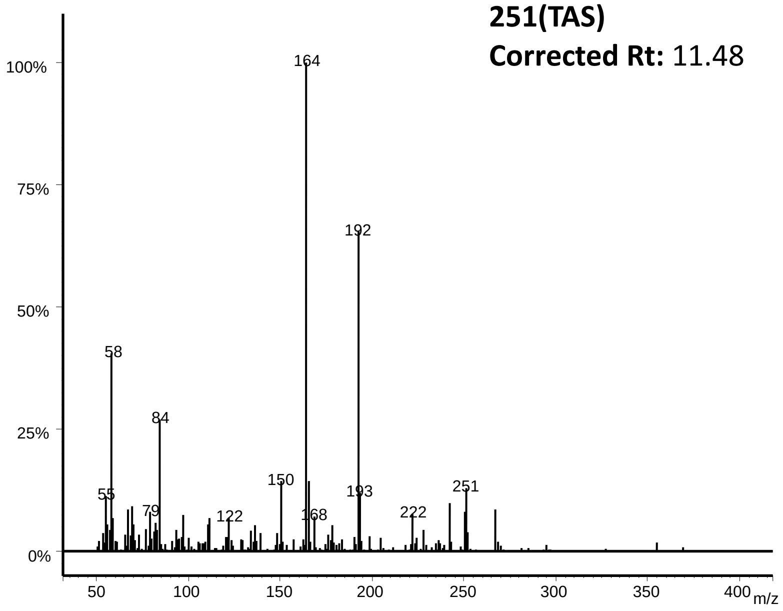
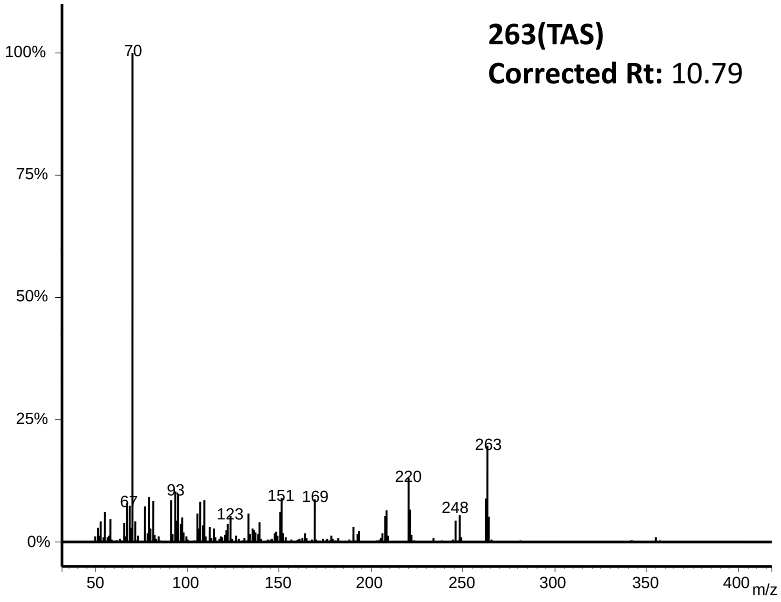


251(TAS)
Corrected Rt: 11.48

Unclass



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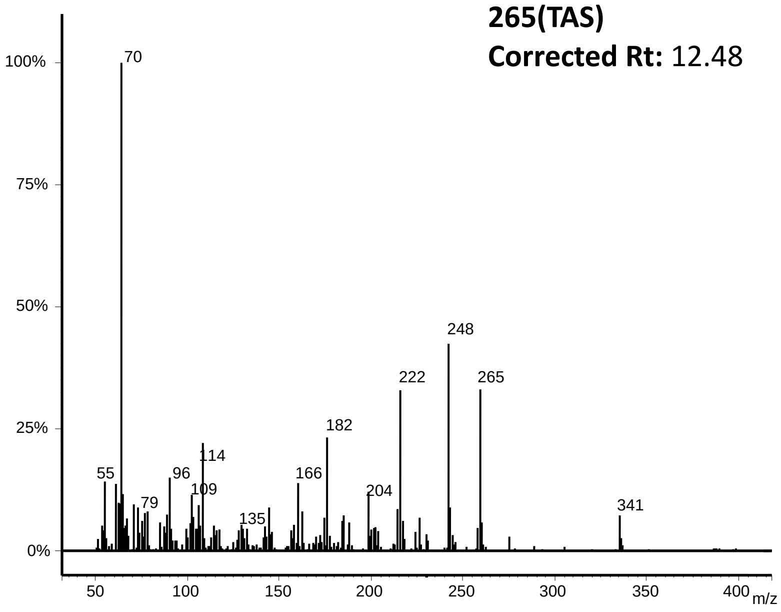

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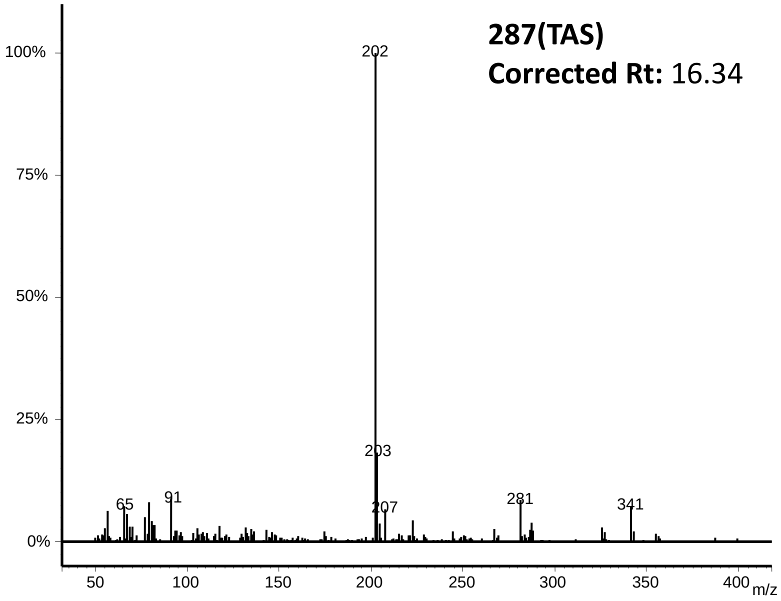

287(TAS)
Corrected Rt: 16.34

DHQ

Appendix 2. The raw values of dorsal brightness, chroma, and hue for *Dendrobates auratus* collected at La Selva, Isais, and the Firestone Center for Restoration Ecology, Costa Rica. Note that "M" refers to males and "F" refers to females within each location.

Location	Frog ID	Brightness	Chroma	Hue
La Selva	M1	60.01	27.42	0.79
La Selva	M2	62.02	29.84	1.46
La Selva	F1	57.73	26.45	1.47
La Selva	F2	85.18	40.48	1.44
La Selva	M3	67.06	33.03	1.46
La Selva	F3	53.65	25.24	1.45
La Selva	F4	69.25	33.97	1.42
La Selva	M4	67.12	32.48	0.50
La Selva	M5	75.92	37.70	1.44
La Selva	M6	71.97	33.44	0.16
La Selva	F5	66.85	32.09	1.48
La Selva	F6	66.40	31.94	-1.04
La Selva	F7	65.26	30.89	1.49
La Selva	M8	70.41	34.51	1.14
La Selva	M7	86.11	40.67	1.49
Isais	M1	36.95	16.49	1.46
Isais	F1	42.73	19.88	1.41
Isais	F2	68.56	30.01	1.19
Isais	F3	75.66	36.06	1.17
Isais	F4	51.50	22.92	-0.86
Isais	M2	60.94	29.67	1.44
Isais	M3	45.45	23.05	0.54
Isais	F5	38.08	21.29	-0.78
Isais	M4	53.92	27.23	1.41
Isais	M5	57.65	29.00	0.50
Isais	M6	67.39	32.45	0.52
Isais	F6	60.90	29.29	1.38
Isais	M7	64.91	28.40	-0.51
Isais	F7	79.96	33.95	-0.14
Isais	M8	73.37	35.48	0.83
Isais	M9	54.32	25.94	1.37
Isais	F8	65.05	30.76	1.49
Isais	F9	54.18	23.61	-1.51
Isais	F10	75.69	36.65	1.37
Isais	F11	64.11	32.16	-1.51
Firestone	M1	68.35	31.11	1.26
Firestone	M2	67.77	34.98	1.16
Firestone	M3	83.97	43.69	1.23
Firestone	M4	80.61	40.18	1.20
Firestone	F1	60.10	30.65	1.34

Firestone	F2	52.90	22.89	1.34
Firestone	M5	92.33	43.65	1.34
Firestone	F3	68.78	29.77	1.30
Firestone	F4	61.01	27.68	1.03
Firestone	M6	84.68	39.63	1.32
Firestone	F5	64.66	30.00	1.25
Firestone	F6	94.13	45.37	1.22
Firestone	F7	60.66	26.68	1.52
Firestone	F8	84.66	43.91	1.08
Firestone	M7	88.96	40.94	1.31
Firestone	F9	66.86	31.13	1.30
Firestone	F10	94.57	46.92	1.23
Firestone	M8	52.03	24.96	1.35
Firestone	M9	68.05	32.89	1.29
Firestone	M10	75.70	39.59	1.26