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FACULTY & STAFF



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Invertebrate Communities and Sporocarp Characteristics of *Ganoderma* Species in a Costa Rican Cloud Forest

Kennan Mighell

Abstract

Woody bracket fungi often provide habitat for communities of invertebrates. The characteristics and composition of these communities are poorly understood, especially in the tropics, despite being widespread hotspots for invertebrate biodiversity. In this study I analyze fruiting bodies of *Ganoderma* in a tropical cloud forest and their insect communities to gather information on the sporocarp characteristics that impact invertebrate community abundance, species richness, diversity, and evenness. I found 123 morphospecies inhabiting these fungi, representing a wide range of invertebrate taxonomy. Spiders, beetles, and immature insects were the most common groups found. The wet weight of fruiting bodies was positively correlated with invertebrate abundance and richness, and fruiting body toughness was negatively correlated with invertebrate abundance, richness, and diversity. None of the measured traits correlated with community evenness. A cumulative catch curve showed that a greater sampling effort will be required to give an accurate estimate of overall invertebrate diversity in these fungi. I show here that these communities are very diverse and, like temperate polypore invertebrate communities, get more complex as a fruiting body decomposes and softens.

Resumen

Los hongos oreja de palo comúnmente ofrecen hábitat para las comunidades de invertebrados. Las características y composición de estas comunidades son poco conocidas, especialmente en los tropicos, a pesar de ser áreas de alta biodiversidad ampliamente conocida para invertebrados. En este estudio analicé cuerpos fructíferos de *Ganoderma* en un bosque nuboso tropical y sus comunidades de insectos para juntar información en las características esporocarpicas que impactan la abundancia, riqueza de especies, diversidad y equidad de las comunidades de invertebrados. Encontré 123 morfoespecies habitando estos hongos, representando una amplia variedad de grupos taxonómicos. Arañas, escarabajos e estadíos inmaduros de insectos fueron los grupos más comúnmente encontrados. El peso húmedo de los cuerpos fructíferos esta correlacionado positivamente con la abundancia y riqueza de invertebrados, y la dureza de los cuerpos fructíferos esta negativamente correlacionado con la abundancia, riqueza y diversidad de invertebrados. Ninguno de los factores medidos se correlacionan con la equidad de las comunidades. Una curva de acumulación de especies muestra que un esfuerzo de muestreo mayor se requiere para dar un estimado apropiado de la diversidad de invertebrados en estos hongos. Mostró aquí que estas comunidades son muy diversas y, como las comunidades de invertebrados en poliporos de zonas

templadas, se vuelven más complejas al suavizarse los cuerpos fructíferos por la descomposición.

Introduction

Fungal insect relationships are poorly studied components of any food web, but especially so for tropical forests (Graf-Peters et al. 2011). Invertebrates interact with fungi in important ways, including as spore dispersers and as fungivores (Tuno 1999, Guevara et al. 2011). Further, insects interacting with tropical fungal fruiting bodies may have to track a resource that is occasional in both time and space. The fruiting body goes through several structural and nutritional changes as it grows and senesces that can alter insect-fungal interactions (Jonsell and Norlander 2004). It is important to study these interactions as fungal fitness may be closely tied to how their insects track and interact with them.

Most fungal fruiting bodies are short-lived, offering an unpredictable resource, but bracket fungi are frequently perennial and can be very large (Arora 1979), providing much more habitat stability to their invertebrate symbionts (Jonsell and Norlander 2004). These microhabitats have even been described as hotspots for insect biodiversity (Komonen 2003), especially for orders Diptera and Coleoptera (Schigel 2012), and have been shown to include rare old-growth insect species (Komonen et al. 2001). In many cases there are entire communities of invertebrates occupying a single sporocarp (Kadowaki 2010). These invertebrate-fungi interactions often involve specialist species, and may be under threat due to habitat loss and fragmentation (Schigel 2012).

Invertebrates may select a fungal host based on species, moisture content, or state of decay (Jonsell and Norlander 2004). Hagvar and Steen (2012) found that dead sporocarps attracted a diverse array of invertebrates, while living sporocarps attracted very few. Jonsell and Norlander (2004) found that initial invertebrate colonizers of a sporocarp were mostly monophagous, and were joined by polyphagous invertebrates as the sporocarp decayed. These findings suggest a succession in the richness and composition of bracket fungi insect community as the woody bracket sporocarp ages and decays. The composition, richness, and succession of these communities have been very poorly studied in tropical forests, despite the high diversity of invertebrates and fungi found there (Kricher 2011).

In this study I examined the communities of invertebrates inhabiting fruiting bodies of *Ganoderma*, a large cosmopolitan woody bracket fungus. Sporocarp collection took place in a tropical lower montane wet cloud forest in Monteverde, Costa Rica. I measured characteristics of fruiting bodies such as size, canopy cover and toughness, and measured the community richness, species abundance, diversity, and evenness of their invertebrate communities. The goal of this study was to explain invertebrate communities in terms of sporocarp characteristics within the

genus *Ganoderma*. I also establish a cumulative catch curve to describe the sampling effort required to accurately estimate the diversity of insects inhabiting *Ganoderma* fruiting bodies.

Materials and Methods

Study Site

This study was conducted in the tropical lower montane wet cloud forest around the Biological Research Station in Monteverde, Costa Rica during the wet season months of October and November. Fruiting bodies of *Ganoderma* species were found near the trails around the station between 1500 and 1600 meters in elevation. The study site was an old-growth forest with dense canopy cover, and colonies of *Ganoderma* were found on large fallen decaying logs or decaying stumps.

Sporocarp Collection and Analysis

Ganoderma was chosen as a study organism because it is one of the largest and most common woody bracket fungi at the study site. Once a colony was selected, the elevation, canopy cover, and substrate type were noted. Nine total colonies were selected for study, and 1-3 fruiting bodies from each colony were harvested for invertebrate community analysis. A total of 23 sporocarp samples were collected. The fruiting bodies selected of a given colony were all within a 1-meter radius, and were chosen to represent the range of size and decay present in that colony (Figure 1). Before harvesting each sample, an aspirator was used to capture visible surface dwelling invertebrates, or any insects flying around the sample. These insects were placed into a vial with alcohol in the field. Then a cotton ball treated with acetone was placed into a large paper bag in order to render any remaining insects immobile. The bag was then held open under the sporocarp as it was pried from the substrate into the bag with a large knife, and then closed tightly.



Figure 1. *Ganoderma* samples collected in a Costa Rican cloud forest at varying degrees of rot. The three major stages include alive (A), recently dead (B), and very decayed (C).

In the lab, each sample was removed from its bag onto a large wooden board. The bag was examined to make sure no insects remained inside. Throughout analysis of the sporocarp any invertebrates found on the board were placed into 95% ethanol for preservation. The length and width of the spore-releasing surface were

measured, and height was measured at the point of attachment to the substrate. The wet weight of each fruiting body was measured, and the volume was calculated by submerging each sporocarp, wrapped tightly in plastic cling-wrap, in a graduated pitcher. Toughness was measured with a soil penetrometer modified with a dissecting needle attached to the end, because the fruiting bodies were generally very tough. The needle was inserted 1 cm into the fruiting body tissue and the reading on the penetrometer was recorded. The top, bottom, and inside of each sample was measured for toughness. The toughness of each surface of the sporocarp was averaged from three measurements from equidistant locations of that surface.

Invertebrate Capture and Identification

Each fruiting body was then cut into cubes of 1-2 cm² in order to find insects living inside. A large, sharp knife and wire-cutting shears were required to cut through the tough tissue of these fruiting bodies, resulting in cubes of somewhat irregular shape and size. After dicing the sporocarp, each cube was carefully examined to find invertebrates living on or inside its tissue. Any invertebrates found throughout the cutting and examining process were placed in 95% ethanol. All invertebrates found on all cubes of a given sample were placed into the same container with all invertebrates found on that sample before dicing for examination. After all the pieces were examined they were placed into a dehydrator along with any smaller fragments that had fallen on the board. The pieces were dehydrated for 24 hours and their dry weight was measured.

The petri dishes with all invertebrates from a sample were examined with a dissecting microscope. Invertebrates were identified as different morphospecies. Each new morphospecies was photographed through the microscope and numbered for later analysis, and was placed in a vial for future reference. After collection from all 23 samples, the invertebrates were identified to a taxonomic group if possible.

Results

In 23 sporocarp samples I found a total of 123 different invertebrate morphospecies. Among these were three orders of Arachnids, eight orders of Insects, five centipedes, six millipedes, three segmented worms, two crustaceans, one mollusk, 20 insect larvae or nymphs including six Diptera and eight Coleoptera juveniles, and 18 unidentified morphospecies, many of which were immature insects (Figure 1). 88 % of invertebrate morphospecies were only found on one or two fruiting bodies, 8% were found on 3-4, and 3% were found on 5-6. The most widespread morphospecies, with 46 individuals found on seven samples, was a minute beetle in the Ciidae family. A cumulative catch curve shows that the rate of new invertebrate morphospecies discovery rises steadily up to 23 samples, and no asymptote is shown (Figure 2).

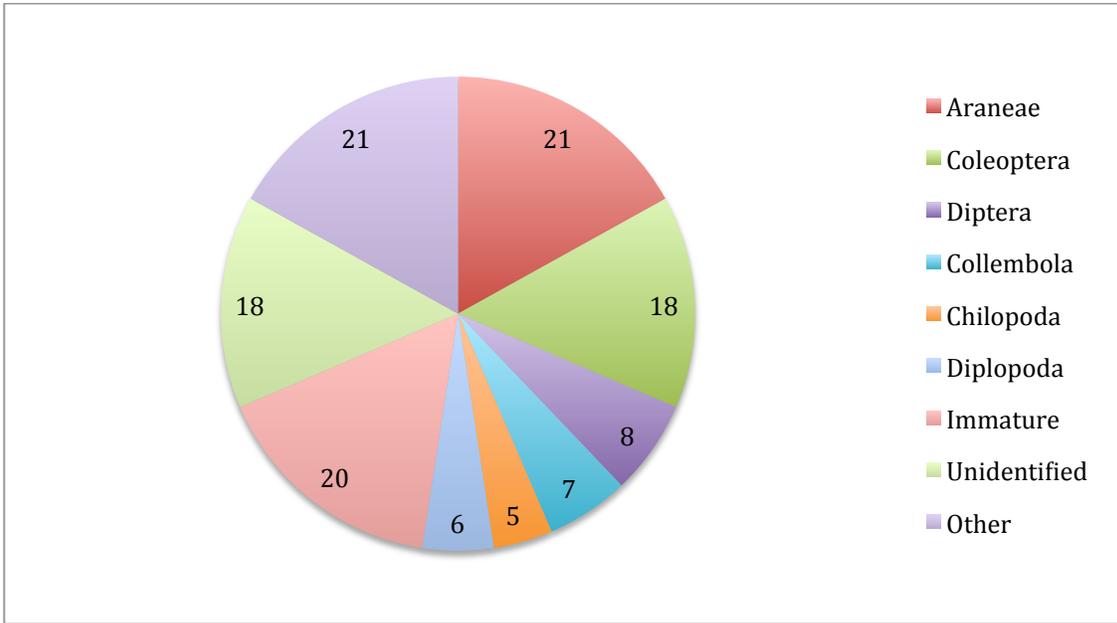


Figure 2. The number of morphospecies found of the most common groups of invertebrates inhabiting *Ganoderma* species in a Costa Rican cloud forest. Groups with four or fewer morphospecies (Pseudoscorpionida, Acari, Orthoptera, Hemiptera, Thysanoptera, Blattodea, Hymenoptera, Mollusk, Isopoda, Amphipoda, and Oligochaeta) make up the “Other” category. Immature insects were found for Diptera, Coleoptera, Hemiptera, Blattodea, Neuroptera, Lepidoptera, and Hymenoptera.

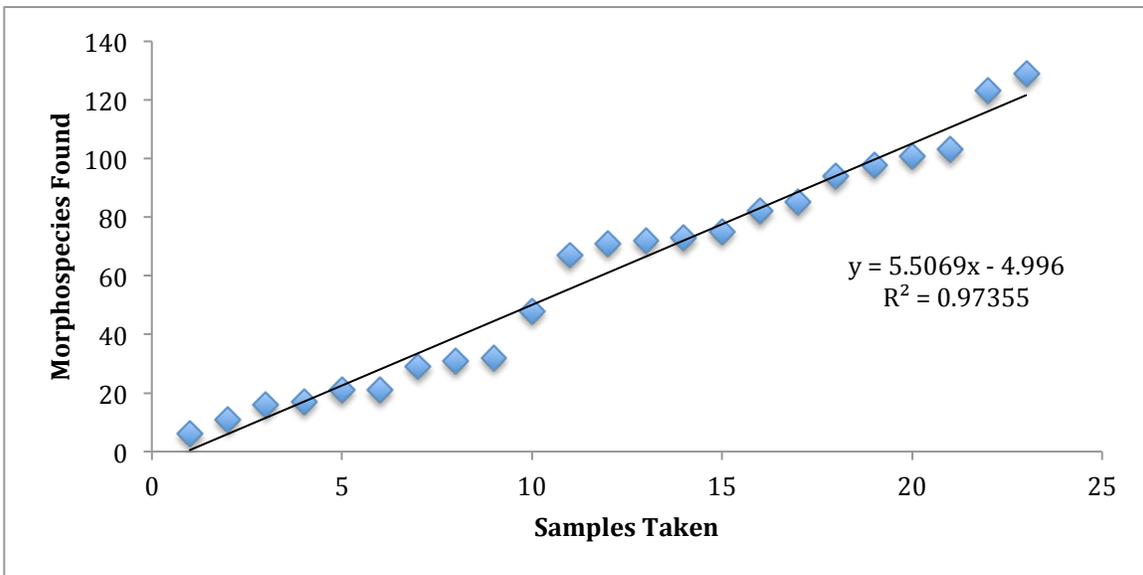
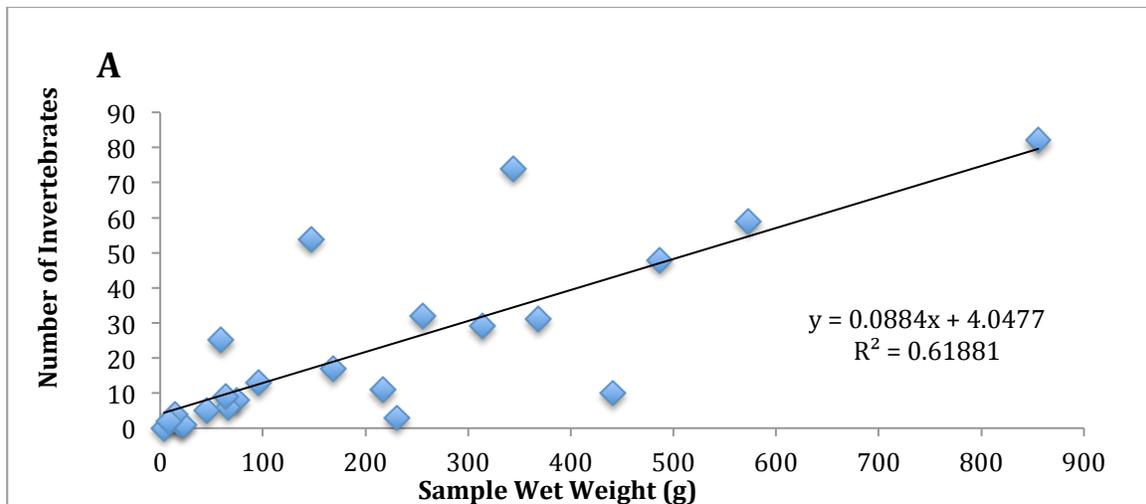


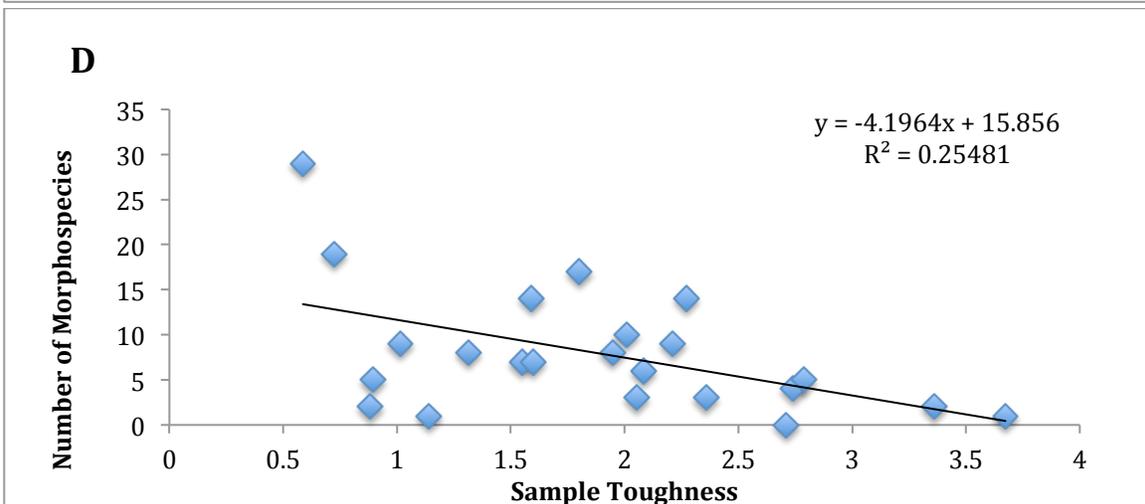
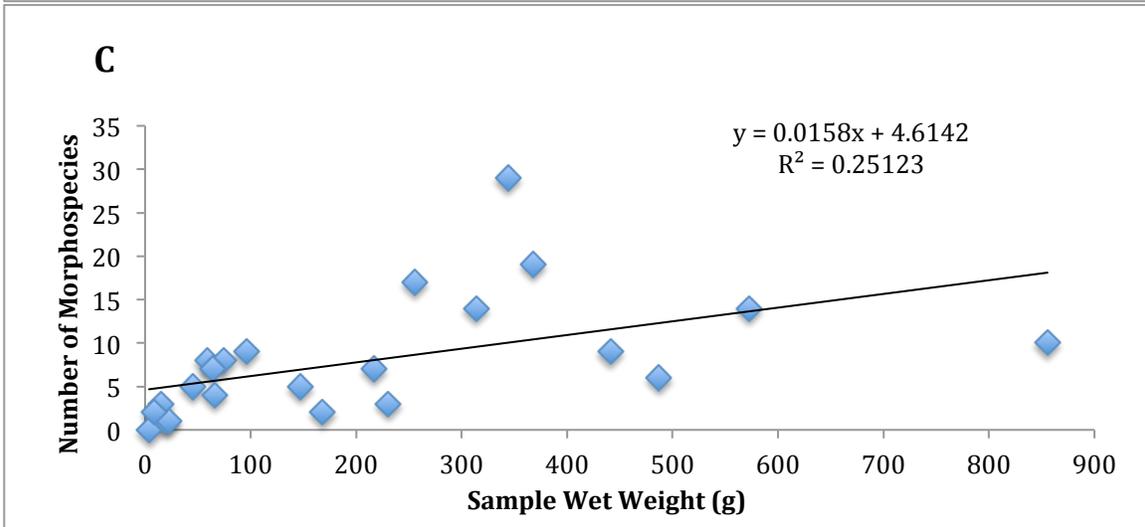
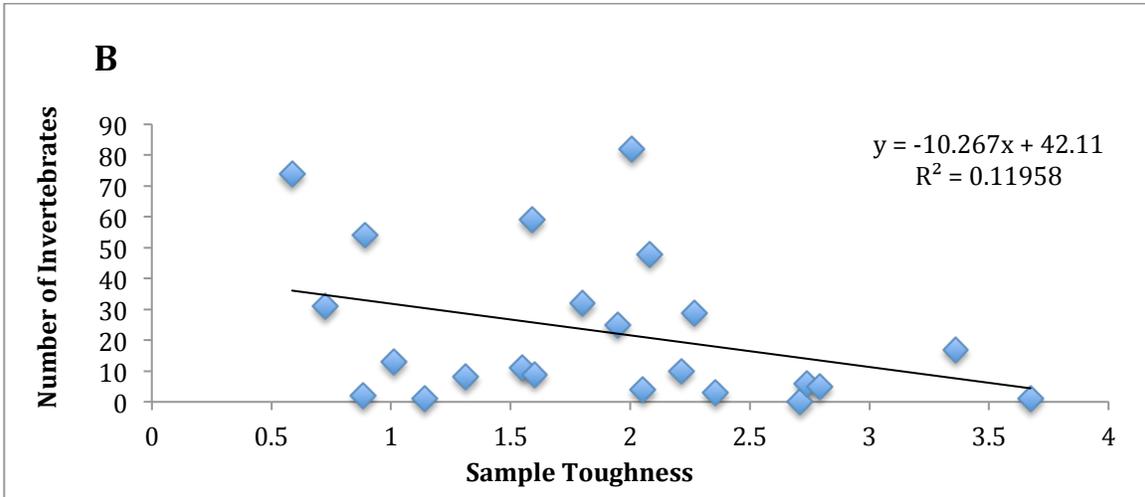
Figure 3. A cumulative catch curve showing the number of morphospecies of invertebrates found in samples of *Ganoderma* collected in a Costa Rican cloud forest. No asymptote is evident after 23 samples, indicating that a greater sampling effort is necessary to accurately estimate *Ganoderma* invertebrate community diversity.

Most of the invertebrates were found on the outside of the fruiting bodies, especially on the pores. Some of the small insects found on the spore-releasing surface used the spore tubes as shelter to avoid capture. Spiders were usually found

with webs built on the underside of a sporocarp. Some insects, most commonly larvae, were found on the inside of the samples. These were usually only found in very soft decaying sporocarps, often inside a small (1 cm) but very dense round compartment.

Canopy cover, percent moisture, average toughness, and wet weight were independent variables in multiple regression analyses describing invertebrate abundance and species richness, Shannon-Weiner Diversity Index (H) and community evenness. Multiple regressions for morphospecies abundance, richness, H, and evenness showed no significant correlation with canopy cover and percent moisture. An adjusted multiple regression model including only average toughness and wet weight had a significant correlation with morphospecies abundance, explaining 65% of abundance ($R^2=0.6524$, $F=21.65$, $DF=20$, $p<0.0001$). Abundance had a positive correlation with wet weight (Figure 3A) and a negative correlation with average toughness (Figure 3B). An adjusted multiple regression model including only average toughness and wet weight had a significant correlation with morphospecies richness, explaining 40% of invertebrate richness ($R^2=0.3982$, $F=8.28$, $DF=20$, $p=0.0024$). Richness had a positive correlation with wet weight (Figure 3C) and a negative correlation with toughness (Figure 3D) as well. A simple regression between average toughness and H showed a strong negative relationship between diversity and toughness (Figure 3E, $R^2=0.1297$, $F=4.279$, $DF=21$, $p=0.0511$). No significant trend was found for community evenness with any of the independent variables.





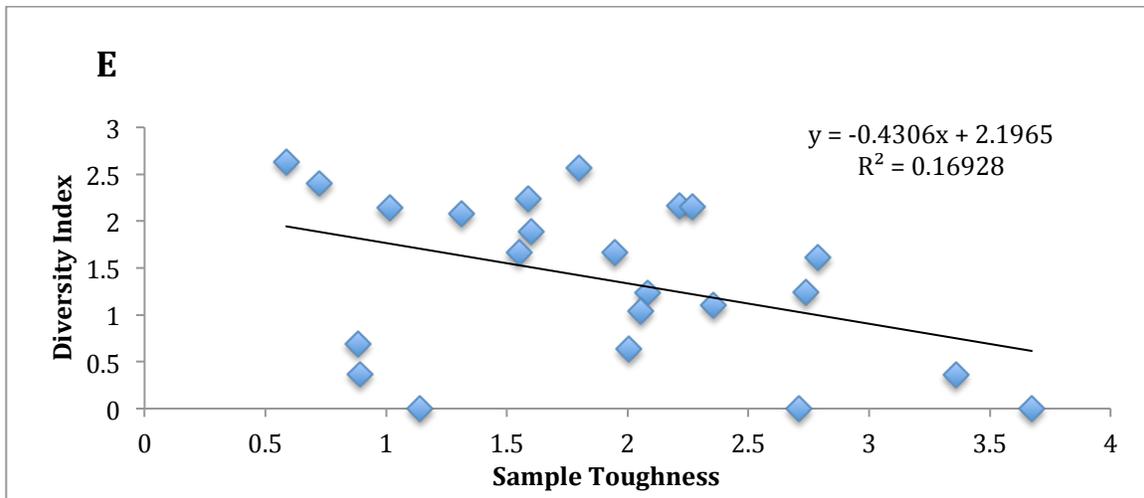


Figure 4. Regression analyses of the *Ganoderma* sporocarp characteristics that impact invertebrate community abundance, species richness, and diversity in a Costa Rican cloud forest. Sporocarp wet weight is positively correlated with community abundance (A) and richness (C). Sporocarp toughness is negatively correlated with community abundance (B), richness (D), and Shannon-Weiner Diversity Index (E).

Discussion

The total number of invertebrate morphospecies found in 23 samples was very high, yet the cumulative catch curve indicates that a greater sampling effort would be required to gain an accurate estimate of total species richness of these *Ganoderma* invertebrate communities. Other studies of invertebrates and bracket fungi found fewer species and clearer patterns of dominance (Kadowaki 2010, Kadowaki et al. 2011b, Maraun et al. 2014). These studies were conducted in Northern Europe, so perhaps the nature of these relationships includes more diversity and less specialization in the tropics, or specialization may simply be harder to discern in the tropics. For example, in one of the few invertebrate-fungi studies conducted in the Neotropics fungivorous beetles were more likely to be oligophagous or polyphagous than monophagous, suggesting low host specificity due to patchy host distribution in the tropics (Graf-Peters et al. 2011). In this study I found few morphospecies more than once, and a species abundance pattern similar to other tropical organisms indicating high species richness requiring a high sampling effort to describe. The most frequent invertebrate was a beetle in the Ciid family, a relatively well-studied family of fungivores (Orledge and Reynolds 2005, Graf-Peters et al. 2011). My findings show that *Ganoderma* species in my study site host a broad taxonomic range of occasional inhabitants that may be rare and widely spaced.

My results also indicate that bracket fungi are important habitats for both adult and juvenile invertebrates, especially adult Dipterans, Coleopterans, Arachnids, Collembolans, and Myriapods, and juvenile Dipterans and Coleopterans. This finding supports reports that Diptera and Coleoptera are among the most frequent fungal symbionts (Schigel 2012), and that fungi have high levels of protein, carbohydrates, phosphorus, and nitrogen, which may allow insect larvae to grow rapidly (Graf-

Peters et al. 2011). The high occurrence of invertebrates on the fungal pores indicates that many of these morphospecies may be consuming spores, as has been shown in temperate polypores (Kadowaki et al. 2011a). Very small insects were also observed going in and out of the pores when I tried to capture them, suggesting that they use them as shelter. The frequency of invertebrates on the fungal pores may also explain the high occurrence of spiders with webs built on the underside of fruiting bodies. Spiders may use these bracket fungi for shelter or as hotspots for prey capture, but I was unable to find any published literature supporting these hypotheses.

I found a statistically significant positive correlation between sporocarp wet weight and invertebrate abundance and richness, providing support for the finding of Thunes and Willassen (1997) that the weight of a sporocarp is one of the most important factors impacting insect community composition. This result makes intuitive sense, because a larger sporocarp has more resources to offer invertebrates. Thunes and Willassen (1997) also found that live vs. dead fruiting bodies was the most important factor impacting insect community composition. My findings that invertebrate abundance, richness, and diversity increase with decreasing toughness of a sample corroborate this finding, as toughness was lowest in the most decomposed dead fruiting bodies. This finding suggests that these communities gradually become more complex and species-rich as decomposition of a sporocarp progresses. This has been described in other studies that found an increase in the number of tunneling insects that attack a bracket fungus as the sporocarp ages and decays (Hagvar and Steen 2012).

My study explains some of the sporocarp characteristics that determine invertebrate symbiont community abundance, species richness, and diversity in Neotropical *Ganoderma* species. Overall I found that these fungi house dynamic and species rich communities of invertebrates that follow similar species abundance patterns as other tropical organisms and are generally rare and widely spaced. The invertebrate use of mainly dead sporocarps suggests that they have a limited impact on the fitness of these fungi, as the fruiting body no longer produces spores after death. Further, by feeding on dead sporocarps invertebrates gain access to a highly nutritional food source while avoiding chemical defenses present in the living sporocarp (Jonsell and Nordlander 2004). The interaction between bracket fungi and spiders should be studied in more detail, as the fungi may be important habitats for a wide array of spiders. Unfortunately these diverse communities may be sensitive to habitat loss and fragmentation (Schigel 2012). Since habitat loss is occurring rapidly in the tropics and few tropical studies of these communities exist, researchers should make an effort in the near future to describe the composition and successional dynamics of these communities before many of them disappear.

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Cyanide allocation with flowering in *Passiflora* spp

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ABSTRACT

Plants have antiherbivore defenses that may be differentially placed to protect particularly vulnerable or highly valued parts. In the Passifloraceae, defense mechanisms include toxic cyanide (CN) in leaves and flowers, and leaf toughness. This study explores floral and foliar CN allocation and leaf toughness in *Passiflora suberosa* with flower age. Flowers of various developmental stages were collected and analyzed for CN concentration, and nearest leaves were collected and analyzed for CN concentration and toughness. Results demonstrated a significant increase in CN in young bud (YB) flowers ($3.69 (\pm) 0.92$), followed by a decrease in CN concentration as flowers matured, suggesting YB flowers are of greater value to the plant. Results displayed that leaf toughness was greatest in leaves nearest to flowers in the female stage. As less CN is allocated to more mature flowers, more energy was allocated to leaf toughness. There was no correlation in CN concentration between flowers and their nearest leaves. This study also examined CN allocation to different flower parts, sepals ($m=0.14 (\pm) 0.03$), petals ($m=0.42(\pm) 0.02$), corona filaments ($m=0.35 (\pm) 0.06$), stigmas ($m=0.48 (\pm) 0.03$), anthers ($m=.25 (\pm) 0.05$), and ovaries ($0.39 (\pm) 0.03$), in *Passiflora miniata*. Findings indicate that flower parts that are more prone to herbivory or have a strong role in increasing plant fitness are more heavily defended. Greater CN content in leaves overall suggests they are of greater value to the plant due to their long lifespan.

INTRODUCTION

Energy used by plants to defend themselves from herbivores is diverted from growth and reproduction (Moles 2013). Therefore, allocating energy to defense should place more defenses in tissues that are particularly prone to herbivory and are of high value to the plant (McCall & Fordyce 2010). For example, young leaves are particularly prone to herbivores because they are tender (McCall & Fordyce 2010) and many plants concentrate defensive compounds in young leaves until they are eventually protected by toughening (Briggs & Shultz 1990). Flowers may also be heavily defended, as they represent a large investment in reproduction (McCall & Fordyce 2010). If so, young leaves may be less well-defended when plants are flowering as energy for defense is allocated toward the flower, as well. Further, different parts of flowers may be more heavily defended than others depending on their role in reproduction and fruit production (Gleadow & Woodrow 1999). Further, pistils and stamens may be more heavily defended than other plant parts as they house gametes (Gleadow & Woodrow 1999).

Vines in the Passifloraceae have both chemical and physical defenses (Briggs & Shultz 1990). Young leaves are laden with cyanide, which is removed as leaves toughen (McCall & Fordyce 2010). Passion vines also invest heavily in their flowers (Hay-Roe & Nation 2007) (Vanderplank 1991). Copious nectar attracts bee and hummingbird pollinators but also flower predators that take nectar as well as eat pollen and other flower parts (Edlund *et al.* 2004). To cope with this florivory, flowers have been found to contain high concentrations of CN (Gleadow & Woodrow 1999).

Florivory, flower herbivory, directly decreases the amount of photosynthetic tissue a plant has and is indirectly related to growth, reproduction, and survival (Cardel & Koptur 2010). Damaged flowers are visited much less frequently by pollinators than are undamaged flowers; florivory may impede successful pollination and reproduction (Cardel & Koptur 2010). In response to this herbivory, changes in resource allocation by the plant occur (Faegri & van der Pijl 1979). Tradeoffs amongst energy for defenses and energy for other processes like growth and reproduction occur, along with tradeoffs between defenses (Moles 2013). A finite amount of resources is being spread across several defenses and these defenses are being spread across different areas of the plant (Moles, 2013). Therefore, the level of cyanide production varies across different plant parts (Moles 2013).

Allocation of CN to different plant parts should reflect their relative value. For example, when not flowering, young leaves should be highest in CN, which is then reabsorbed by the plant as the leaves toughen (Moles 2013). When flowering, CN levels may be lower in all leaf ages as CN is diverted to flowers. Further, flowers may have more CN earlier, when their value is greatest, eventually losing CN as pollen is removed and the flower senesces (Moles 2013). Plants may increase their fitness by heavily defending young flowers, ensuring they make it to reproductive age. Higher concentrations of CN may reside in flower parts that are more susceptible to herbivory based on their function and placement within the flower (Gleadow & Woodrow 1999). Plants may increase their fitness by heavily defending more reproductively valuable flower parts. Here, I examine changing cyanide concentrations and leaf toughness in leaves and flowers of the passion vine *Passiflora suberosa* throughout flower development. I also examine CN allocation in different flower parts of mature flowers in *Passiflora miniata*.

MATERIALS AND METHODS

Sample Collection:

I studied cyanide production in *P. suberosa* and *P. miniata* at the Monteverde Butterfly Garden in Costa Rica. I located several vines of *P. suberosa* currently flowering. I looked at flowers in five different developmental stages- young bud (YB), old bud (OB), opening (OPEN), female stage (FEMALE), and male stage (MALE) YBs and OBs were differentiated based on size with YBs being smaller than OBs. OPEN flowers were buds that had begun opening their petals. FEMALE and MALE

stage flowers were determined by the position of their stamens and pistils. The FEMALE flowers displayed five stamens in an upright position above three dangling pistils. The MALE flowers displayed three pistils in an upright position above the five dangling stamens. I removed 15 flowers in each developmental stage. I also removed each flowers' nearest adjacent leaf to see if CN was changing with floral CN concentrations. I collected flowers with leaves of different ages, determined by leaf toughness. Older leaves felt tougher and were often a darker green than younger, more tender leaves. I made a point of collecting both old and tough leaves, and young and tender leaves nearest to flowers at each developmental stage. I also made sure the flower bunches/nearest leaves were found at different locations in the vine, not just near the growing tip, to avoid collecting only younger leaves. In total, I collected 75 flowers, 15 in each of the five different developmental stages, and 75 adjacent leaves of various ages. Next, I collected ten mature *P. miniata* flowers. These were chosen because the flowers are large and individual flower parts could be analyzed.

Flower and leaf measurements in P. suberosa

I weighed each *P. suberosa* flower to the nearest .001 g using a Fisher-Scientific XT Top Loading Scale. Next, I measured area, weight, and toughness in each of the adjacent leaves. I began by measuring the area of each leaf using a transparent grid of 0.25 cm squares. Squares that were 50% or more covered by leaf were counted as one, and squares that were covered by less than 50% were counted as 0. The sum of squares covered by a leaf accounted for the leaf area measurement. Next, I weighed each leaf to the nearest .001 g. I then used a penetrometer to measure the toughness of each leaf.

***P. miniata* flower parts**

After weighing each *P. miniata* flower to the nearest .001 g, I separated them into six different parts- sepal, petal, corona filament, anther, stigma, and ovary (Figure 1).

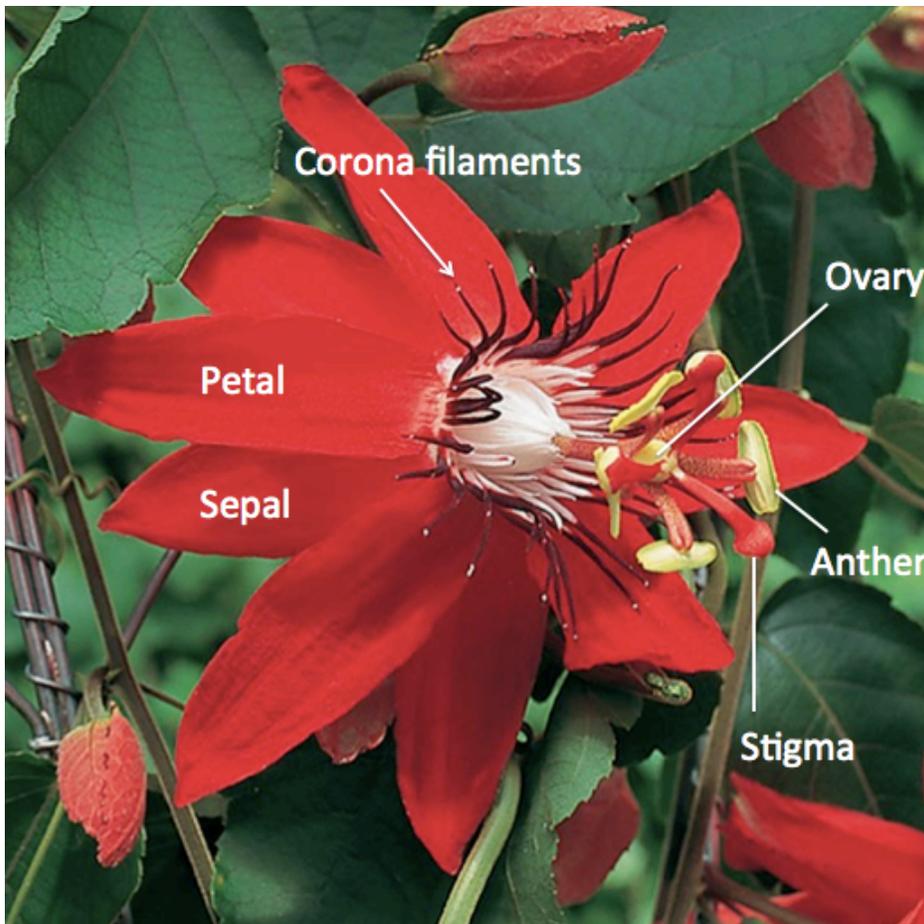


Figure 1. *Passiflora miniata* flower with labeled parts (Source: Logee's Plants for Home and Garden) collected at the Monteverde Butterfly Garden in Costa Rica and examined.

Cyanide Concentration:

Next, I determined the cyanide concentration of each leaf using the Sodium-Picrate Test (Haque & Bradbury 2002). I measured .1 g of each leaf and added 3 drops of toluene. The leaves were then pulverized for 30 seconds using a glass stir rod. Picrate test strips, prepared using strips of filter paper 0.5 cm by 6 cm soaked in sodium picrate and then allowed to sit in a Petri dish until the excess fluid had evaporated, were then suspended in each vial and the vials capped. The vials then sat for 60 minutes at approximately 25 °C. After sitting for one hour, the test strips were removed and dipped in a cuvette with 10 mL of water for 10 seconds. A blank was created using a picrate test strip that had not been exposed to any leaf tissue. The cuvettes containing the leaf tissue extract were read in a MRC UV-200-RS ultraviolet and Visible spectrophotometer at 550 nm, where their transmittance was recorded. The transmittance value was converted to absorbance using the formula $ABSORBANCE = 2 - \text{LOG}_{10} (\% \text{ TRANSMITTANCE})$. A standard curve of absorption (Arthur 2009) was used to calculate μg cyanide from absorbance ($y=0.0284\ln(x) + 0.1665$). I divided CN amount by the tissue's sample weight to get the concentration.

I repeated the above process for each of the 75 *P. suberosa* flowers weighed out to .016 g. I repeated the above process again for 10 of each of the six different flower parts weighed to .016 g.

RESULTS

Cyanide production in P. suberosa flowers

A one-way analysis of variance was conducted to compare mean CN concentrations in *P. suberosa* flowers at different developmental stages. The means for flowers at each stage fell between .30 and 3.70 (Figure 2). There was a significant difference in CN concentrations at the $p < .05$ level for the different developmental stages of flowers ($p = 5.397e-05$, $df = 4$, $\chi^2 = 24.8483$). The results indicate a significant difference in CN production between YB and all other stages. Post hoc comparisons indicate that YB flowers had a significantly greater concentration of CN than OB, OPEN, FEMALE and MALE stage flowers (Figure 2). YB and OB ($p = 1.348889e-02$), YB and OPEN ($p = 9.666667e-04$), YB and FEMALE ($p = 6.333333e-04$), and YB and MALE ($p = 4.444444e-05$).

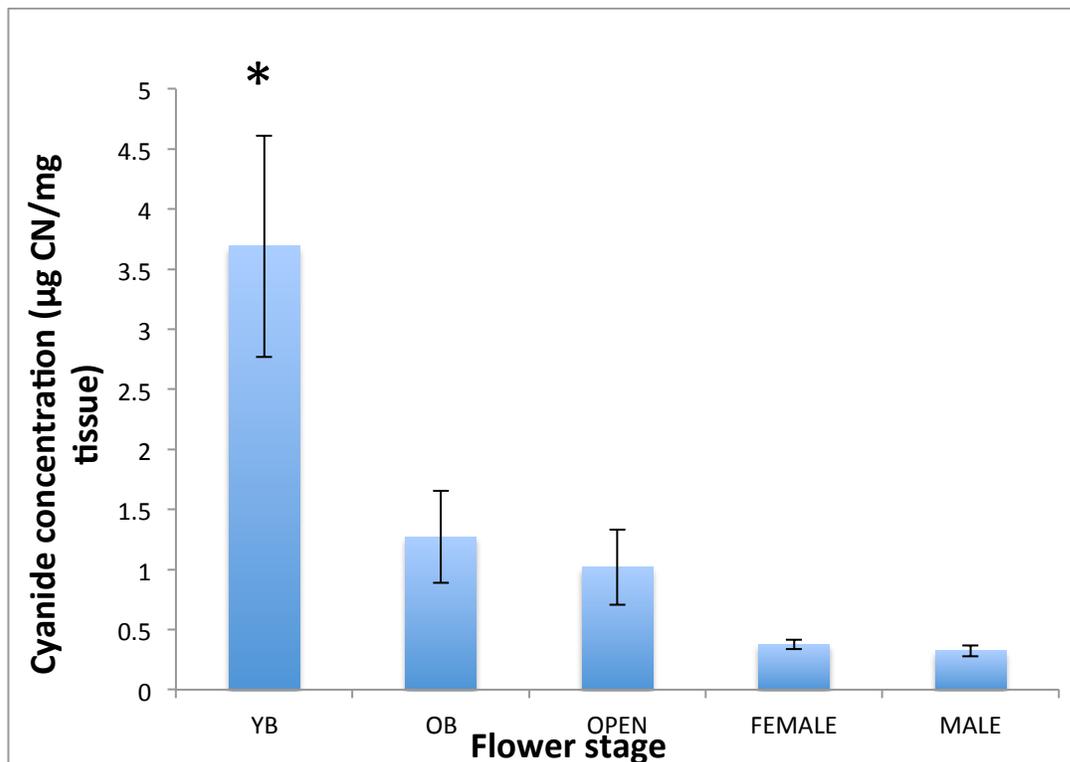


Figure 2. Mean cyanide concentration in *P. suberosa* flowers at different developmental stages- YB, OB, OPEN, FEMALE, MALE. Each flower stage is represented by 15 flowers in the same developmental stage found in the Monteverde Butterfly Garden in Costa Rica. The Asymptotic Kruskal-Wallis Test indicates a significant difference in cyanide concentration between the different

flower stages ($p= 5.397e-05$, $df= 4$, $\chi^2= 24.8483$). Means with asterisks are significantly different (Kruskal-Wallis' post hoc comparison, $p < 0.05$). Standard error bars included.

Leaf toughness in P. suberosa at different flower stages

My results indicate a significant difference in the toughness of the nearest leaves to *P. suberosa* flowers in various developmental stages ($p=.0296$). The results show a significant difference in leaf toughness between YB and FEMALE flower stages ($p=.0296$, $F= 2.86$, $df=4$). Leaves nearest to FEMALE phase flowers had the highest mean toughness, and leaves nearest to YB phase flowers had the smallest mean toughness (Figure 3). There was not a significant difference in leaf toughness between YB, OB, OPEN, and MALE stages or between FEMALE, OB, OPEN, and MALE stages (Figure 3).

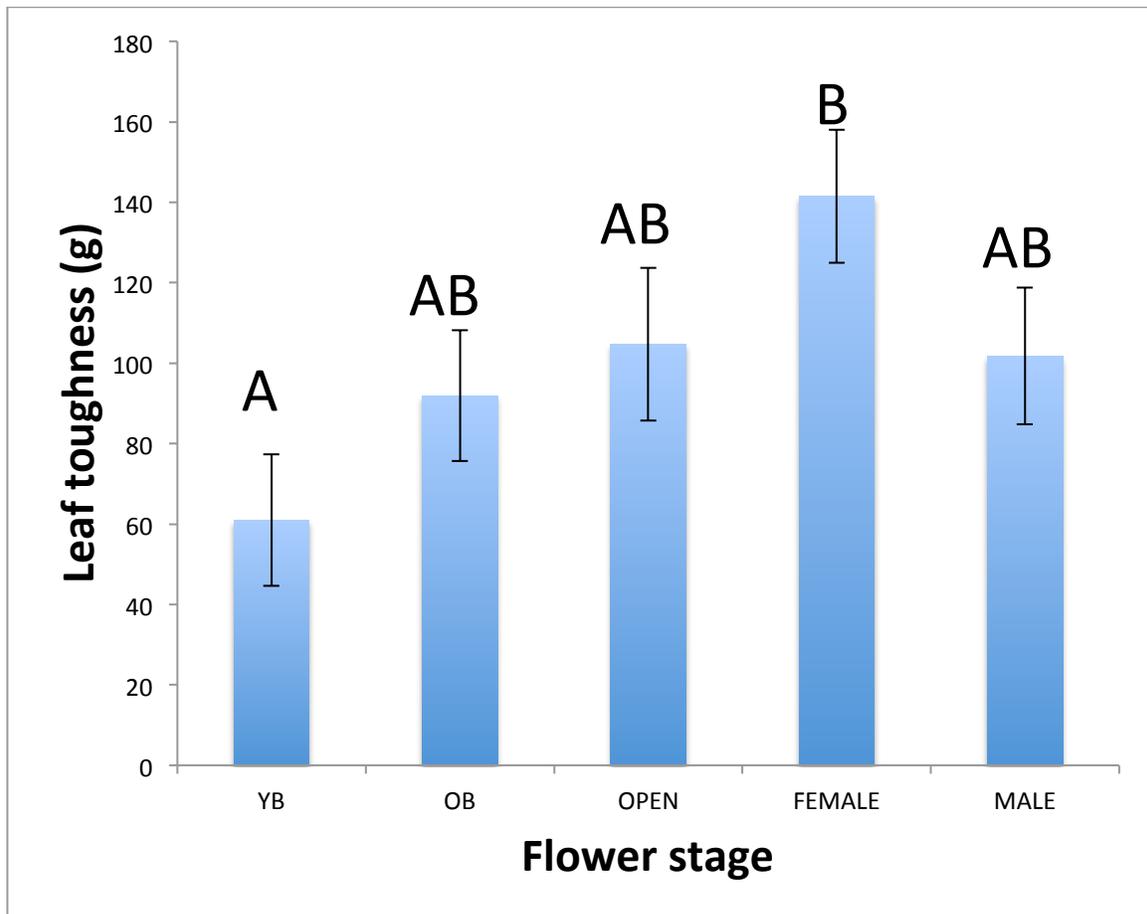


Figure 3. The mean toughness of nearest leaves to *P. suberosa* flowers in different developmental stages- YB (Young Bud), OB (Old Bud), OPEN (just open), FEMALE, and MALE- collected at the Monteverde Butterfly Garden in Costa Rica. Each leaf was removed adjacent from its nearest flower. Each flower stage is represented by 15 nearest leaves of various size and age. Toughness was measured using a penetrometer. Tukey's post hoc comparison shows a significant difference in leaf

toughness at different flower stages ($p=0.0296$, $F= 2.86$, $df=4$). Means with different letters are significantly different (Tukey's post hoc comparison, $p<0.05$). Standard error bars included.

Leaf toughness and leaf CN content in *P. suberosa*

There is not a significant correlation between leaf toughness and leaf CN content ($p=0.0889$). However, there is a strong negative trend (-0.02328).

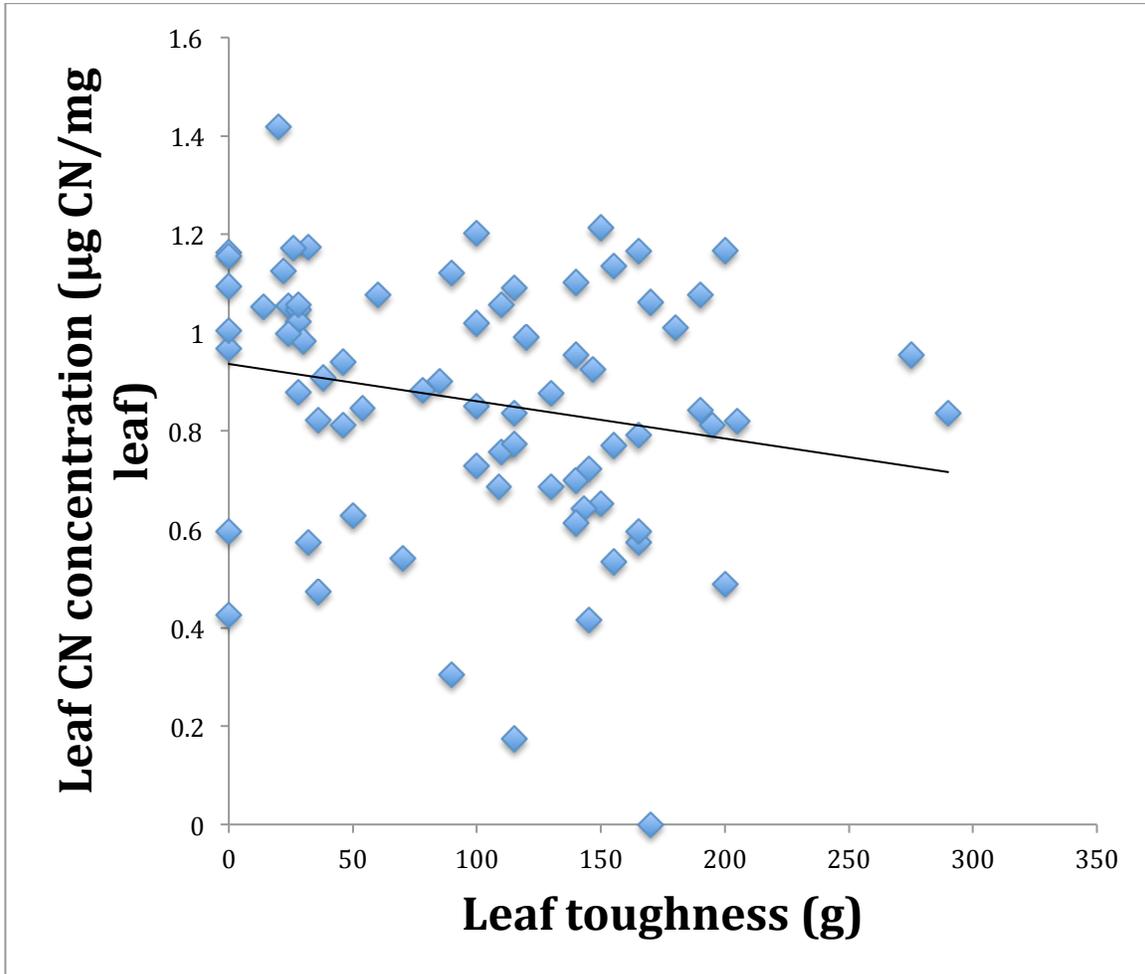


Figure 4. Leaf toughness and cyanide concentration in *P. suberosa* leaves nearest to flowers collected at the Monteverde Butterfly Garden in Costa Rica. There was not a significant correlation between leaf toughness and CN content ($p=0.0889$). There is a negative trend between leaf toughness and CN content (Spearman correlation; -0.2328). Best-fit line included for visual clarity.

CN concentration in *P. suberosa* flowers and their nearest leaves

Results indicate that in *P. suberosa* there is not a relationship between CN concentration in flowers and CN concentration in their nearest leaf ($p=0.2635$)

(Figure 5). The CN concentration in flowers is independent from CN concentration in their nearest leaf. There was a slightly positive trend (Spearman's rank correlation 0.1307562).

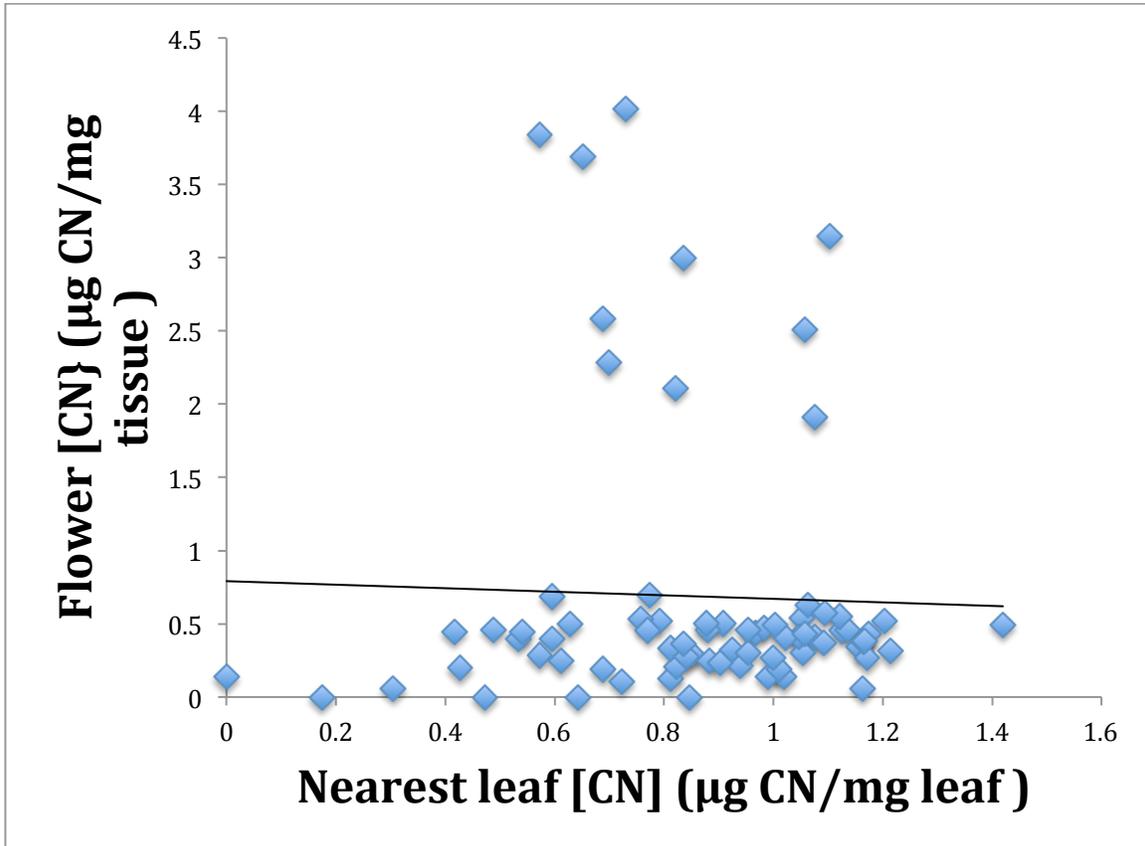


Figure 5. Cyanide concentration in *P. suberosa* flowers and their nearest leaves, collected at the Monteverde Butterfly Garden in Costa Rica. 75 leaves and 75 flowers were represented, regardless of flower stage. Spearman's rank correlation test indicates no relationship between CN concentration in flowers and their nearest leaves ($p=.2635$). Best-fit line included for visual clarity.

CN concentration in different parts of P. miniata flowers

Results indicate a significant difference in CN concentration between different flower parts of *P. miniata* ($p=2.38e-05$, $F=7.407$, $df=5, 54$). The CN concentration means fell between .1 and .5 µg CN/mg tissue for all flower parts (Figure 6). A multiple comparison of means indicated significant differences between STIGMA and ANTHOR (p= .00948), SEPAL and CORONA FILAMENTS (p=.02657), SEPAL and OVARY (p=.00285), SEPAL and PETAL (p=.001), and STIGMA and SEPAL (p=.001).

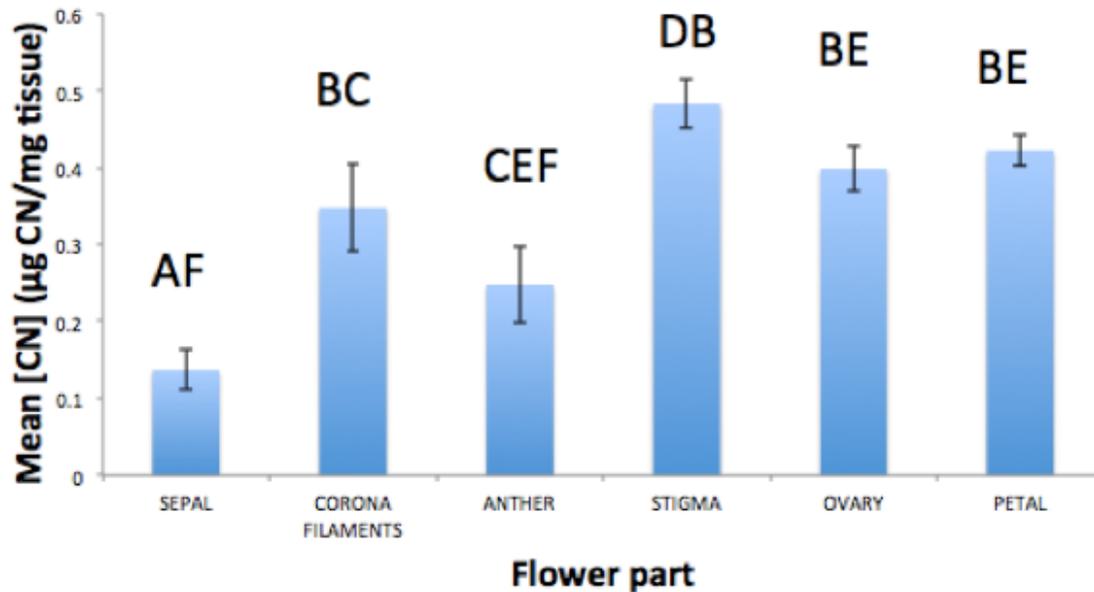


Figure 6. Mean cyanide concentration in six different flower parts- SEPAL, CORONA FILAMENTS, ANTHOR, STIGMA, OVARY, and PETAL of *P. miniata*. Each flower part is represented by 10 of the same flower parts collected in the Monteverde Butterfly Garden in Costa Rica. An ANOVA indicates a significant difference in CN concentration between flower parts ($p=2.38e-05$, $F=7.407$, $df=5, 54$). Means with different letters are significantly different (Tukey contrasts, $p<0.05$). Standard error bars included.

DISCUSSION

Cyanide content was greatest in YB flowers. As the flower progresses from young bud to mature flowers and fruits, the flower loses CN. More CN might be allocated to young flowers because the plants want to grow mature flowers capable of reproduction, fruit production and then dispersal. If the flower makes it to reproductive age, the plant is more likely to be pollinated, fruit, and then be dispersed. By heavily investing in the protection of young flowers, the plant may increase its fitness. It may also be possible that flowers face a tradeoff between CN and toughness defenses as well. Perhaps as the young buds mature, their tissue becomes tougher, and thus they need less CN protection.

There was a significant difference in leaf toughness depending on the developmental stage of its nearest flower. Leaves nearest to FEMALE flowers were the toughest while leaves nearest to YB phase flowers were the most tender. Perhaps because less energy is being allocated toward CN production in flowers at older developmental stages, more energy can be diverted toward leaf toughness. If this is true, leaves nearest to more mature flower stages may have greater leaf toughness, because the flowers have less CN. Leaves nearest to YB flowers may be less tough as the flowers have greater CN content, and thus require energy allocation to produce it.

There was a strong negative trend between leaf toughness and leaf CN content. This suggests that there is a tradeoff in defense energy allocation. As leaves increase in toughness, they lose CN. There was no relationship in cyanide content between flowers and their nearest leaves. This may be because nearby leaves are not always young and do not always have lots of CN. Therefore, leaves are not a reliable source of CN for nearby flowers. Because I selected the nearest leaves over a range of age and toughness to flowers in every developmental stage, leaf toughness in nearest leaves was not just developmental.

A significant difference in CN concentration was found between different flower parts in *P. miniata*. Differences in CN allocation to different parts of the flower suggest that the flower may value some parts more than others. Stigmas had the greatest CN concentration perhaps because they are the landing platform for pollen and may be the most vulnerable to herbivory as they stick out farthest on the flower. Sepals had the lowest concentration of CN. They initially serve as protection for the young bud (Edlund *et al.* 2004), but in a mature flower, they no longer serve as protection for the bud, and may therefore be allocated less CN. Sepals may also have low CN levels because they are less susceptible to herbivory, due to their placement on the bottom of the flower. Petals also had a high concentration of CN. Petals aid in the attraction of pollinators, but also the attraction of flower predators (Edlund *et al.* 2004), and therefore should be highly protected. The ovary is where the new plant forms, and is in charge of production of eggs, polar nuclei, seeds, and fruits (Edlund *et al.* 2004), and thus may have had a high CN concentration because of its multiple functions. Anthers, which house the male gametes (Harrison 2013), had the lowest levels of CN after the sepal. Differences in CN levels between anthers and ovaries might suggest relative investment in plants as their susceptibility to herbivory is similar, but their CN concentrations were different. Female gametes are larger, fewer, and more energy expensive to produce than male gametes (Harrison 2013) and thus may be allocated more CN defense in order to protect this higher investment. Whereas only one egg is produced, lots of pollen is produced (Harrison 2013). Anthers therefore have multiple expendable units, pollen, and therefore may require less protection. Corona filaments also had high CN concentrations. Corona filaments are believed to act as nectar guides for pollinators, so their protection would increase the flower's chance of being pollinated. My results suggest that reproductive function was the determining factor in how much CN was allocated to each flower part.

By looking at my data, I determined there is a greater CN content in leaves than flowers overall, possibly suggesting that plants favor leaves because they are more valuable over their lifetime. I found that *P. suberosa* did not take CN away from leaves to give to flowers. Leaves may be more greatly valued because they last longer; leaves can last for several months whereas flowers last only for a few days (Primack 1985). Because flowers have a shorter lifespan, they face less time exposed to herbivory. Damage caused by herbivores in flowers only lasts for the few days the flower is alive, while damage in leaves lasts the entire several months the

leaf lives. In leaves, herbivory means loss of photosynthetic tissue, which means a loss in energy production (Moles 2013). However, damaged flowers can still be pollinated and therefore reproduce. Therefore, it is more beneficial for the plant to allocate more energy toward defense in leaves than in flowers.

Evidence of tradeoffs reflects the overall value of the tissue to the plant, to some degree. CN has nitrogen in it and may be a limited resource for plants. Toughness is expensive in a different sense in that it takes lots of photosynthetic products to produce and it takes a longer time. It makes sense, then, for Passion vines to use CN only on younger tissues until toughness takes over. Keeping CN in the pistil is an added defense for a particularly valuable part of the plant.

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Leaf Nutrients, Anthocyanin Concentration, and Leaf Cutter Ant (*Atta cephalotes*) Foraging Preference in Young and Old leaves of *Zygia palmana* and *Inga punctata* (Fabaceae: Mimosoidea).

Lisa M. Colombo

ABSTRACT

Young understory leaves often lack chlorophyll but may have photosynthetically active, red anthocyanins. Such leaves have been shown to inhibit herbivory by leaf cutter ants (*Atta cephalotes*) but it is not clear if this is because they are nutritionally inferior or the anthocyanins have antiherbivore properties. I examine the effects of toughness, water content, nitrogen, and anthocyanin concentration on *A. cephalotes* foraging preference. To do this, I use young and old leaves in two species with delayed greening: *Zygia palmana* whose young leaves are dark red and *Inga punctata* whose young leaves are nearly white. Significant differences were found between leaf types in toughness, percent water weight, and anthocyanin concentration. *Z. palmana* young leaves were the most tender (M= 0.0, SD= 0.0) followed by *I. punctata* young (M= 48.93, SD= 77.69) with both old leaves being much more tough (*Z. palmana*: M= 222.11, SD= 61.55 & *I. punctata*: M= 180.87, SD= 51.40). Both young leaves had about 72% water weight and old leaves had about 52%. *Z. palmana* young leaves (M= 3.85×10^{-5} mg/g, SD= 1.03×10^{-5} mg/g) had 20 times more anthocyanins than the three other leaf types. No significant differences were found in nitrogen between the four leaf types, indicating high variability between individuals and possible ant preference on a leaf-to-leaf basis. Trials with *A. cephalotes* showed preference for *Z. palmana* young leaves, indicating that anthocyanins do not inhibit ants and may be preferred. Ant foraging preference is very variable because leaf preference may come from inside the colony through semiochemicals based on the fungus's needs, which can vary day to day. It is also hypothesized that ants collect a variety of leaves, which are either accepted or rejected in the colony. Because anthocyanins do not inhibit herbivory, it is hypothesized that the plant may use them to lower its investment in young leaves because anthocyanins have some photosynthetic activity and can contribute to leaf growth. Because anthocyanins help the young leaves self-sustain, the plant experiences a less detrimental loss in fitness when red leaves are herbivorized as compared to white or very light green young leaves.

Resumen

Las hojas juvenes del sotobosque normalmente carecen de clorofila pero pueden ser antocianinas fotosintéticamente activa. Dichas hojas han mostrado inhibir la herbivoria por zompopas (*Atta cephalotes*) pero no es claro si esto es debido a que son nutricionalmente inferiores o porque las antocianinas tienen propiedades contra herbivoros. Examine los efectos de dureza, contenido de agua, nitrógeno, y concentración de antocianinas en las preferencias de forrajeo de *A. cephalotes*. Para realizar esto, usé hojas juvenes y maduras en dos especies con enverdecimiento retardado: *Zygia palmana* las cuales tienen hojas juvenes de color rojo oscuro e *Inga punctata* cuyas hojas juvenes son casi blancas. Diferencias significativas se encontraron entre los tipos de hojas en dureza, porcentaje de contenido de agua, y concentración de

antocianinas. Las hojas juvenes de *Z. palmana* fueron las más tiernas (M= 0.0, SD= 0.0) seguidas por las hojas juvenes de *I. punctata* (M= 48.93, SD= 77.69), con ambas hojas maduras siendo mucho más duras (*Z. palmana*: M= 222.11, SD= 61.55 & *I. punctata*: M= 180.87, SD= 51.40). Ambas hojas juvenes tuvieron un contenido de agua de alrededor del 71% y las hojas maduras de alrededor del 52%. Las hojas juvenes de *Z. palmana* (M= 3.85×10^{-5} mg/g, SD= 1.03×10^{-5} mg/g) tuvieron 20 veces más antocianinas que los otros tres tipos de hojas. No hay diferencias significativas en el contenido de nitrógeno entre los cuatro tipos de hojas, indicando una gran variabilidad entre individuos y una posible preferencia de las hormigas basada entre hojas. Los tratamientos con *A. cephalotes* muestran una preferencia por las hojas juvenes de *Z. palmana*, indicando que las antocianinas no inhiben a las hormigas y pueden ser preferidas. La preferencia de forrajeo por parte de las hormigas es muy variable debido a que la preferencia puede deberse a la selección por parte de las hormigas dentro de la colonia basada en las necesidades semi-químicas del hongo, la cual puede variar día a día. Se cree también que las hormigas colectan una variedad de hojas que pueden ser aceptadas o rechazadas en el nido. Debido a que las antocianinas no inhiben la herbivoría, se cree que la planta puede usarlas como una inversión menor en hojas juvenes ya que las antocianinas poseen capacidad fotosintética y pueden contribuir al crecimiento de la hoja. Debido a que las antocianinas ayudan a las hojas juvenes a auto-sostenerse, la planta experimenta una menor pérdida en el éxito reproductivo cuando las hojas rojas son depredadas en comparación con las hojas juvenes de color blanco o verde claro.

Young leaves of tropical shade-tolerant plants suffer on average twenty times more herbivore damage than older leaves, most likely because they are more tender (Coley & Kursar 1996). Understory plants may delay greening of new leaves, only investing chloroplasts and nitrogen in them once they toughen (Queenborough et. al. 2013; Coley & Kursar 1991). This strategy may protect young leaves by keeping them devoid of nutrients and unappealing to herbivores (Pomar & Barcelo 2007). Plants with delayed greening often have anthocyanins, red photosynthetic flavonoids that typically break down with age (Lee & Collins 2001). Anthocyanins allow some photosynthetic activity in young leaves and appear to have antifungal properties that may restrict leaf cutter ant (*Atta cephalotes*) herbivory (Coley & Kursar 1996). However, it is unknown if leaf cutter ants avoid red young leaves because they are repelled by anthocyanins or because the leaves are low in nutrients.

Leaf cutter ants are important herbivores that collect leaves high in nutrients, like nitrogen (Coley & Kursar 1991), and low in antifungals (Coley & Aide 1989; Leal et al. 2014). They use the leaf pieces to cultivate fungus, which is their primary food source (Leal et al. 2014). It may be important for the ants to select leaves with low levels of

antifungals to avoid damaging their fungus (Leal et al. 2014). Young leaves with higher concentrations of anthocyanins are avoided more by leaf cutter ants than other young leaves, however nutrient content was not evaluated (Coley & Aide 1989). Direct applications of various concentrations of anthocyanin to oat flakes showed a trend for higher ant preference as concentration decreased (Coley & Aide 1989). Hertel (2010) found that when presented with oat extracts of various ages of leaves with delayed greening the ants selectively avoided extracts of young leaves with anthocyanins and in general found older leaf extracts more appealing. After additional study, it is still unclear if *A. cephalotes* selectively avoid red young leaf extracts because they are high in anthocyanins or because they are low in nutrients (Hertel 2010).

This study will examine ant foraging preference based on toughness, water content, nitrogen, and anthocyanin concentrations in young and old leaves of *Inga punctata* (Mimosaceae) and *Zygia palmana* (Mimosaceae). Both species exhibit delayed greening, but only *Z. palmana* has anthocyanins in its red young leaves. Over the four leaf combinations of species and leaf age tested, a natural range of each chemical leaf property will be established. By presenting the leaf samples to the ants in both leaf disk form and as extracts on oats, this experiment will aim to answer the question of which chemical properties of the leaf are most influential in ant preference, specifically focusing on the dynamics of nutritious value and anthocyanin concentration in foraging decisions.

METHODS

STUDY SITE AND PLANT SPECIES.— This study was conducted in Monteverde, Costa Rica from October to November 2014. This area is classified as a Premontane Wet Forest (2-4 m of rain/yr) by the Holdridge Life Zone System (Holdridge et al. 1971). Two plant species with delayed greening *I. punctata* (Mimosaceae) and *Z. palmana* (Mimosaceae) were selected and collected at an elevation of 1530 m. *I. punctata* has very light green young leaves and *Z. palmana* displays red young leaves. Leaves of both species age to a similar dark green as the plant starts allotting chlorophyll to the leaves as they toughen (Figure 1).



FIGURE 1. Young and old leaves of *Inga punctata* (Mimosaceae) and *Zygia palmana* (Mimosaceae) collected in a premontane cloud forest in Monteverde, Costa Rica at 1530 m. From left to right: *Z. palmana* young, *Z. palmana* old, *I. punctata* young, *I. punctata* old. *Z. palmana* young leaves exhibit red delayed greening with anthocyanins and *I. punctata* young leaves exhibit delayed greening without secondary chemical defenses. Both leaves gain chlorophyll and age to a similar dark green.

CHEMICAL TESTING.—Young and old leaves from ten individuals of each species were collected for chemical testing. Leaf age was determined by color and placement on plant. Young leaves displayed the colors described above and old leaves were selected as the closest fully dark green leaves to the young leaves. Selected individuals were healthy and of similar size. Leaves with herbivory were avoided and only healthy leaves were selected for analysis.

TOUGHNESS.—Toughness was measured using a penetrometer. The leaves were placed between the two tiles of the penetrometer; the plate with the needle was placed on top, and leaf veins were avoided. A beaker was placed on top of the penetrometer, water was added until the leaf was punctured, and the volume was recorded. This procedure was repeated three times for each leaf age of each individual, with different leaves being used in each trial, and averaged.

WATER CONTENT.—Water content was measured for each sample by measuring and recording about 1.5 g of fresh sample (wet weight) and placing it in a dry box for two

days at 69.9 °C. The dry weight was recorded and the weight lost from wet to dry and the wet weight were used to calculate the percent water content.

ANTHOCYANIN CONCENTRATION.—For each sample, 2.0 g of fresh leaf were weighed then cut up into about 1 cm² pieces to aid grinding. Samples were ground in 80% methanol containing 1% HCl (40 ml) and pH 7.0 phosphate buffer (2 ml) (to mimic chloroplast conditions) in a mortar and pestle for ten minutes to ensure all of the secondary compounds were extracted (Figure 2A). The sample was decanted into a test tube and centrifuged for 5 minutes at 5000 rpm (Coley & Aide 1989). The sample was transferred into three cuvetts and percent transmittance was measured in a Model UV-200-RS Ultraviolet and Visible Spectrometer at 663 nm, 653 nm, 646 nm, and 532 nm. The readings were converted to absorbance using the following equation:

$$\text{Abs} = -\log(\%T/100)$$

The level of absorbance was then used to calculate the amount of anthocyanin in the sample. To get the concentration, the following equation was used (Hertel 2010):

$$\text{Anthocyanin (mg/g)} = \text{Abs}_{(532)} - 0.25\text{Abs}_{(653)} / 2.45 \times 10^4 (\text{leaf mass(g)})$$

NITROGEN.—The nitrogen content of each leaf sample was determined using a Model Sth Series LaMotte soil kit according to the manufacturer's instruction manual. To prepare the leaves for sampling, two 4 cm² pieces from each leaf sample were cut and then cut again into smaller 1/8 cm by 1/8 cm pieces. The pieces were funneled to a vial containing Nitrogen Extracting Solution (10 ml) and shaken for 5 minutes. The solution was filtered and the extract tested for nitrate following the instructions outlined in the LaMotte SMART2 Colorimeter Operator's Manual (Figure 2B). Mixed Acid Reagent (5 ml) were added the extracted solution (5 ml) and mixed for two minutes. Then Nitrate Reducing Reagent (0.2 g) was added to the solution and the vial was inverted approximately every second for four minutes. The vial was left to stand for 10 minutes to allow maximum color development, then tested using a SMART2 colorimeter, using the 64 Nitrate-N LR test. The colorimeter measured the concentration of Nitrate Nitrogen in the sample, which was multiplied by 4.4 to convert the data to Nitrate concentration in parts per million, as instructed by the manual (Bakker 2014).

FEEDING TRIALS WITH *A. CEPHALOTES* (LEAF CUTTER ANTS).—One large colony of *A. cephalotes* was selected for analysis in Bajo del Tigre close to the Children's Eternal Rain Forest (Figure 3A). To determine if leaf cutter ant preference is affected by the chemical properties analyzed above, feeding trials were conducted. Leaf disks of both ages of each species were presented to measure ant preference of all leaf properties. Oats were presented to eliminate factors of toughness and water content, measuring ant preference based only on concentrations of anthocyanins and nitrogen. The ants were presented with the leaf disks for one day and the oats the following day.

LEAF DISKS.—About 100 pieces were cut out of each leaf age and species with a standard 5 mm diameter hole punch. Various individuals contributed to the leaf disk sampling. The hole punch was cleaned with water before switching to a different leaf type. Three different stations were set up around the ant colony. One started 2 meters from the colony entrance on a trail and the other two were placed on a different trail, one 4 m from the entrance and the other 15 m from the entrance. Each station had 5 disks of each age of each species laid out 5 cm apart on one side of the trail in the following order: *I. punctata* young, *I. punctata* old, *Z. palmana* young, and *Z. palmana* old (Figure 3B). On the other side of the trail the disks were laid out in the same pattern except backwards to control for ant preference for leaf disks closest to the colony. The number of leaves removed, including picking up or pushing, was recorded for 2 hours. Once a disk was removed, it was replaced by a new one to preserve the availability of five disks of each age of each species.

OAT FLAKES.—Methanol extractions were created for young and old leaves of *Z. palmana* and *I. punctata*, and again leaves came from a mix of individuals. The extracts were made by grinding 5 g of fresh leaves with 80% methanol (40 ml) in a mortar and pestle for 10 minutes. The mixture was filtered and the solution was applied to the oats. About 50 oats were placed in petri dishes, one for each leaf type, and about 2 drops of extract was applied to each oat and left to dry. A control was made by applying pure 80% methanol to the oats. The oats were presented to the ants in the same way as the leaf

disks; 3 stations, 5 oats of each type, 5 cm apart, and order reversed on the other side of the trail. The control oats were added before or after *I. punctata* young (Figure 3C). The number of oats removed, including picking up or pushing, was recorded for 1.5 hours. Once a disk was removed, it was replaced by a new one to preserve the availability of five oats of each age of each species and control.



FIGURE 3. (A) The *A. cephalotes* colony used for feeding trials in Bajo del Tigre, Costa Rica. (B) The leaf disks placed 5 cm apart on either side of the trail at the station 2 m from the colony entrance. (C) The oats with leaf extracts placed 5 cm apart along the trail at the station 15 m from the colony entrance.

OBSERVATIONS.—Some individuals of *Z. palmana* had redder young leaves than others. These red leaves generally had higher levels of anthocyanins. Some young *I. punctata* leaves had traces of red in them, which translated to the highest anthocyanin concentrations for *I. punctata* young, but still much lower than *Z. palmana*. During the two days with the ants it was observed that they were carrying pieces of red leaf, which looked very similar to *Z. palmana*.

RESULTS

CHEMICAL TESTING.—Correlations were found between percent water weight and toughness (df= 40, $r = -0.7226$, $p < 0.0001$) and anthocyanin concentration and toughness (df= 40, $r = -0.4979$, $p = 0.0076$). These correlations are negative, indicating that as the leaves get tougher with age, the amount of water and anthocyanin decrease (Table 1).

TABLE 1. Spearman correlations (n= 40) for each chemical test performed. Correlations are in italics and p-values are in bold, showing there are statistically significant correlations between percent water and toughness and anthocyanin and toughness.

	Nitrogen	Toughness	Anthocyanin	Percent Water
Nitrogen	<i>1.000</i>	<i>0.0366</i>	<i>-0.0339</i>	<i>0.0858</i>
Toughness	1.000	<i>1.000</i>	<i>-0.4979</i>	<i>-0.7226</i>
Anthocyanin	1.000	0.0076	<i>1.000</i>	<i>0.3612</i>
Percent Water	1.000	<.0001	0.2204	<i>1.000</i>

A MANOVA test run over all variables and sample types indicated that there were statistical differences that were pinpointed using post-hoc tests (df= 6, 31 $F = 22.7$, $p = 4.47 \times 10^{-10}$).

TOUGHNESS.—The toughness levels of old leaves of each species are statistically significantly higher than young leaves of each species with *Z. palmana* old (M= 222.11, SD= 61.55) being the toughest and *Z. palmana* young (M= 0.0, SD= 0.0) being the least tough (MANOVA, df= 1, F= 6.52, p= 0.01503; Figure 4) No statistically significant difference was found between the two species within the age groups, although averages and observation indicate that *Z. palmana* young is much less tough than *I. punctata* young (M= 48.93, SD= 77.69) and *Z. palmana* old was tougher than *I. punctata* old (M= 180.87, SD= 51.40).

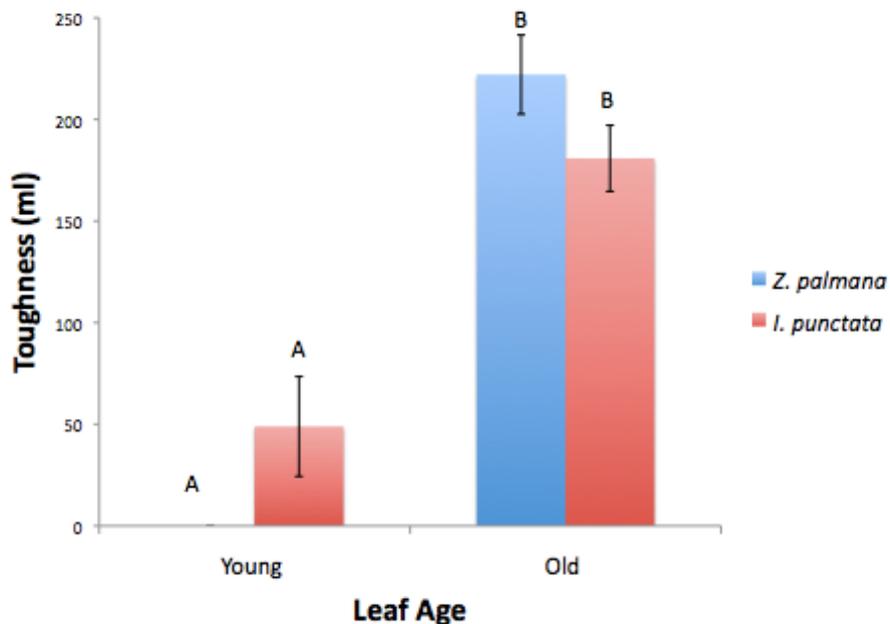


FIGURE 4. Average measures of toughness in 10 young and old leaf samples of *Z. palmana* and *I. punctata* collected in a premontane cloud forest in Monteverde, Costa Rica (means with standard error bars). Means with different letters are statistically different (Tukey Post Hoc, $p < 0.05$).

PERCENT WATER WEIGHT.—The percent water weight in young leaves was found to be statistically significantly higher than percent water in old leaves (MANOVA, $df= 1$, $F= 7.45$, $p= 0.00974$). However, no statistically significant difference was found between the species within the age groups. Young leaves had about 72% water weight and old leaves had about 52% water weight (Figure 5).

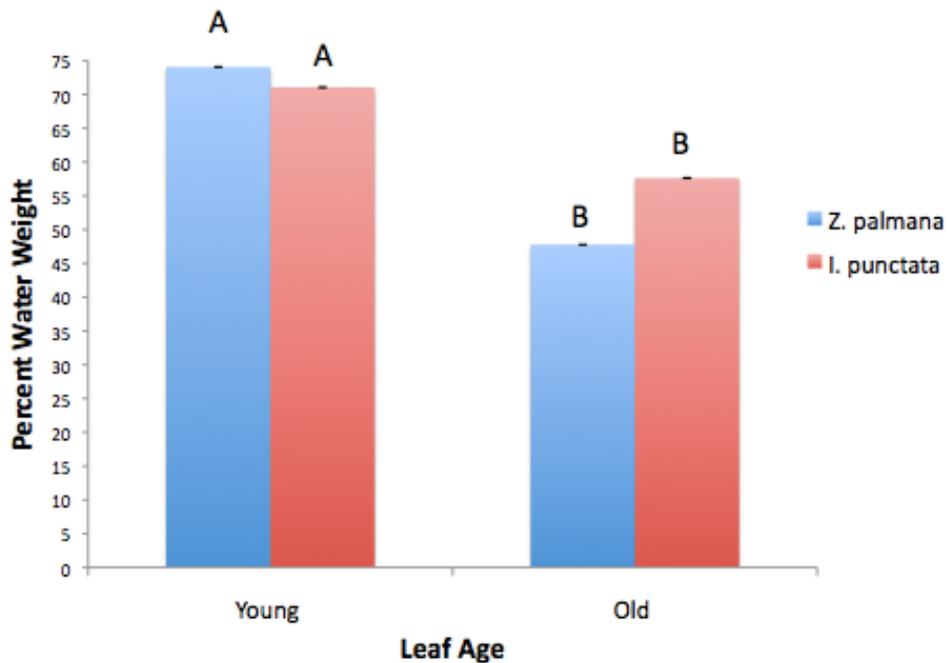


FIGURE 5. Average measures of percent water weight in 10 young and old leaf samples of *Z. palmana* and *I. punctata* collected in a premontane cloud forest in Monteverde, Costa Rica (means with standard error bars). Means with different letters are statistically different (Tukey Post Hoc, $p<0.05$).

ANTHOCYANIN CONCENTRATION.—The concentration of anthocyanins in *Z. palmana* young leaves ($M= 3.85 \times 10^{-5}$ mg/g, $SD= 1.03 \times 10^{-5}$ mg/g) was statistically significantly higher than the concentrations in the other leaf samples (MANOVA, $df= 1$, $F= 132.90$, $p= 1.22 \times 10^{-13}$; Figure 6). *I. punctata* old ($M= 3.08 \times 10^{-6}$ mg/g, $SD= 6.90 \times 10^{-7}$ mg/g) and young ($M= 2.01 \times 10^{-6}$ mg/g, $SD= 1.00 \times 10^{-6}$ mg/g) had statistically the same anthocyanin concentration (Tukey, $p= 0.966$). The anthocyanin concentration in *Z. palmana* dropped ten-fold from young leaves to old leaves ($M= 1.92 \times 10^{-6}$ mg/g, $SD= 4.95 \times 10^{-7}$ mg/g).

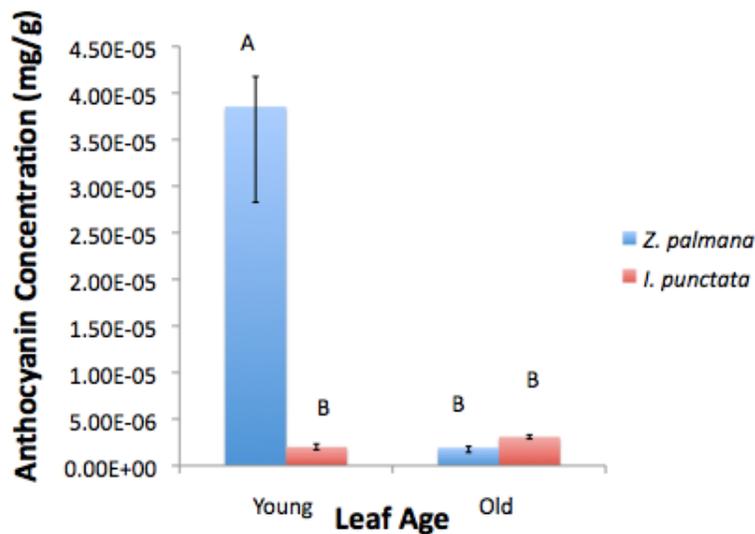


FIGURE 6. Average measures of anthocyanin concentration in 10 young and old leaf samples of *Z. palmana* and *I. punctata* collected in a premontane cloud forest in Monteverde, Costa Rica (means with standard error bars). Means with different letters are statistically different (Tukey Post Hoc, $p < 0.05$).

NITROGEN.—The nitrogen levels in each leaf sample are not statistically different from one another (MANOVA, $df= 3$, $F= 0.396$, $p= 0.7567$; Figure 7). Each leaf category had a range of about 2.0 ppm from the highest to the lowest, indicating that individuals vary considerably in nitrogen concentration.

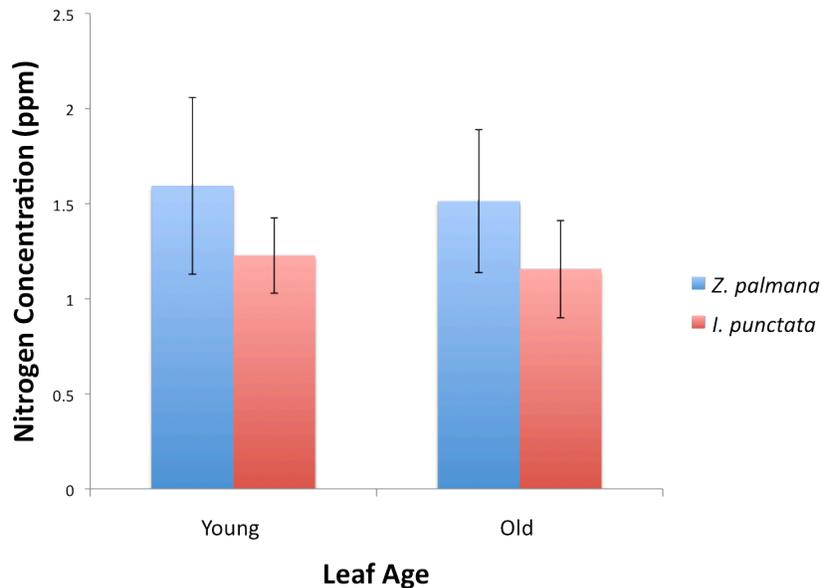


FIGURE 7. Average measures of nitrogen concentration in 10 young and old leaf samples of *Z. palmana* and *I. punctata* collected in a premontane cloud forest in Monteverde, Costa Rica (means with standard error bars). The nitrogen levels are not statistically different from one another (MANOVA, $df= 3$, $F= 0.396$, $p= .7567$).

FEEDING TRIALS WITH *A. CEPHALOTES* (LEAF CUTTER ANTS).—When presented with leaf disks the ants picked up a total of 50 disks, showing a statistically significant preference for young *Z. palmana* leaves and avoidance of young *I. punctata* leaves (Chi-square, $\chi^2= 29.2$, $p= 2.033 \times 10^{-6}$, $df= 3$, $n=50$; Figure 8).

When the experiment was run again with leaf extracts on oats the ants picked up 53 oats. The ants showed a statistically significant preference for *Z. palmana* old leaves (Chi-square, $\chi^2= 9.925$, $p= 0.0417$, $df= 4$, $n= 53$; Figure 9).

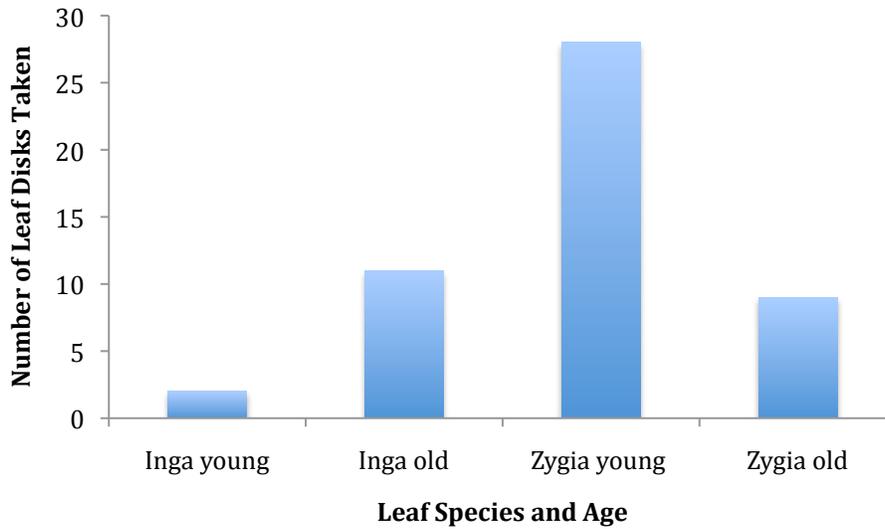


FIGURE 8. Number of leaf disks removed by *A. cephalotes* for each leaf species and age in Bajo del Tigre, Costa Rica. The results are statistically significant showing a preference for young *Z. palmana* (Chi-square, $\chi^2 = 29.2$, $p = 2.033 \times 10^{-6}$, $df = 3$, $n = 50$).

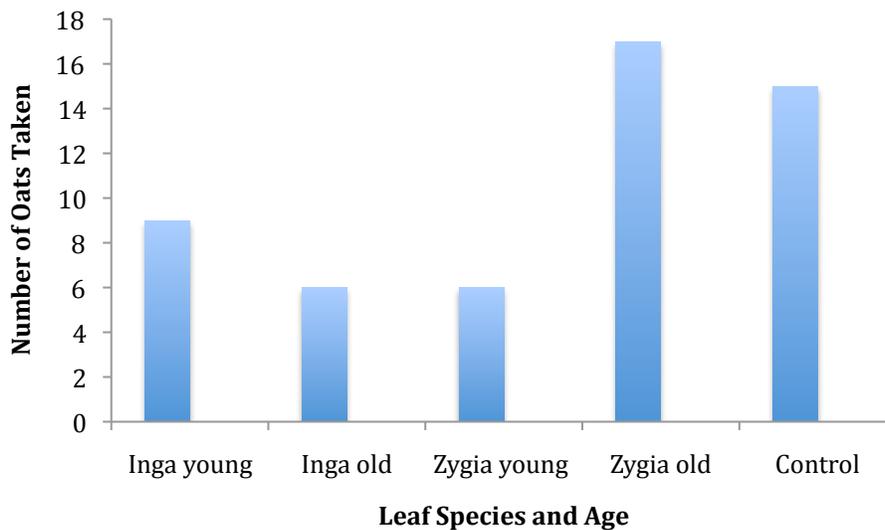


FIGURE 9. Number of oats removed by *A. cephalotes* for each leaf species and age in Bajo del Tigre, Costa Rica. Oats were treated with methanol extracts made from fresh leaves with the control being pure methanol. The results are statistically significant with a preference for *Z. palmana* old leaves (Chi-square, $\chi^2 = 9.925$, $p = 0.0417$, $df = 4$, $n = 53$).

DISCUSSION

Chemical testing of old and young leaves of *Z. palmana* and *I. punctata* revealed statistical differences between leaf types for toughness, anthocyanin concentration, and

percent water weight. *Z. palmana* young leaves were the most tender (M= 0.0, SD= 0.0) followed by *I. punctata* young (M= 48.93, SD= 77.69) with both old leaves being statistically more tough (*Z. palmana*: M= 222.11, SD= 61.55 & *I. punctata*: M= 180.87, SD= 51.40). Both young leaves had about 72% water weight and old leaves had about 52%. *Z. palmana* young leaves (M= 3.85×10^{-5} mg/g, SD= 1.03×10^{-5} mg/g) had 20 times more anthocyanins than the three other leaf types. Nitrogen, however, was not statistically different between the ages and species, indicating that nitrogen levels are highly variable between individual leaves, regardless of species or age, at least for the ten individuals tested. This high variability may indicate ant preference on a leaf-to-leaf basis.

Ant preference for leaf disks of young *Z. palmana* leaves indicates that ants are not inhibited by anthocyanins and prefer tender leaves with high water content. It could be possible that anthocyanins are even preferred in some cases because the ants were observed carrying young red leaves and preferred the young red leaf disks. On the other hand, ant preference for extracts of old *Z. palmana* leaves indicates indifference to anthocyanins. The range of oats taken is narrower than the range for leaf disks, suggesting that the background oat nutrients may have over-powered the leaf extract and that the leaf disk trial is a more compelling test. Both trials with the ants indicate that they are not inhibited by anthocyanins, and they could even be desired.

Studies have shown that leaf cutter ant foraging preference is highly variable and depends on many factors. Ants sometimes do not attack palatable species and often do not attack nearby individuals of species they are consuming at farther distances (Rockwood & Hubbell 1987). Toughness has been defined as the most influential factor on ant preference because it slows the rate of cutting, and this was also found here. However, other chemical properties are certainly involved (Nichols-Orians & Schultz 1989). This could help explain the observed cutting and carrying of red leaves as well as preference for *Z. palmana* young disks.

Foraging decisions may also come from inside the colony, depending on the fungus's needs (North et. al. 2002). The fungus releases a volatile semiochemical, which is used to communicate to the workers and change their foraging preferences (North et. al. 2002). During the days of this test, the fungus may have needed tender leaves or

leaves high in water content or anthocyanin concentration. It is also hypothesized that leaf fragments are either accepted or rejected in the colony, making it hard to pinpoint what leaf properties the ants are looking for. Leaf cutter ant foraging preference is difficult to study because much of the leaf screening takes place in the colony, but it seems to be dominated by leaf toughness and the fungus's needs (North et. al. 2002).

Because anthocyanins are not effectively inhibiting herbivory, plants may be using them to lower their investment in young leaves while still having photosynthetic activity. Because anthocyanins are photosynthetically active, they can help contribute to their own growth instead of relying completely on the mother plant (Lee & Collins 2001). Anthocyanins provide a cheaper alternative for the plant instead of investing in chloroplasts early in leaf development that are more costly to lose. Herbivory on young leaves with anthocyanins is less detrimental to plant fitness than herbivory on light green or white delayed greening leaves because the anthocyanins have been contributing energy to leaf growth and the plant is less invested. Because the light levels in the understory are so low and it takes time for leaves to toughen and become protected from herbivory, anthocyanins help young leaves capture energy and possibly toughen more quickly than young leaves without these secondary chemicals.

In this study anthocyanins did not inhibit leaf cutter ants, and they were in fact preferred in intact leaf fragments. This could be because leaf preference is coming from inside the colony based on the fungus's needs or because ants are collecting a variety of leaves to be accepted or rejected once inside the colony. My data support that toughness is the largest driver of leaf preference. In regards to the mother plant, it is hypothesized that anthocyanins lower the investment in young leaves while still being photosynthetically active, perhaps allowing the leaf to toughen more quickly.

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Sexual dimorphic coloration of frog *Isthmohyla pseudopuma* and predation rates using clay models

Grace Corrigan

ABSTRACT

Sexual selection was first described by Charles Darwin to explain inconsistencies in evolution that did not line up with natural selection. Many studies have shown how conspicuous coloration or ornamentation usually displayed by males to attract females increases male mortality. However, little is known about predation on *Isthmohyla pseudopuma* in adult stages and investigation is necessary for a better understanding of its species interactions. I constructed a total of 250 clay models representing the coloration of *Isthmohyla pseudopuma* frog during breeding season in order to test how sexual dimorphism affects predation. Equal numbers of brown models which represent female *I. pseudopuma* and yellow models representing male coloration during breeding season were placed in the field. Research sites were trails surrounding the Monteverde Biological Station in the Monteverde Cloud Forest of Costa Rica. Sites were both open and forested areas, habitats where *I. pseudopuma* are commonly found. Results showed that predators more often attacked frogs with brighter yellow colorations than brown colorations. Attacks were categorized as missing, chewed up, teeth mark, and beak mark. It appeared that predators were more likely to carry off prey to different locations when attacking in the open area. Clay models were more often left in their original sites when placed in forest areas and attacked with teeth marks associating forest predators with mammals. Camera trap and physical evidence showed that one main forest predator was *Nasua narica*, or the coati. In conclusion, this study shows that male coloration in *I. pseudopuma* although more attractive to females, puts them more at risk for predation than their female counterparts.

RESUMEN

La selección sexual fue descrita primeramente por Charles Darwin para explicar las inconsistencias en evolución que no se explican con selección natural. Muchos estudios han mostrado como las coloraciones conspicuas u ornamentadas usualmente mostradas por machos para atraer hembras aumentan la mortalidad en los machos. Sin embargo, poco se conoce sobre la depredación en *Isthmohyla pseudopuma* en los estadios adultos e investigación es necesaria para un mejor entendimiento de las interacciones de estas especies. Construí un total de 250 modelos de plastalina representando la coloración de *Isthmohyla pseudopuma* durante la época reproductiva para determinar como el dimorfismo sexual afecta la depredación. Igual número de modelos de color café que representan las hembras de *I. pseudopuma* y modelos amarillos representando la coloración de los machos durante la época reproductiva se colocaron en el campo. Los sitios de investigación fueron los senderos alrededor de la Estación Biológica de Monteverde en el bosque nuboso de Monteverde, Costa Rica. Los sitios fueron tanto en bosque como en área abierta, sitios donde normalmente *I. pseudopuma* se encuentra. Los resultados muestran que los depredadores atacan más frecuentemente las ranas con coloraciones más brillantes. Los ataques fueron catalogados como perdido, masticado, mordido y picoteado. Parece que los depredadores gustan más de mover las presas a otro lugar al atacar en el área

abierta. Los modelos de plasticina fueron más comúnmente dejados en su sitio original al ser colocados en el bosque y atacados con mordiscos asociando la depredación en el bosque a mamíferos. Evidencias físicas y con cámaras trampa muestran que uno de los mayores depredadores en el bosque fue *Nasua narica* o pizote. En conclusion, este estudio muestra que la coloración de los machos en *I. pseudopuma* aunque es más atractivo para las hembras, los pone en un mayor riesgo de ser depredados que la contraparte de las hembras.

INTRODUCTION

Darwin first used the idea of sexual selection to describe inconsistencies in organisms within the theory of natural selection. Heavy ornamentation, extravagant coloration or overt behaviors in many species hold a significance that differs from mere survival (Darwin 1871, Andersson 1994). Often, these traits have importance when it comes to mating preference and selection for an organism. While sexual dimorphic traits can help to transmit the genes of an individual within a species to the next generation, it important to recognize how these ornamental traits or behaviors can also be disadvantageous to survival (Promislow *et al.* 1992). A balance between sexually attractive traits and physically disadvantageous ornamentation is necessary for the success of a species.

While sexual dimorphism is advantageous for mating purposes, it is often disadvantageous for survival, especially for mortality from predation. A study investigating the North American passerine bird species analyzed sex-based mortality rates in conjunction with male plumage brightness (Promislow *et. al* 1992). The genetic attempts to impress their female counterparts lead males to be more susceptible to predation because the brighter the plumage on the male passerine birds, the higher their mortality rates. Another study shows the female preference for more brightly colored male poison dart frogs *Oophaga pumilio* and that predator visual wave-lengths favored predation of brightly colored male *O. pumilio* frogs despite their aposematic colorations that warn of their poisonous nature upon ingestion (Maan and Cummings, 2009). In combination, these studies show sexually selected conspicuous characteristics in males can result in both higher predation rates and therefore lower survival. To the best of my knowledge there are no studies on the effect of color in frog predation besides aposematic poisonous species that are not sexually dimorphic. Therefore, there are no studies on the effect of color ion survival in sexually dimorphic species. This study is examining the predation rates in the Meadow Treefrog *Isthmohyla pseudopuma* which is not an aposematic species, or non-poisonous. Since it is difficult to observe predation on frogs, such as the *I. psuedopuma*, I made clay frog models resembling both the male and female mating colorations of this species of frog. Pre-colored plasticine clay models, if made convincingly enough, have been shown to accurately measure predation rates by counting the number of attacks on the model (Sapiroto *et al.* 2007).

The *Isthmohyla pseudopuma*, more commonly known as the Meadow Treefrog is a small frog found abundantly throughout Costa Rica and Panama in both open and pristine areas. The slightly larger female has a relatively uniform brown coloration year round, while the males are brown but turn a bright mustard yellow with brown or tan splotches during the mating season (Savage 2002). Presumably, the bright coloration in males during breeding season is a result of the sexual dimorphism in an effort to attract females. There have been no studies done as to whether the coloration of the *I. pseudopuma* has an affect on predation levels. While tadpoles of

this species are consumed by either insects or cannibalistically, there is little known about what species specifically predate upon *I. pseudopuma* (Crump, 1984). However, barn owls, and omnivorous crab-eating foxes are predators of *Hyla faber*, a close relative to *I. pseudopuma* (Martins *et al.* 1993). For reference, *I. pseudopuma* was formerly a part of the genus *Hyla* before being separated. Hence, it is reasonable to assume that *I. pseudopuma* is also at least occasionally predated by mammals and birds. In this study I tested whether yellow male frogs are more susceptible to predation than brown females during the mating season which spans from May to October. After the mating season males revert back to the brown coloration that the females have. The use of clay models of species has in the past shown to be a viable technique for recording predation in anurans (Saporito *et al.* 2007). In addition, I evaluated the differences in predators and predation attacks in both open and forest areas to show whether there was a difference in predation rates in these areas.

MATERIALS AND METHODS

Study Site

This study was performed in both open and canopy covered areas of Monteverde, Costa Rica close to the Monteverde Biological Station at elevations ranging between 1420 and 1450 meters. The open areas were considered forest edge but had little to no tree cover while the canopy covered areas ranged from 30-100 meters from forest edge.

Clay Models

In both open and forested areas I placed 100 clay frog models for a total of 200 models (Fig. 1). The brown model resembles the color morph of the female year round while the yellow model resembles that of the male frog during mating season (Fig. 2). Studies have shown that color contrast with landscape has an effect on predation (Sapiroto *et al.* 2007) and for this reason I placed frog models on relatively similar appearing ground cover, whether that be short green grass in the open area or leaves and brown dirt in the forest. The bodies of male *I. psuedopuma* is between 37.6-42.9mm on average and that of females is 41.1-45.6mm on average. I made all clay models 42mm in body length for consistency.

General Approach

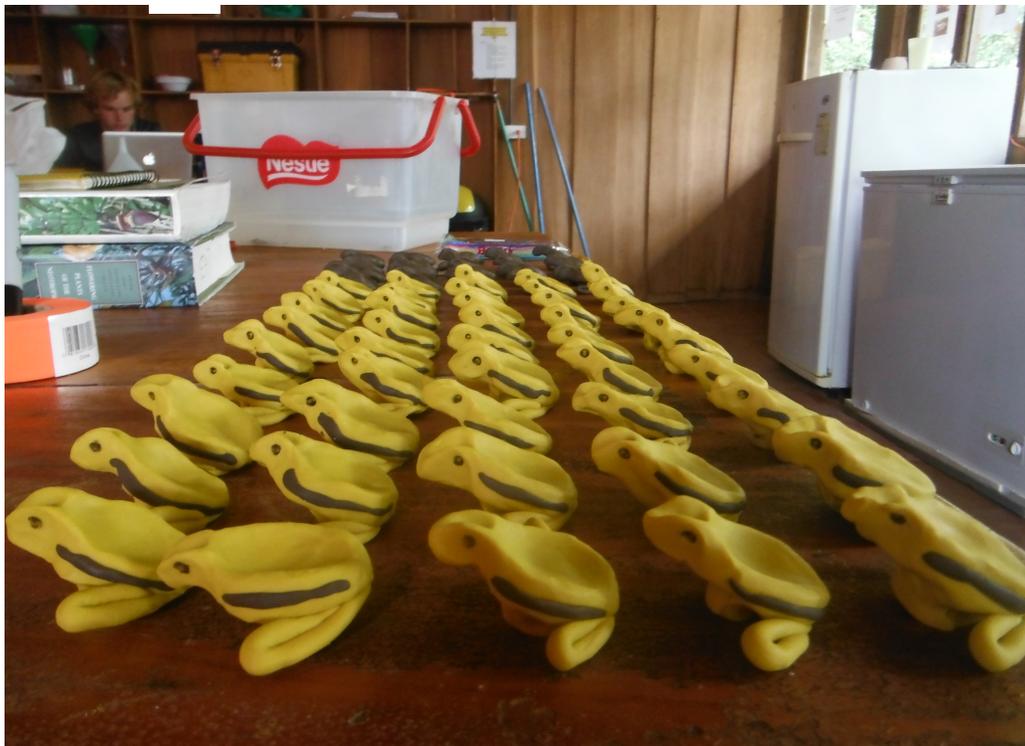
Following trails surrounding the Monteverde Biological Station I placed model pairs approximately every two meters. Models were placed approximately a meter off the trail and the brown and yellow models were placed 50 cm apart. 100 models were placed in each research area for a total of 200 models. The models were in the field for a total of three weeks. Every four days after placement the models were checked for marks of predation. If there was a mark, it was smoothed over or the frog was replaced with a new model. Marks of predation were categorized as either missing, chewed up, teeth mark, or beak mark. While each of these indicated an act of predation, the categories allowed for different patterns to be drawn. As far as the categories are described, missing indicates that when checking on the model it had gone missing, which was assumed to be carried off by a predator. To consider a model missing, I searched a 5 meters in every direction of the original site. Chewed up indicates that the model was 50% or more destroyed by a predator attack. A teeth mark indicates a bite on the model, differing from a beak

mark which is distinctly singular. As far as marks on the models, one mark will be counted as one act of predation because predators will, in theory, realize the model is not true prey and abandon it.

In addition, I used two camera traps to record any potential predation attempts on the clay models; one in the open site and one in the forest site. These camera traps were in the field for a total of 2 weeks.

Statistical Analysis

I determined whether there was a difference in the frequency of predation between yellow and brown frog models using chi-square tests or Fisher Exact t-test, both in contingency tables. Fisher's Exact Tests were used when expected values were <5 . I made contingency tables for open vs. canopy areas, types of predation marks (beak vs. teeth), and how the color variation effected predation.



Figure

1. Male and female frog models before being placed in the field.



Figure 2. Yellow breeding males were given brown stripes on the torso and eyes to make as realistic as possible. Uniform brown clay models represented the female frogs as they have no distinct markings. In addition, the legs were formed to intentionally mimic those of a sitting treefrog, making the model convincing to terrestrial, arboreal, and aerial predators.

RESULTS

Clay Models

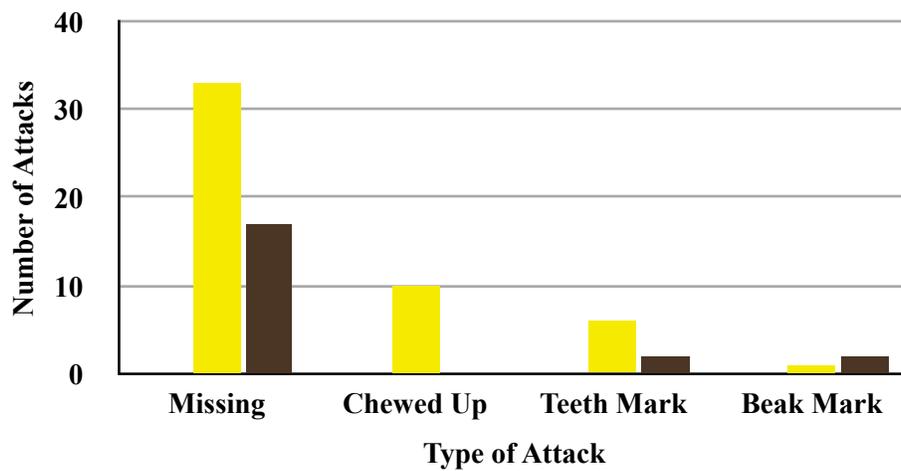
In total, 50 yellow frog models were attacked and 21 brown models were attacked. This showed a statistically significant trend that yellow frogs were more likely to be predated upon (Chi-square 11.84, $df = 1$, $p\text{-value} = 0.0003$).

Missing models, presumed to be avian predation that were carried away, were the most frequent attack recorded on the clay model frogs. The next most common attack was the chewed up predation, presumably caused by terrestrial mammals because of teeth markings (Fig. 3). When looking at the predation rates of the yellow male models versus the brown female models, while not statistically significant there is a strong trend showing higher attack rates in yellow models across predation types. (Fig. 4). A total of 3 beak marks were found out of 71 attacks. In models that had been chewed up or more than 50% destroyed by predators there was obvious teeth imprints from mammals (Fig.5) In one model, it was evident that a coati skull and intact teeth provided by the Monteverde Biological Station matched up almost perfectly with one teeth mark (Fig. 6). In addition, a camera trap near where the frog had been placed had recorded several coatis roaming the open area as well as personal observations of coatis in all areas of study.



Figure 3. An example of a chewed up predation. Model is missing front legs as well as a chunk out of the backside. The small teeth marks shown are most likely from those of a small mammal.

Chi-square analysis of significant differences of predation in open and forest areas showed p-values of 0.03 and 0.04 respectively with degrees of freedom at 1. However, a Fisher's Exact test for count data showed a p-value of 0.06 showing a strong trend toward predation of yellow frog models but data was not significant.



■ Yellow ■ Brown

Figure 4. Number and Type of Predation Attacks on Yellow and Brown Models. Of a total 71 attacks, 50 were found on yellow while 21 were on brown models. While not significant, there is a strong trend showing a predator preference for yellow males during the breeding season.

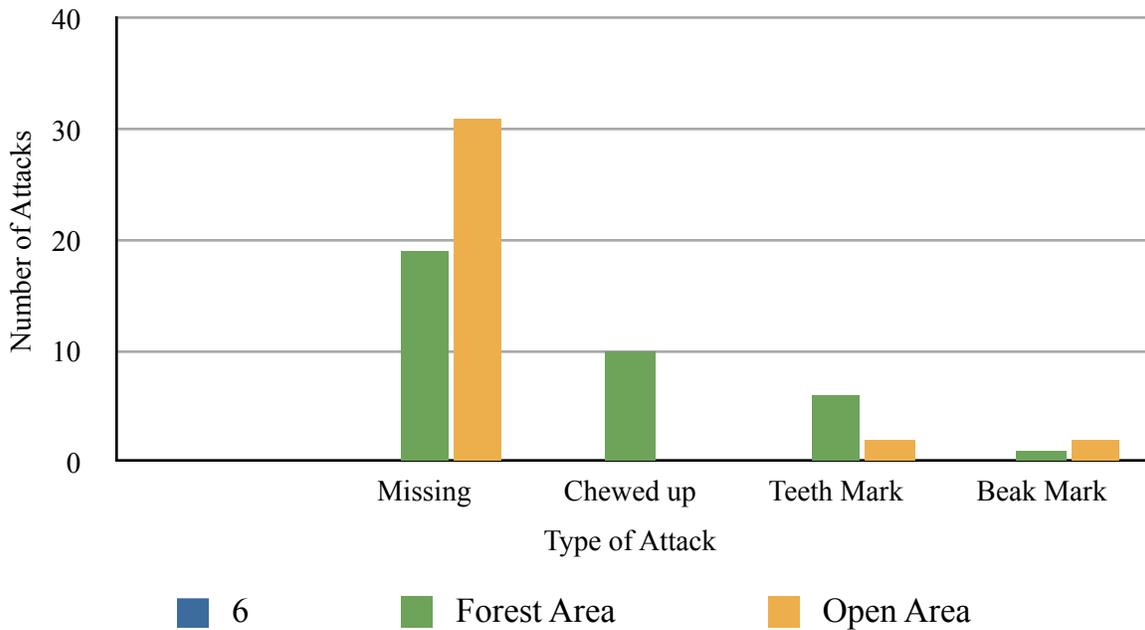


Figure 5. Number and Type of Predation Attacks in Forested and Open Research Areas. The forest area had a total of 36 attacks while the open area had a total of 35. However, predation attacks that caused the model to go missing were found to be higher in open areas. (Fisher’s Exact Test for Count Data, p-value= 0.0002). Although the total number of attacks in forest and in open were virtually identical, more models were missing from the open area and more were chewed up in the forest.



Figure 6. Teeth mark predation in one brown female frog model matched up almost perfectly with the dental imprint of a coati skull available for comparison at The Monteverde Biological Station.

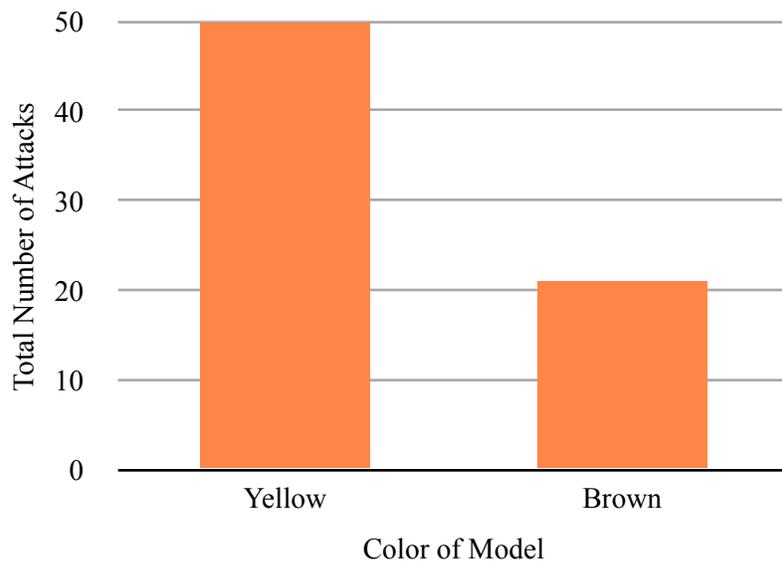


Figure 7. This graph shows the number of attacks on both brown and yellow models.

DISCUSSION

There was a statistically significance in the data showing predator's attacks more commonly on yellow frog models that represented male *I. pseudopuma* in the midst of breeding season. This shows substantial, significant, evidence that in this experiment predators attacked males showing a brighter color during breeding season and did not view them as a threat based on coloration. Females were often left untouched and less often predated, showing that they are less predated than males because of their coloration. The bright color in males presumably increases reproductive success although they suffer higher predation rates than females during the breeding season. This shows that although given the option of both colors of models in the same area predators most often chose to attack the male yellow models- this could be because of their brighter coloration such as those studied elsewhere (Promislow *et. al* 1992). It only makes sense that the female brown models were more often ignored in the presence of what predators consider more appetizing prey. In addition, it can be assumed that predators, even if they saw female frog models, did not return to attack them because they had already realized prey in that area was not palatable plasticine.

In addition, models were most likely to be predated in open areas where they were easy seen without the obstruction of trees. The increased attacks in the form of missing models shows that the most common open area predators for the frogs took their prey to another location, While little is known about predation on *I. pseudopuma* the attacks on *H. faber* were often made by barn owls or crab-eating foxes (Martins *et al.* 1993). In the Monteverde area the aviary attacks may have been committed by many species of birds that consume frogs. The forest attacks were most likely a result of coatis because of the camera trap evidence as well as the teeth mark that

matched the coati skull. Some other predators may have been smaller mammals based on teeth marks on some models. In conclusion, brighter colored males of *I. pseudopuma* were attacked more often showing that while sexual dimorphism can be beneficial to reproductive success, it can also be detrimental to individual survival.

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Nectarivorous bat preferences for flower placement and morphology in model *Mucuna spp.* (Fabaceae: Papilionoideae)

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ABSTRACT

The theory of pollination syndromes states that plants often exhibit morphological characteristics that are associated with the pollinators they have evolved to attract. Bat pollinated plants in particular are assumed to have certain characteristics, such as being large, dull-colored, and low in number. Though studies have shown that these syndromes do not necessarily apply to all plants that are pollinated by the same pollinator, it is still possible that certain plants can have a specific set of characteristics unique to them that will make them more attractive to bat pollinators, particularly in the case of plants in the *Mucuna* genus. To test this hypothesis, several unique characteristics of plants in the *Mucuna* genus were studied in relation to bat preference in a bat enclosure located in Monteverde, Costa Rica, using hummingbird feeders and artificial flowers. The characteristics tested included inflorescence height (with the feeders presented at three different heights), inflorescence cover (having each feeder at a different level of obstruction by vegetation), individual flower size (attaching flowers of either size small, medium, or large to each feeder), and inflorescence size (attaching either six small flowers, four medium flowers, or two big flowers to each feeder). Number of visits and nectar drunk were counted and measured for each different characteristic as a way to measure bat preference, and inflorescences at greater heights (over 40 more visits and 8mL more nectar drunk compared to low and middle feeders), uncovered (over 19 more visits and 5mL more nectar drunk), with small flowers (over 15 more visits and 3mL more nectar drunk) arranged in a cluster (over 19 more visits and 4mL more nectar drunk) were found to be preferential. The results on size and inflorescence size and number in particular differ from the typical pollination syndromes associated with bat-pollinated plants, since it is often assumed that their flowers are large and few in number. These findings provide insights on the particular pollination syndromes of *Mucuna spp.* and support the theory that a plant species or genus may have its own unique pollination syndromes that work best in concert to attract its pollinator.

RESUMEN

La teoría de síndromes de polinización dice que las plantas normalmente exhiben características morfológicas que están asociadas con los polinizadores que ellos han evolucionado para atraer. Las plantas polinizadas por murciélagos en particular se asume tienen ciertas características, como ser largas, de colores pálidos y bajas en número. Aunque estudios muestran que estos síndromes no se aplican necesariamente para todas las plantas que son polinizadas por el mismo polinizador, es posible que ciertas plantas puedan tener un set específico de características únicas a estas que las hagan más atractivas a murciélagos polinizadores, particularmente en el caso de plantas

en el género *Mucuna*. Para probar esta hipótesis, varias características únicas de las plantas en el género *Mucuna* fueron estudiadas en relación a las preferencias por murciélagos en cautiverio en Monteverde, Costa Rica, utilizando comederos de colibrí y flores artificiales. Las características probadas incluyen altura de la inflorescencia (con comederos presentados a tres alturas diferentes), cobertura de la inflorescencia (colocando los comederos con tres niveles diferentes de obstrucción por la vegetación), tamaño de flores individuales (adjuntando flores ya sea de tamaño pequeño, mediano o grande a cada comedero) y tamaño de la inflorescencia (adjuntando ya sea seis flores pequeñas, cuatro flores medianas, o dos flores grandes a cada comedero). El número de visitas y el néctar consumido se midió para cada característica diferente para determinar la preferencia por los murciélagos, y las inflorescencias a alturas mayores (sobre 40 visitas más y 8 ml más de néctar consumido comparado con los comederos a altura media y baja), descubiertos (más de 19 visitas y 5 ml más de néctar consumido) con flores pequeñas (más de 15 visitas y más de 3 ml néctar consumido) agrupadas (más de 19 visitas más y 4 ml más de néctar consumido) fueron encontrados de ser preferidos. Los resultados en tamaño de flor y número de flores por inflorescencia en particular difieren de los síndromes de polinización típicos de plantas asociadas con murciélagos, ya que se asume que estas flores son grandes y en números pequeños. Estos resultados proporcionan ideas en el síndrome de polinización particular de *Mucuna* spp. y apoya la teoría de que una especie o género de planta puede tener su síndrome de polinización único que trabaja mayor para atraer a su polinizador.

INTRODUCTION

Flowers and plants vary morphologically and often exhibit pollinator “syndromes”, which are characteristics directly associated with the type of pollinator they have evolved to attract. These differences are often morphological, relating to the flower’s color, size, shape, number, and arrangement, and affect how attractive they are to a specific pollinator (Faegri & van der Pijl 1979). Bat-pollinated plants are good examples, as they have a variety of well-known and presumably shared characteristics across most species. The flowers of these plants are typically designed in ways that make them attractive to bats, such as having a strong musky smell, being of a dull color, protruding away from the plant, and having a tendency to be large and few in number (Fleming *et. al.* 2009). These characteristics are meant to make the flower easy to find and easily accessible for bats, thereby increasing their chances of being pollinated. This is mainly due to bats’ need to conserve energy; they tend to expend a large amount of calories a day, often restricting their time for foraging and affecting their choice in flower, making the easier ones to access and find the most popular (Arbol 2005).

Though the theory of pollination syndromes is widely accepted and used, studies have shown that the theory has not been subjected to much assessment, and when tested it has been shown to be inadequate for describing the diversity of plant morphologies that occur in relation to one pollinator (Ollerton *et. al.* 2009). Ollerton *et. al.* (2009) were able to identify the pollinators of only one third of the plant species they analyzed using the pollinator syndrome theory as an identification guide, suggesting that morphological traits across all flower types can vary widely despite their being related to the same pollinator. One example of this would be plants in the *Mucuna* genus. Plants in the

Mucuna genus are classified as woody lianas, and they produce pendant inflorescences that hang down into the canopy (Hammerton 2008). While they are all bat-pollinated, these flowering vines can vary in distance from the ground (due to the variability in the length of each flowering vine), amount of cover from other vegetation in the canopy and understory, flower size, and number of flowers (Hammerton 2008).

Despite this variation, studies have shown that bats can have preferences in terms of the aforementioned characteristics of the plants in this genus. Vincent (2003) found that bats preferred *M. urens* inflorescences that were high in the canopy, as opposed to inflorescences that were closer to the ground. *Mucuna* species have also been shown to have evolved to be easily found by echolocation, due to their development of a petal that acts as an “acoustic nectar guide” for echolocating bats (von Helversen & von Helversen 1999). Studies have shown that removing or covering this petal can reduce the chances of the flower being pollinated, and that increasing its size can make the flower more attractive to pollinators (von Helversen & von Helversen 1999, Macedo 2002). These preferences would seem to indicate that, despite the fact that bat pollination syndromes may not apply to all bat-pollinated plants as stated by Ollerton *et. al.* (2009), plants in the *Mucuna* genus do exhibit certain syndromes that make certain inflorescences more preferable to bats.

Though bat preference in terms of specific species have been evaluated in previous studies, the genus as a whole has not been studied, and certain characteristics have also been neglected when identifying preference. The purpose of this study was to determine whether plants in the *Mucuna* genus exhibit certain syndromes that make certain inflorescences more preferable to bats and, if they do, whether those syndromes differ from the bat-pollinated plant syndromes as suggested by Faegri and van der Pijl’s theory. For the study, several characteristics of plants in the *Mucuna* genus were tested for bat preference in captivity using artificial flowers, including inflorescence height, inflorescence cover, individual flower size, and inflorescence size.

MATERIALS AND METHODS

Study site

I conducted this study at the Bat Jungle, a bat enclosure exhibit located in Monteverde, Costa Rica. The enclosure contains 90 bats which represent a total of 8 bat species, including two which are nectar feeding: *Anoura geoffroyi* and *Glossophaga commissaris*. It should be noted that the majority of the nectar feeding bats are of the latter species. Most of the bats in the enclosure were raised in captivity or have spent the majority of their lives in captivity. The bats are kept in a reversed day-night cycle, which made it possible to conduct this experiment in the daytime. They are released into the enclosure from their sleeping room between 8 and 8:30 in the morning, and in the enclosure there are usually two hummingbird feeders filled halfway with nectar, hanging from hooks located in the middle of the room. When conducting the experiments on flower placement and arrangement, the flowers were simulated by the hummingbird feeders, and access to the feeding holes located at the top of the feeders was manipulated in order to create four different experimental treatments.

Study organism: Mucuna spp.

Plants of the genus *Mucuna* are widespread, ranging from Nicaragua to South America. There are about 100 species in the genus, seven of which are found in Costa Rica. They are classified as lianas, with flowers arranged in a chandelier cluster at the end of a long stem that dangle down in the canopy. Fruits are brown wrinkled pods that contain around 3 seeds (Zuchowski 2007). Since this study focuses on the genus in general rather than specific species, three common neotropical species of *Mucuna* were selected when creating petals of different sizes: *M. fawcetti*, with a flower length of up to 95mm, *M. sloanei* with a flower length of up to 65mm (Sea Bean Guide), and *M. urens*, with an average flower length of 30-40mm (Acevedo-Rodriguez 2005) (Figure 1).



Figure 1: Inflorescence of *Mucuna urens* in typical growth form, with flowers not yet mature (Photo by Papoulias 2013). Flowers hang down from the canopy as pendant inflorescences and are made up of five petals: two lateral ones forming the wings, two forming the keel, and a raised petal called the vexillum (von Helversen and von Helversen 1999).

Study materials

Three hummingbird feeders were used for this experiment. All three had six feeding holes available, however one had slightly larger holes than the other two. For this reason, feeders were rotated to different positions and with different treatments every day to spread the effect that the feeder with larger holes may have had on bat preferences for specific flower morphology treatments.

Height experiment

The first treatment tested whether bats show preference for flowers at a specific height. All feeders contained 100ml of a nectar mix that is prepared at the jungle and fed regularly to the bats. Data were collected by counting the number of visits each feeder received in a half hour period and the amount of nectar that was drunk from each in the same half hour period. Three feeders were hung at three different equidistant heights: one close to the floor (hanging at approximately 0.35m), one hanging in the middle of the room (approximately 1.1m), and one close to the ceiling (approximately 1.85m). Once the feeders were up, I observed each feeder for a period of 10 minutes, ending up with a total of 30 minutes spent observing. After this half hour period, I entered the bat enclosure and removed the feeders in order to measure the amount of nectar that had been drunk. After pouring out the nectar into a graduated cylinder and measuring, feeders were placed back into the enclosure. This process was repeated three times in one day for four days.

Cover experiment

The second treatment tested whether bats show preference for flowers that are not obscured by other vegetation. I used the same three feeders, all three of which were placed at the height for which bats showed preference in the first experiment and were equidistant from each other. To cover the feeders I used large leaves taken from a tree outside of the bat jungle, which were then tied from their stems to the feeder with twine so that the leaf portion was hanging down onto the feeder. The leaves were then cut and positioned according to how much they were to cover the feeder. One feeder was almost completely obscured, with leaves hanging directly on top of the feeding holes (Figure 2). The second was moderately covered, with leaves cut and positioned so as to be hovering right above the feeding holes, leaving just enough space for access (Figure 3), and one was completely uncovered, with no leaves attached (Figure 4). I followed the same procedure used for the first experiment, observing each feeder for a period of 10 minutes within a half hour and then removing the feeders to measure the amount of nectar that had been drunk, repeating this three times a day for four days.



Figure 2: First version of the well-covered feeder hanging in the bat enclosure, deployed on day 1 of the four-day experiment. Leaves were taken from a tree near the enclosure (unidentified, possibly *Piper auritum*) and tied to the feeder using twine. All feeders in this experiment were hanging at approximately 1.85m from vines. For this feeder, leaves were adjusted so that most of the holes were covered, leaving two only half covered on either side of the feeder where the leaves met.



Figure 3: First version of the medium coverage feeder hanging in the bat enclosure, deployed on day 1 of the four-day experiment. Leaves for this experiment were left hovering above the holes, covering the entrance while still leaving enough space for the bats to access the holes. While this version was left like this, later versions were cut and modified so that they did not extend farther out from the top of the feeder, as they do in this picture.



Figure 4: No coverage feeder hanging in the bat enclosure.

Size experiment

The third treatment tested the theory that bats show preference for flowers of a specific size. I used the same three feeders, which were all hung at the same preferred height, indicated by experiment one. For this experiment, I created models of *Mucuna* flowers out of purple construction paper. While there is a species of *Mucuna* with purple flowers (*Mucuna cyclocarpa*), the flowers were not modeled after flowers of this species, and the color chosen was arbitrary. These flowers were simple, made out of two petals, one of which was placed on the outside of the feeding hole, above and perpendicular to it, and one which was placed at the bottom rim of the feeding hole, the petal also pointing outwards (Figure 5). These petals were meant to mimic the structure of an open *Mucuna* flower, with the upright echolocation guide and the tube-like structure (von Helversen & von Helversen 1999). The petals did not cover the feeding hole, just surrounded it in order to simulate an actual flower with nectar. The feeders have six holes, however only four were used for this experiment, and the two unused ones were covered with tape so as to render them inaccessible. One of the feeders was equipped with large flowers (approximately 95mm in size, approximately one of the largest sizes in the natural range for *M. fawcetti*), another had medium sized flowers (approximately 65mm), and the third had small flowers (approximately 35mm, the average size for *M. urens* flowers). I followed the same procedure of observing visits and measuring nectar used for the first experiment.



Figure 5: All three feeders shown with flowers of different sizes: big (front right), medium (front left), and small (back).

Inflorescence experiment

The fourth treatment tested whether bats show preference for certain sizes of inflorescences. I used the same three feeders, which were all hung at the same preferred height, indicated by experiment one. I also used the same material to make petals and the petal sizes. The first feeder had two large flowers (95mm) attached to feeding holes on opposite ends of the feeders, with the other wholes taped in order to be inaccessible. The second feeder had four medium sized flowers (65mm), with the other feeding holes taped over. The third feeder had all six feeding holes attached with small flowers (35mm). I followed the same procedure of observing visits and measuring nectar used for the first experiment.

Each data set was also analyzed separately using a one-way analysis of variance (ANOVA) in order to determine whether differences between the values existed.

RESULTS

Height experiment

Bats visited feeders at different heights with significant differences in frequency (Two way ANOVA, $df = 2$, $P = 1.85e-07$, $n = 12$). Mean visits for the high feeder over four days were relatively high. The high feeder was visited more often than either the medium or low feeders (Tukey post hoc, $P < 0.05$ in both cases). There was also a non-significant trend showing that the medium feeder was visited more often than the low feeder (Tukey post hoc, $P = 0.06$; Figure 6).

The differences in means for the amount nectar drunk were also significant (Two way ANOVA, $df = 2$, $P = 9.25e-06$, $n = 12$). The nectar data mirrored the visits data, where the high feeder also had the most nectar drunk from it, compared to the other two feeders (Tukey post hoc, $P < 0.05$), and a non-significant trend showed that the medium feeder had more nectar drunk from it than the low feeder (Tukey post hoc, $P = 0.09$; Figure 7).

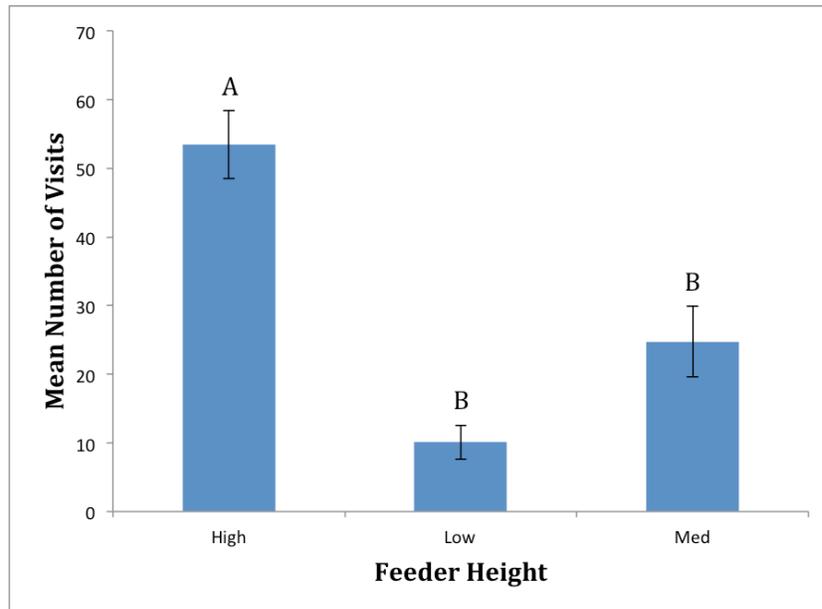


Figure 6: Mean number of visits (\pm SE) of high (53.42 ± 4.92), medium (24.75 ± 5.13), and low (10.08 ± 2.47) feeders hung in the bat enclosure, observed over three half hour increments every day for four days. Means with different letters are significantly different (Tukey post hoc pairwise comparison).

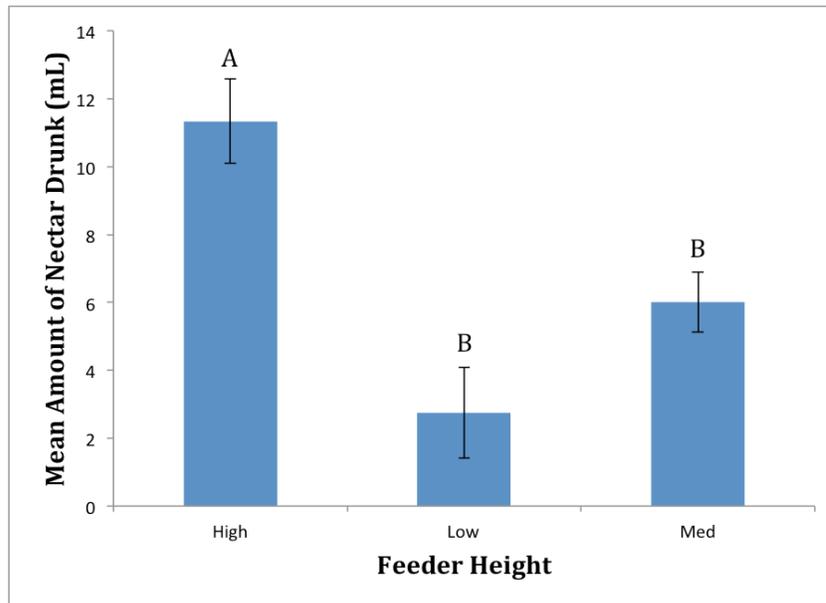


Figure 7: Mean number of nectar drunk (\pm SE) from the high (11.33 ± 1.24), medium (6.00 ± 0.88), and low (2.75 ± 1.34) feeders hung in the bat enclosure, measured every half hour increment three times for four days. Means with different letters are significantly different (Tukey post hoc pairwise comparison).

Cover experiment

Differences in means for number of feeder visits in the leaf cover experiment were significant (Two way ANOVA, $df = 2$, $P = 0.0269$, $n = 12$). The feeder with no coverage (NC) had the highest visits on average, followed by the feeder with medium coverage and then by the feeder with total coverage. The NC feeder was visited significantly more often than the TC feeder (Tukey post hoc, $P < 0.05$), but other means showed no significant differences (Figure 8).

The results from the nectar data reflected the results from the visits data. Differences in means were significant (Two way ANOVA, $df = 2$, $P = 0.0269$, $n = 12$), and the NC feeder had the highest amount of nectar drunk from it on average compared to the MC feeder and the TC feeder. The trend showed a significant difference in means between the NC feeder and the TC feeder (Tukey post hoc, $P < 0.05$; Figure 9).

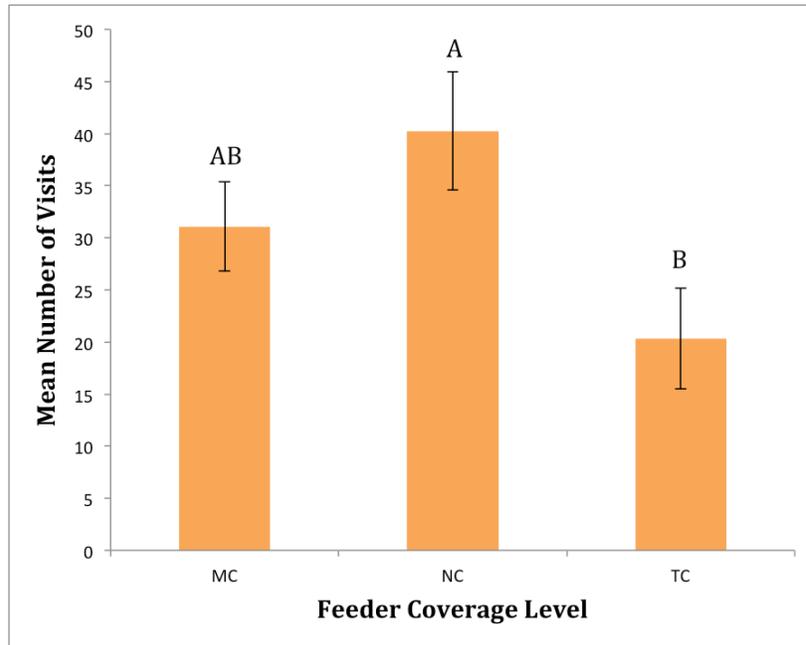


Figure 8: Mean number of visits (\pm SE) of total coverage (TC; 20.33 ± 4.83), medium coverage (MC; 31.08 ± 4.29), and no coverage feeders (NC; 40.25 ± 5.66) hung in the bat enclosure, observed over three half hour increments every day for four days. Means with different letters are significantly different (Tukey post hoc pairwise comparison).

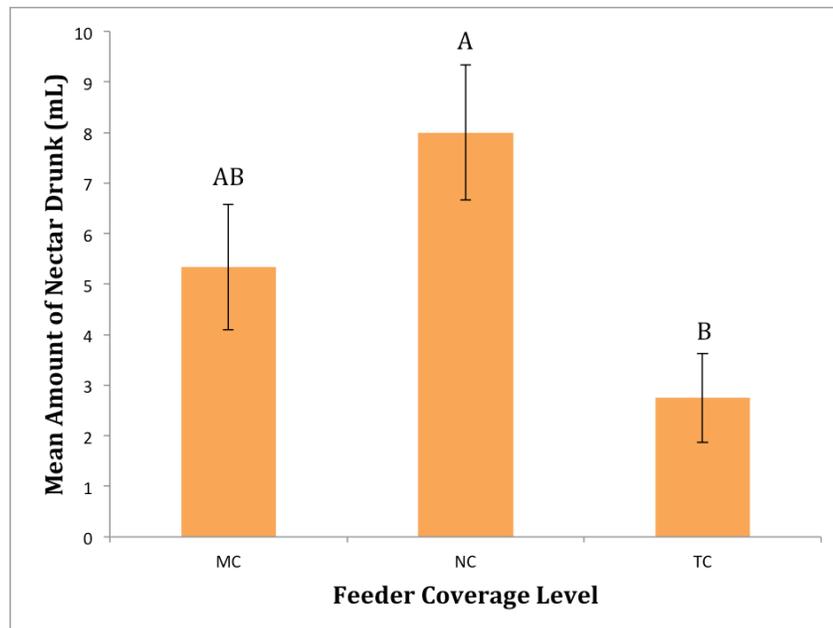


Figure 9: Mean number of nectar drunk (\pm SE) from total coverage (TC; 2.75 ± 0.88), medium coverage (MC; 5.33 ± 1.24), and no coverage (NC; 8 ± 1.34) feeders hung in the bat enclosure, measured every half hour increment three times for four days. Means with different letters are significantly different (Tukey post hoc pairwise comparison).

Size experiment

Statistical analysis showed that the differences in the mean number of visits were significant (Two way ANOVA, $df = 2$, $P = 0.0065$, $n = 12$). The feeder with the small flowers (S) had the highest average number of visits, however the average was only slightly higher than that of the feeder with the medium flower (M). The mean number of visits for the feeder with big flowers (B) was substantially lower than that of both the medium and small flowers (Tukey post hoc, $P < 0.05$ for both; Figure 10).

However, the results from the nectar analysis did not completely reflect those of the visit analysis. The differences in means from the nectar data showed a non-significant trend (Two way ANOVA, $df = 2$, $P = 0.0815$, $n = 12$ (except for the big flowered feeder, for which $n = 11$)). This trend was caused by the significant difference between the small flowered big flowered feeder, with bats drinking more nectar from the small flowered feeder on average but also showing a non-significant trend (Tukey post hoc, $P = 0.106$; Figure 11).

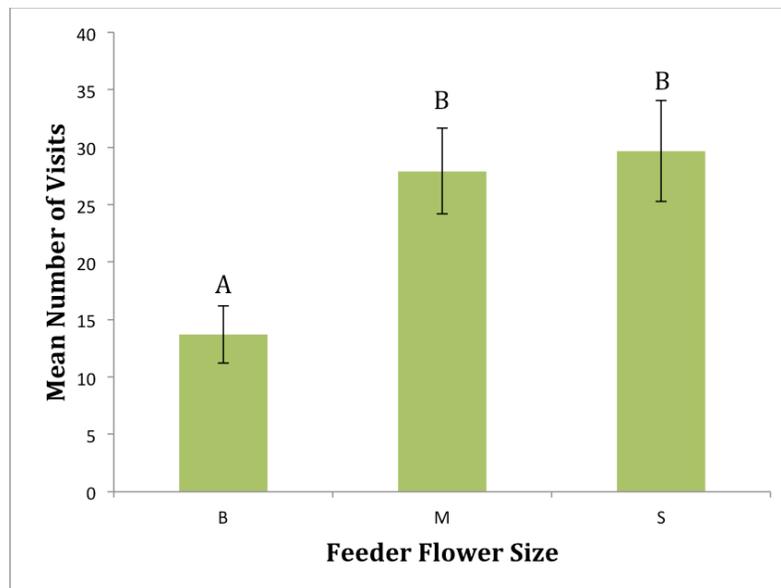


Figure 10: Mean number of visits (\pm SE) of big flowered (B; 13.67 ± 2.49), medium flowered (M; 27.92 ± 3.72), and small flowered (S; 29.67 ± 4.39) feeders hung in the bat enclosure, observed over three half hour increments every day for four days. Means with different letters are significantly different (Tukey post hoc pairwise comparison).

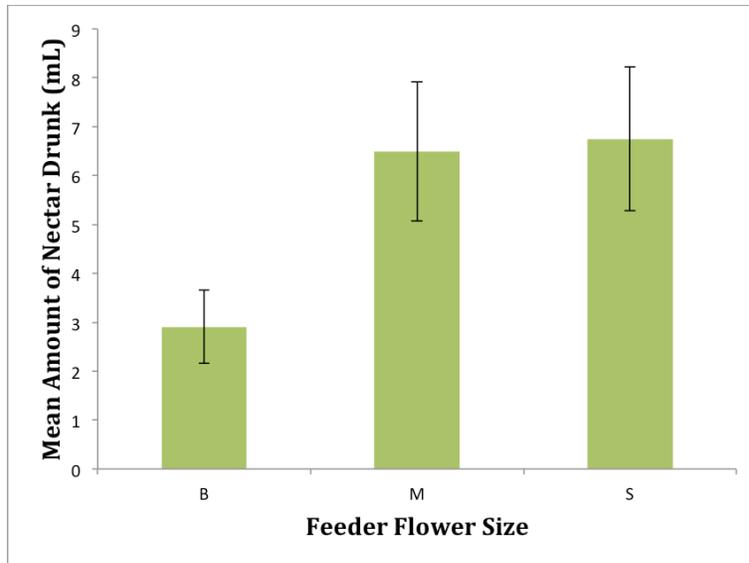


Figure 11: Mean number of nectar drunk (\pm SE) from big flowered (B; 2.91 ± 0.76), medium flowered (M; 6.5 ± 1.43), and small flowered (S; 6.75 ± 1.47) feeders hung in the bat enclosure, measured every half hour increment three times for four days. Means with different letters are significantly different (Tukey post hoc pairwise comparison).

Inflorescence size experiment

Differences in means for number of feeder visits in the inflorescence size experiment were significant (Two way ANOVA, $df = 2$, $P = 0.00459$, $n = 12$). The feeder with six small flowers (6S) had the highest average amount of visits compared to the feeder with four medium flowers (4M) and the feeder with two big flowers (2B). However, the differences were only significantly different for 6S and 2B (Tukey post hoc, $P < 0.05$), though the differences in the means seem large (Figure 12).

Analysis of the nectar data also showed significant differences in the means (Two way ANOVA, $df = 2$, $P = 0.0102$, $n = 12$). The means followed the same trend as those for the visits, with 6S having the highest amount of nectar drunk. The differences between 6S and 2B were also found to be significant (Tukey post hoc, $P < 0.05$; Figure 13).

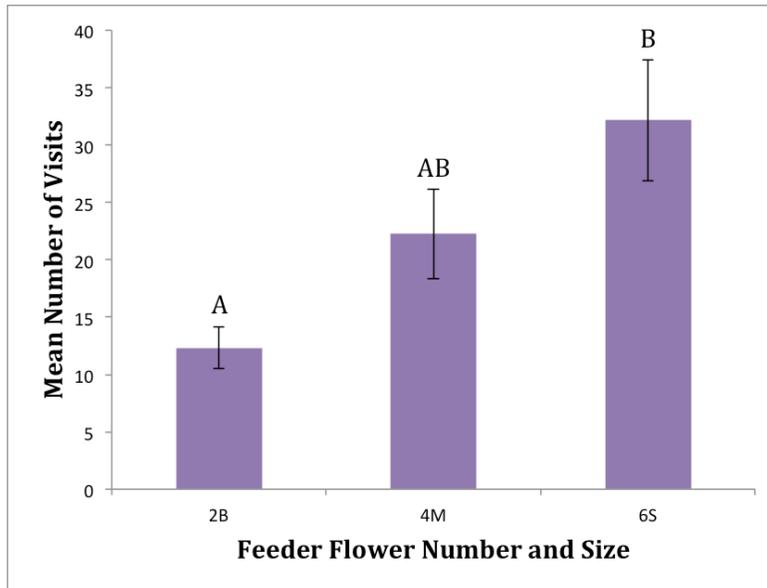


Figure 12: Mean number of visits (\pm SE) to the feeder with two large flowers (2B; 12.33 ± 1.81), the feeder with four medium flowers (4M; 22.25 ± 3.9), and the feeder with six small flowers (6S; 32.17 ± 5.29) hung in the bat enclosure, observed over three half hour increments every day for four days. Means with different letters are significantly different (Tukey post hoc pairwise comparison).

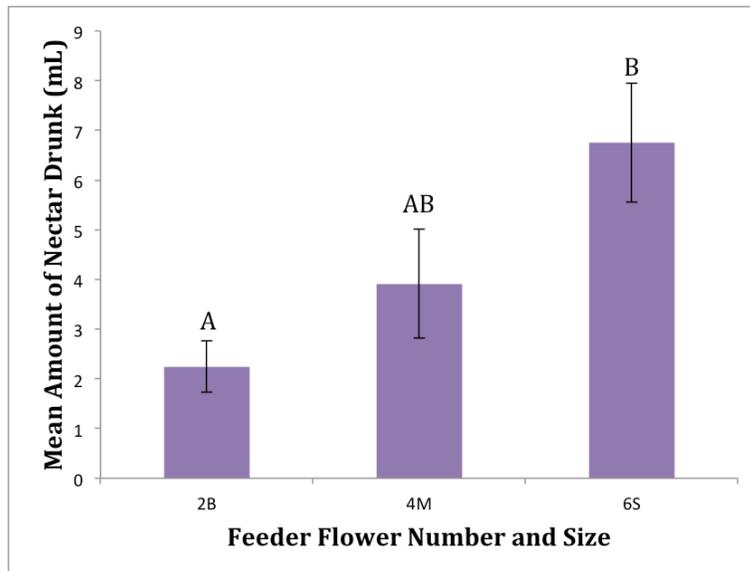


Figure 13: Mean number of nectar drunk (\pm SE) from the feeder with two large flowers (2B; 2.25 ± 0.52), the feeder with four medium flowers (4M; 3.92 ± 1.10), and the feeder with six small flowers (6S; 6.75 ± 1.20) hung in the bat enclosure, measured every half hour increment three times for four days. Means with different letters are significantly different (Tukey post hoc pairwise comparison).

DISCUSSION

These findings suggest that when it comes to flowers in the genus *Mucuna*, bats prefer flowers that are higher in the canopy, relatively uncovered, with small flowers arranged in a clustered inflorescence. Preference for flowers at high elevations could be caused by a couple of different factors. The first is predation. Predators of bats include snakes and small terrestrial mammals (Kasso and Balakrishnan 2013), and it is possible that in flying low bats would be placing themselves at high risk for predation from those terrestrial animals, whereas in higher areas they would have the advantage. Though the bats in this study were raised in captivity, it is possible that they retain an instinctual fear and resulting avoidance of flying low. This would explain why these bats, who have never known the presence of a predator, would still be averse to flying so low in the enclosure to feed. This hypothesis, however, has not been proven by any previous studies. Another possible explanation for the avoidance of the low hanging feeders is wing damage: in flying low, there is a higher chance that bats might damage their wings against the ground or other objects, and it would also be harder to maneuver around the flower area to find the right angle (Kimbel 2002). This, coupled with the constant search for ways to diminish energy expenses (Arbol 2005), would make foraging for low hanging flowers very inefficient (given the greater amount of energy needed in order to maneuver more) and dangerous. This hypothesis also has not been tested. In the wild, another factor that could explain these results is that flowers that are higher tend to have less vegetation covering them, compared to flowers that are hanging in the understory, which, according to the results of the second experiment in this study and other studies, could have a large effect on preference (Vincent 2003, Terborgh 1992). However this hypothesis would apply more to bats in the wild, given that there was no obstruction due to vegetation on this portion of the overall experiment and resulting bat behavior would probably be learned, rather than innate. It is also worth noting that studies have shown that when inflorescences are not pollinated, they will continue to grow downwards until they are, at which point they will stop growing (Papoulias 2013). Therefore, it is possible that flowers that are higher in the canopy can be seen as younger by the bats and therefore more likely to contain nectar, whereas flowers lower in the canopy are more likely to have been pollinated or to have less nectar (due to the fact that they have been exposed for a longer period of time).

As stated above, preference for flowers that are not obstructed by vegetation or other objects has been supported by other studies (Vincent 2003, Terborgh 1992, Kimbel 2012). Obstruction of a *Mucuna* flower and, in turn, its vexillum, which is designed to facilitate its discovery by echolocating bats, can make it much more difficult to find and access (von Helversen et. al. 2002). Studies have also shown that *Mucuna* flowers tend to protrude from areas with a large amount of foliage (Tschapka and Dressler 2002), further supporting the results from this study. It is interesting that there were no statistical differences in means with relation to medium coverage, though it could just be seen as an in-between point between complete obstruction and no obstruction, with bats taking a non-preferential approach to it. It is possible that one species of bat handled the obstruction better than another, or that the feeder with larger holes may have had an effect on the bats' choice.

Preference for smaller flowers may be due to the fact that in flowers with larger petals, the nectar is more difficult to access, since bats have to maneuver around the petals, get into the proper position, and extend their tongues a lot more (personal observation). This is costly energy-wise, so it would make sense for bats to go for flowers with nectar that are less obstructed by petals when the choice presents itself. In this case, it is also possible that a larger amount of flowers in an inflorescence advertises more available nectar than would two flowers, which would in turn attract more bats.

The main morphological and spatial characteristics that make plants in the genus *Mucuna* more attractive to nectarivorous bats do not necessarily adhere to (or, in the case of elevation from ground, are not even mentioned in) the theory of pollination syndromes for bats; while the theory of pollination syndromes states that bat pollinated flowers are large and few in number in order to attract bats, the characteristics that were preferred in this study were small flowers in large numbers. This suggests that other flowers may have their own unique syndromes, a possibility that should be further explored in order to gain a better understanding of the complex plant-animal relationships in the tropics.

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Epiphytic lichen species diversity and coverage across an altitudinal gradient with understory trees

ABSTRACT

Tropical forests are known to contain diverse and dynamic epiphytic communities yet they remain poorly researched. Previous studies on epiphytic lichens have identified them as the first organisms to be seen in forest succession (Plitt 1927; Benzing 1995) This study focuses on how lichen composition and diversity changes across an altitudinal gradient. Fifty four trees were surveyed near the Monteverde Cloud Forest Preserve between 1550 m and 1700 m. The lichen coverage was measured using a laminate grid with an area of 18 x 21 cm at breast height and base for nine trees across six elevational bands of 25 m. The lichen coverage at breast height and base decreased with increasing altitude yet the area of lichen coverage at breast height was always greater than the base across the entire gradient. The lichen morpho species diversity decreased with increasing altitude yet the number of lichen morpho species was always greater at breast height than at the base. Lichen coverage never differed significantly between young, medium aged, and older trees across the gradient. The conclusions of this study demonstrate that previous studies on the patterns of canopy lichen growth, abundance, and diversity cannot be applied to understory trees.

RESUMEN

Los bosques tropicales son reconocidos por contener comunidades epifíticas diversas y dinámicas pero aún son poco estudiadas. Estudios previos en líquenes epifíticos los han identificado como los primeros organismos vistos en la sucesión del bosque (Plitt 1927; Benzing 1995). Este estudio se enfoca en como la composición y la diversidad de líquenes cambia a lo largo de un gradiente altitudinal. Cuarenta y tres árboles se estudiaron cerca de la Reserva del Bosque Nuboso de Monteverde entre los 1550 y 1700 m. La cobertura de líquenes se midió usando una cuadrícula laminada con un área de 18 x 21 cm a la altura del pecho y la base en nueve árboles a lo largo de seis bandas altitudinales de 25 m. La cobertura de líquenes a la altura del pecho y en la base disminuye al aumentar la elevación y el área de la cobertura de líquen a la altura del pecho fue siempre mayor que en la base a lo largo del gradiente entero. La diversidad de morfoespecies de líquenes disminuye con un aumento en la elevación y el número de morfoespecies fue siempre mayor a la altura del pecho que en la base. La cobertura de líquenes nunca difirió significativamente entre árboles jóvenes, medianos y maduros a lo largo del gradiente. Las conclusiones de este estudio demuestran que estudios previos en los patrones de crecimiento, abundancia y diversidad de los líquenes en el dosel no se aplican para árboles en el sotobosque.

INTRODUCTION

As tropical forests continue to be impacted by human disturbance through deforestation, fragmentation, and other destructive actions, the understanding of forest succession is paramount to conservation efforts. Forest succession has been defined as the directional change with time of the species composition and vegetation physiognomy of a single sight where climate remains effectively constant (Finegan 1984) There are two types of

succession primary and secondary; primary succession occurs on previously unvegetated land while secondary successions occur on disturbed areas. (Finegan 1984) Epiphytic lichens are often the first organisms to be seen in the beginning stages of succession and have even been described as “pioneers” of succession. (Plitt 1927) Lichens are a composite of a symbiotic relationship between a fungus (mycobiont) and a photosynthetic symbiont such as cyanobacteria or green algae. Within this symbiotic relationship the photosynthetic symbiont provides the mycobiont with a source of food while the mycobiont provides the photosynthetic symbiont with protection, a steady supply of moisture, and the capability to colonize broader habitats. (Brodo 2001)

The first lichen growth forms to appear in successions are crustose, followed by foliose which then give rise to the bryophyte formations of mosses, liverworts, and hornworts. (Plitt 1927) This was exemplified in a study conducted in a temperate forest that demonstrated that lichens are early colonizers in epiphytic succession that are eventually replaced by bryophytes, which include mosses, liverworts, and hornworts (Benzing 1995). However, the diverse and dynamic epiphyte communities in tropical forests have rarely been studied directly. Even though the tropics are known as hotspots of rich biodiversity, research and documentation on the patterns of species diversity of nonvascular plants is lacking. Researchers have previously examined the shifts in biodiversity along altitudinal gradients but have rarely studied non-vascular epiphytes on understory trees. The first study entirely focused on the flora of the canopy in a tropical forest was conducted by Wolf in 1993 in the Andes on species richness, distribution, and biomass along an altitudinal gradient.

This project focuses on the distribution, abundance, and diversity of lichens across an altitudinal gradient. This study will specifically focus on how the lichen community composition changes across an altitudinal gradient with a lower montane wet tropical forest in Monteverde, Costa Rica. According to Barr’s research lichen coverage should decrease as the DBH and elevation increases. Based on Barr’s results bryophytes will outcompete lichens at higher elevations and on trees with larger DBH’s.

MATERIALS AND METHODS

This study was conducted in Monteverde, Costa Rica in the lower montane rain forest near the Monteverde Cloud Forest Preserve along an altitudinal gradient from 1,550 m to 1,700 m. The fieldwork was collected from October 19th to November 15th 2014. The altitudinal gradient was subdivided into 6 elevational belts every 25 meters in elevation, using an altimeter. In total, nine trees were surveyed each elevational belt with a diameter at breast height (DBH) around 10 cm, 20 cm, and 30 cm (three for each) for lichen coverage using a laminate grid. The laminate grid was 18 x 21 cm with a total of 378 grid squares (1 cm x 1 cm) The lichen coverage was surveyed by counting up the total number of grid squares filled at least 50% by lichen growth. The same laminate was used to survey the tree at breast height and the base of the tree. The laminate was placed with the bottom side of the sheet touching the ground for base. Trees were selected haphazardly based on whether or not they fit the size requirements not by observed lichen coverage. There were no other requirements for tree selection other than DBH. Lichen individuals were classified and recorded by their growth form (crustose, foliose, squamulose, and gelatinous) and morphology (color.)

An analysis of covariance was used to compare the lichen coverage and morpho species present between breast height and base along an altitudinal gradient. A Shannon-Wiener Index was calculated for each elevational band using the total number of morpho species and their frequency at each elevational band. A correlation test was used to examine if there were patterns of diversity along the elevational gradient.

RESULTS

Coverage

Spearman Correlation Test between tree diameter and coverage at Breast Height: $r_s = -0.16$, $P = 0.71$, and the same test for base $r_s = -0.15$, $P = 0.71$. So, there is no relationship between tree diameter and coverage.

Lichen coverage varied with elevation and position on the trunk (Analysis of variance: $F = 9.107$, $df = 2, 105$ DF, $p = 0.0002253$; Figure 1). Specifically lichen coverage at breast height and base decreased at increasing elevational belts (Analysis of Covariance: $t = -2.961$ $p = 0.00379$). The area of lichen coverage at breast height was always higher than at the base across all elevational belts (Analysis of covariance: $t = 3.073$ $p = 0.00270$).

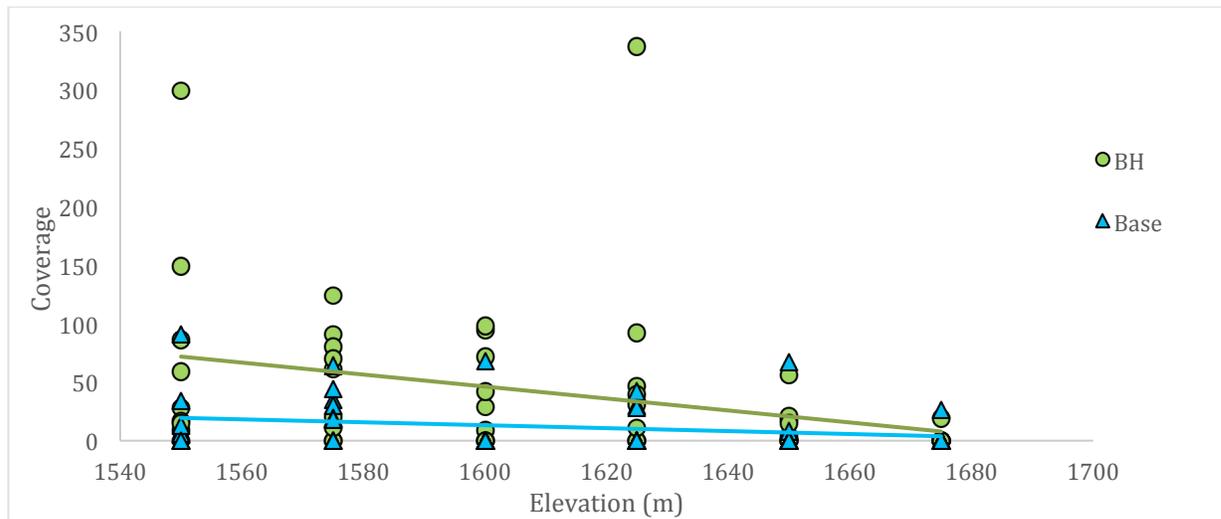


Figure 1: Lichen coverage over an altitudinal gradient from 1550 m to 1700 m in a tropical lower montane wet forest of Monteverde, Costa Rica. A total of 54 trees were sampled with each elevational belt is represented by 9 tree samples. Trees were selected based on having a DBH closest to 10 cm, 20 cm, and 30 cm. Three trees were measured per DBH increment.

Morpho species

Morpho species present at breast height and base varied with elevation (Analysis of Variance: $F = 21.1$, $df = 2, 105$ DF, $p = 1.97e-08$; Figure 2) Specifically, morpho species present at breast height and base decreased at increasing elevational belts (Analysis of Covariance: $t = -5.132$

$p=1.32e-06$ The number of morpho species present was always higher at breast height than at the base (Analysis of covariance $t=3.984$ $p=0.000125$)

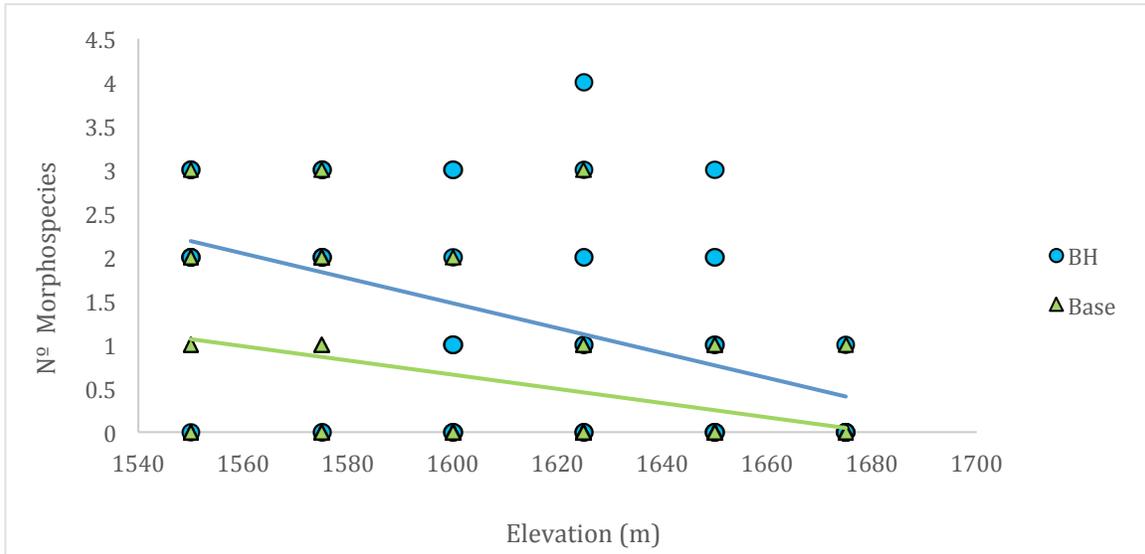


Figure 2: Morpho species count over an altitudinal gradient from 1550 m to 1700 m in a tropical lower montane wet forest of Monteverde, Costa Rica. A total of 54 trees were sampled with each elevational belt is represented by 9 tree samples. Trees were selected based on having a DBH closest to 10 cm, 20 cm, and 30 cm. Three trees were measured per DBH increment. Morpho species were identified using the CIEE lichen laminate.

Shannon-Weiner Index

There was no statistical trend in the diversity indices along the altitudinal gradient. The highest diversity index was recorded at the 1650-1675 m elevational belt with the lowest diversity index was recorded at the 1675-1700 m elevational belt.

Shannon H	1.747	1.905	1.979	1.75	2.098	0.6931
Equitability	0.898	0.916	0.9518	0.8992	0.955	1

Figure 3: Morpho species count over an altitudinal gradient from 1550 m to 1700 m in a tropical lower montane wet forest of Monteverde, Costa Rica. A total of 54 trees were sampled with each elevational belt is represented by 9 tree samples. Trees were selected based on having a DBH closest to 10 cm, 20 cm, and 30 cm. Three trees were measured per DBH increment. Morpho species were identified using the CIEE lichen laminate.

Diversity Indices

There is no statistical significance between changes in diversity as measured by the Shannon Weiner-Index along the altitudinal gradient (Spearman correlation $r_s=-0.0286$ $N=6$ $p=0.9572$)

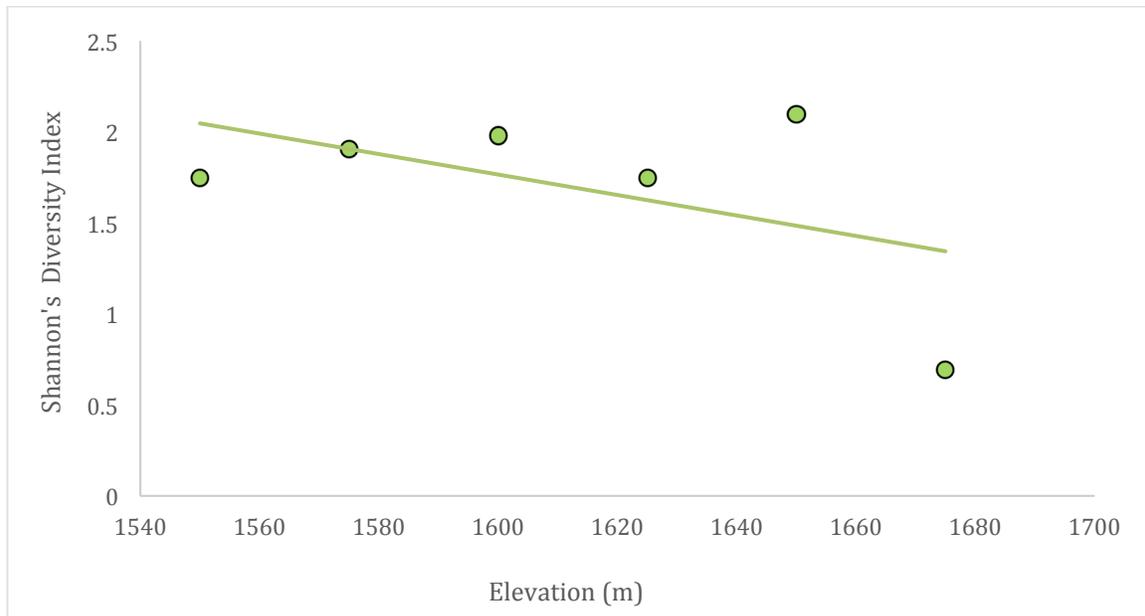


Figure 4: Morpho species count over an altitudinal gradient from 1550 m to 1700 m in a tropical lower montane wet forest of Monteverde, Costa Rica. A total of 54 trees were sampled with each elevational belt is represented by 9 tree samples. Trees were selected based on having a DBH closest to 10 cm, 20 cm, and 30 cm. Three trees were measured per DBH increment. Morpho species were identified using the CIEE lichen laminate

DISCUSSION

The results of the study showed that lichen coverage decreased at higher elevations, which supports previous studies that have found that lichens are eventually replaced in succession by vascular epiphytes. A previous study examining the relationship between the succession of corticolious lichens to epiphytic bryophytes at two altitudinal zones (1,350 m and 1,650 m) in Monteverde, Costa Rica (Barr 2007) demonstrated that as trees aged they gained more bryophyte coverage at the expense of lichens. The results of the study showed a relationship between the area of coverage for lichens depending on the diameter at breast height (DBH) of the tree. According to the study lichen coverage on trunks generally tended to decrease as DBH increased. In addition, a study on epiphytic colonization in Monteverde, Costa Rica found that epiphytic succession begins with fruticose or crustose lichens, which eventually die and leave a layer of dead organic material that then serves as a substrate for vascular epiphytes (Nadkarni 2000) However this pattern was not observed in this study as the Spearman correlation test found no significant difference between the lichen coverage on young, medium aged, or older trees across the elevational gradient. In regards to the area at breast height having greater lichen coverage than the base, this can possibly be attributed to a few factors. It is possible that different amounts or intensities of light reach the tree at breast height then at the base, which would greatly impact the ability of lichen to grow. In addition based on field observation, many tree bases were covered in dust or splattered dirt that may suffocate lichens or create an unfavorable substrate for them to grow on.

There are a few possible reasons why the diversity of morpho species did not significantly change across the altitudinal gradient. The first being that previous studies on

epiphytic succession and rates of colonization were conducted over much wider altitudinal gradients (Nadkarni 2000; Wolf 1993) One study found that found that foliose and crustose lichen taxa numbers tend to drop with altitude while fruticose lichen morpho species increase up to an altitude of about 3400 m (Wolf 1993) It could be that changes in lichen diversity can only be seen over altitudinal gradients spanning a wider range than what was measured in this study. Humidity and moisture are often limiting factors for epiphytic growth and can therefore impact species diversity. It is possible that the humidity and moisture inputs were similar across the entire gradient providing ideal moisture inputs for certain species. Future studies should measure differences in moisture input and temperature to assess how they potentially impact lichen diversity. Finally, data was collected along an established and maintained trail (human disturbance), which could have impacted the overall measure of diversity. A study conducted in the Monteverde cloud forest demonstrated that disturbed epiphyte communities take significantly longer to recolonize (Nadkarni 2000) Therefore the lichen coverage observed along the trail may not have been accurate representations of the entire undisturbed forests.

In conclusion, this study demonstrates that previous findings on lichen growth, abundance, and diversity found in previous studies on the canopy cannot be generalized and applied to the understory.

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Nest distribution, soil hardness, and vegetation cover in *Crawfordapis luctuosa* (Colletidae)

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ABSTRACT

Different bee species show varying levels of sociality, and species exhibiting more primitive forms of sociality are considered to show evolutionary stepping-stones on the path to eusociality. One possible precursor to cooperative behavior in bees is aggregated nesting in species that are otherwise solitary. As *Crawfordapis luctuosa* (Colletidae) show aggregation behavior in their nesting, previous studies have examined these large, ground nesting, Central American black bees for possible indicators of primitive sociality based upon individuals' behavior. This study focused instead on the nesting aggregation and soil to show whether or not the bees preferred the nest aggregation site simply due to abiotic conditions: in three plots with nests, patterns in *C. luctuosa* nest dispersion were investigated, soil hardness was tested by penetrometer, and observed percentage of vegetation cover was recorded around nest holes, around clumps of nest holes, and in areas with no nests. In terms of dispersion, some significant clumping and trends toward clumping were found in study plots. Though soil hardness was not significantly different in the plots with nests vs. three additional plots without nests that were tested for soil hardness and vegetation cover, it was found to be significantly different for the different nest types in the plots with nests, specifically that areas with no nests had significantly higher mean soil hardness ($4.282 \pm \text{S.E. } 0.0509$) over all plots than areas with nests (Three types ranges in mean from 3.614 ± 0.147 to 3.95 ± 0.133). Mean percent vegetation cover was significant, with no-nest plots showing significantly lower vegetation cover (0.078 ± 0.015) than plots with nests (0.335 ± 0.015). Overall, the bees somewhat prefer softer soils, but areas on the same road with no bees had very similar soil hardness to areas with *C. luctuosa* nests, so other nest-site selection factors such as social behaviors or their clear preference for areas with higher vegetation may influence the bees to clump nests together and aggregate in certain places.

RESUMEN

Diferentes especies de abejas muestran diferentes niveles de socialidad, y las especies de formas más primitivas de socialidad son consideradas de mostrar eslabones en el paso hacia la eusociabilidad. Una causa precursora posible al comportamiento cooperativo en las abejas es el anidamiento agregado en especies que son de otra manera solitarias. Como muestra el comportamiento de anidación agregado en *Crawfordapis luctuosa* (Colletidae), estudios previos han examinado esta abeja grande Centroamericana por posibles indicadores de socialidad primitiva basado en el comportamiento de los individuos. Este estudio se enfoca por otra parte en la anidación agregada y el suelo para mostrar si las abejas prefieren o no anidar agregadamente debido a condiciones abióticas: en tres parcelas con nidos, los patrones de dispersion de los nidos de *C. luctuosa* se investigaron, la dureza del suelo se probó con un penetrometro, y se observó el porcentaje de cobertura de vegetación alrededor de los nidos, alrededor de grupos de nidos, y en áreas sin nidos. En terminos de dispersion, un agrupamiento significativo y tendencias al agrupamiento se encontraron en los sitios de estudio. Aunque la dureza del suelo no fue significativamente diferente en las parcelas con nidos versus tres parcelas adicionales sin nidos que se utilizaron para probar la dureza del suelo y cobertura de la vegetación, se encontró una diferencia significativa para los diferentes tipos de nidos

en las parcelas con nidos, específicamente que las áreas sin nido tienen una dureza del suelo mayor ($4.282 \pm \text{S.E. } 0.0509$) sobre las áreas con nidos (tres tipos en promedio 3.614 ± 0.147 to 3.95 ± 0.133). El porcentaje promedio de cobertura de vegetación fue significativa, con las parcelas sin nido mostrando una menor vegetación (0.078 ± 0.015) que las parcelas con nidos (0.335 ± 0.015). En resumen, estas abejas prefieren en algún grado suelos suaves, pero las áreas en el mismo camino sin abejas tienen una dureza de suelo similar a las áreas con *C. luctuosa*, así otros factores como el comportamiento social pueden afectar la selección del sitio de anidamiento o una clara preferencia por áreas con mayor cobertura vegetal puede influenciar el agrupamiento de las abejas y las agregaciones en ciertos lugares.

INTRODUCTION

Species of the order Hymenoptera show a wide range of social behaviors: for example, advanced eusocial bees, wasps, and ants have a clearly delineated system of cooperative brood care, division of labor, and overlapping generations. Apart from advanced eusocial behaviors, species may be primitively eusocial, parasocial, subsocial, or simply solitary, depending upon the extent to which individuals cooperate or live communally (Kocher and Paxton 2014). Species exhibiting primitive sociality are considered to show evolutionary stepping stones on the path to eusociality, so understanding species and higher taxa that show these different levels of sociality plays a key role in understanding the evolution of eusociality (Danforth 2002).

One behavior considered a possible precursor to cooperative behavior in bees is aggregated nesting in species that are otherwise solitary. Nests are constructed in close proximity to one another by independently reproducing females to form aggregations, and this is common in ground nesting bees from a great number of families. They may carry out this behavior for a number of reasons: limited areas with preferred substrate for nest building, or to reduce individual probability of parasitism through safety in numbers, the selfish herd effect (Kocher and Paxton 2014).

A Central American species of ground nesting bee, *Crawfordapis luctuosa* (Colletidae), lives in nest aggregations of up to hundreds of nests along road edges and exposed ridges with low vegetation or exposed soil at elevations from 1540 m to 1680 m (Otis et al. 1982, Roubik & Michener 1984). These bees have been described as solitary, but observations of activities like visits by individuals to multiple nests have led to studies exploring amounts of nest hole sharing or amounts of competition between individuals as a possible indicator of a primitive sociality (Jang et al. 1995, Hubbard 1997, Blackwell 2003).

What these studies have not examined in depth are abiotic qualities of *C. luctuosa*'s nest sites and any patterns in nest layout within nesting aggregations. If the bees are showing sharing behaviors or competitive behaviors, this could affect the bees' nest placement choices, causing them to be more regularly distributed or clumped closer together. In this study, I evaluated the existence of these patterns or lack thereof by mapping different sections of the nesting aggregation. Clumping could also indicate a patch of preferable soil for nest building, and it is possible that a very specific substrate preference leads the bees to aggregate in specific areas. For that reason, I also examined preferences of the bees in terms of abiotic conditions by measuring soil hardness throughout the nesting aggregation and outside of the nesting aggregation.

MATERIALS & METHODS

STUDY SITE—The borders of the clay road to the TV towers near Cerro Amigos (Monteverde, Costa Rica), approximately 1750m in elevation, where the local *C. luctuosa* population has an aggregation of nests. Cloud forest surrounds the clay road. This nesting aggregation was studied from October 20 to November 14, 2014.

STUDY ORGANISM—*C. luctuosa* is a dark-haired, short-tongued (Wuellner & Jang 1996) bee that can be as long as 24 mm (Otis et al. 1982). Females construct underground nests, which are found in aggregations of up to several hundred nests on road edges and exposed ridges with low vegetation cover or exposed soil (Otis et al. 1982), with openings from 10-12 mm in diameter (Roubik & Michener 1984) and tunnels extending from 22-120 cm into the soil in which they create cells to lay eggs and raise larvae (Otis et al. 1982, Roubik & Michener 1984).

EXPERIMENT 1: MAPPING NESTS TO DETERMINE NEST DISTRIBUTION PATTERNS—Three sites were chosen along one side of the clay road, out of the middle of the road (where motorcycles and all-terrain vehicles occasionally pass) in areas where bees were observed to be actively flying around the aggregation and entering and leaving nest holes. In each site, a 1 x 4 m plot was marked (by placing thin twine on the ground) around the nesting area. These plots were used throughout all experiments. All existing nest holes were mapped within the borders of the plots. Nearest-neighbor distance (NND) was measured for each nest within the plots in order to determine any dispersion patterns using the Clark and Evans test (Krebs 1989). Some nest holes had a nearest neighbor excluded from the plot—these outside-plot nearest neighbors were used to record NND for nests inside the plot, but were not included in nest maps or used in the analysis of NNDs for each plot.

EXPERIMENT 2: SOIL HARDNESS AND VEGETATION COVER AS INDICATORS OF PREFERRED NEST-DIGGING CONDITION—Soil was measured in the aggregation area using two penetrometers to test soil hardness: one was modified as in Figure 1, with a dissecting pin attached to the end for greater sensitivity, while the other was unmodified (red tip as seen in Fig. 1 enters the soil rather than the pin). The modified penetrometer was necessary as some soils were too hard for the regular penetrometer to measure, and a value of 4.5 (maximum reading on regular penetrometer) was added to all measurements taken with the modified penetrometer.



FIGURE 1: Modified penetrometer with dissecting pin attached to tip, used to measure soil hardness in three 1 x 4 m plots created within a *C. luctuosa* nesting aggregation as well as three additional plots with no *C. luctuosa* nests, along the clay road to Cerro Amigos at 1750 m in elevation. Penetrometer measurement scale is from 0 to 4.5, with markings every 0.25 (units arbitrary). Modified penetrometer was used to measure soils too hard to be measured with an unmodified penetrometer, which has only the blunt red cap pictured (no pin attached), but softer soils were measured with an unmodified penetrometer and readings from modified penetrometer were adjusted by adding 4.5 to each.

Three plots in addition to those used in Experiment 1 were marked:

One each was created within 50 m of each original plot in areas along the same side of the clay road, but the new plots were placed in areas with no *C. luctuosa* nest holes, to compare soil hardness and vegetation cover in areas with and without nests. Soil was measured with the appropriate penetrometer throughout the plots with no nest holes by taking 3 measurements each at 15 evenly spaced coordinates throughout the plot. For the plots with nest holes, penetrometer measurements were classified as being taken in areas with no nest holes, in an area with a single isolated nest hole, an area with a small clump of two to three nest holes, or an area with a medium-sized clump of 4-6 nest holes. Initial data was collected with the same coordinate system as the plots with no nest holes, and additional data was collected in areas with no nests (in each plot, 5 measurements in ten 10 x 10 cm squares with no holes) and targeting isolated nest holes (in each plot, 5 measurements around 10 isolated holes), small clumps (in each plot, 5 measurements around 10 clumps of 2-3 nest holes) and medium clumps (Clumps of 4-6 holes, 5 measurements at each: in Plot A, 4 clumps; in Plot B, 2 clumps; in Plot C, 3 clumps). Amount of vegetation directly around each spot where soil was measured (approximately 5 x 5 cm centered on where the penetrometer entered the ground) was estimated and recorded as closest to 0%, 25%, 50%, 75%, or 100% vegetation cover.

RESULTS

EXPERIMENT 1: MAPPING NESTS TO DETERMINE NEST DISTRIBUTION PATTERNS—The spatial pattern of the nests was analyzed using the Clark and Evans test (Krebs 1989) to determine significant deviation from random distribution.

Out of the three mapped plots, one plot, Plot C (mean NND = 0.1089 m, density 12.75 nests per m²) was shown to have a significantly clumped nest hole distribution (Clark and Evans test: $p=0.0012$). Plot B showed a trend toward clumping though it was not significant (Clark and Evans test: $p=0.0574$), and Plot A was randomly distributed (Clark and Evans test: $p=0.3085$).



FIGURE 2. Maps of *C. luctuosa* nest holes in three 1 x 4 m plots in a nesting aggregation at 1750 m elevation on the clay soil road to Cerro Amigos near Monteverde, Costa Rica. Clay road runs parallel to the long side of each rectangle on the left of each plot, and the tops of these maps represent the end of the plot further uphill. Plot A (left) contains 71 nest holes, Plot B (center) contains 28 nest holes, and Plot C contains 51 nest holes.

EXPERIMENT 2: SOIL HARDNESS AND VEGETATION COVER AS INDICATORS OF PREFERRED NEST-DIGGING CONDITIONS—Comparing all three plots with nests to all three plots without nests, there is no significant difference in mean soil hardness (Figure 3; ANOVA: $F = 0.0817$, $df = 1, 473$, $p = 0.7752$).

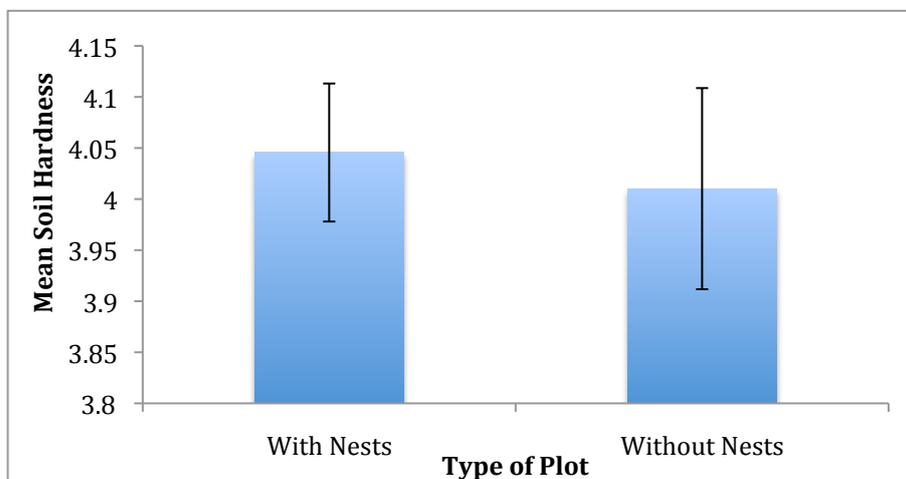


FIGURE 3. Comparison of three 1 x 4 m plots with *C. luctuosa* nests to three 1 x 4 m plots without nest holes on the clay road to Cerro Amigos at 1750 m elevation, in

terms of mean soil hardness throughout all plots of each type (± 1 SE), as measured throughout the plots with penetrometers.

Soil hardness in the three plots with nests, separated into the classifications of different nest distribution types around site measured, (Figure 4) show that the soils measured near isolated nests, small clumps of nests, and medium-sized clumps were significantly softer than the soil measured in areas with no nest holes nearby (ANOVA: $F = 19.931$, $df = 3, 328$, $p < 0.0001$).

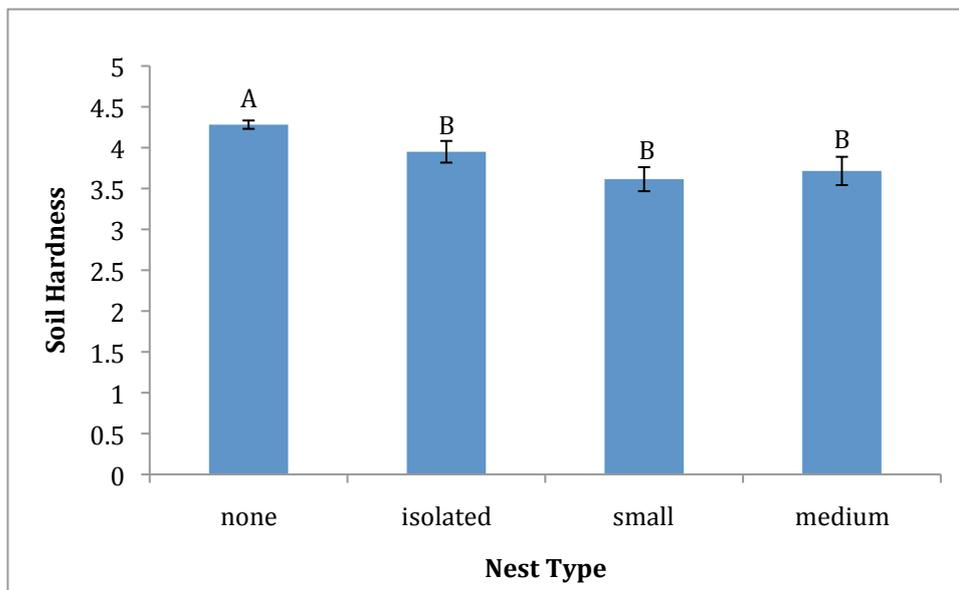


FIGURE 4. Comparison of mean soil hardness (± 1 SE) by type of nest clump (or lack thereof) where measurements were taken, in three 1 x 4 m plots within a nesting aggregation of *C. luctuosa* along the clay road to Cerro Amigos above Monteverde, Costa Rica around 1750 m in elevation. ‘None’ indicates no nests in the vicinity of measurements, ‘isolated’ indicates measurements taken near one nest hole, ‘small’ indicates measurements near a clump of two to three nests, and ‘medium’ indicates measurements near a clump of four to six nest holes. Means labeled with different letters are significantly different (Tukey *post-hoc* pairwise comparison, $p < 0.05$).

Differences between the three plots with nests and their inconsistencies with Figure 4 become apparent when separating the soil hardness data for each specific plot with nests (ANOVA: $F = 13.690$, $df = 6, 328$, $p < 0.0001$). Plot A differs from the overall pattern for all plots in that the mean soil hardness is highest in soil near medium sized nest clumps, though means for isolated nests and no nests are not significantly different from the medium sized clump mean (Figure 5). Plot B data (Figure 6) very closely resemble all-plot data, with highest mean soil hardness in no-nest areas and lower mean soil hardness near isolated nests, small nest clumps, and medium sized nest clumps. In plot C, areas with small clumps and medium clumps showed softer mean soil hardnesses than isolated nest areas and areas with no nests. Isolated nest areas in plot C show slightly higher mean soil hardness than areas with no nests, but the two do not significantly differ (Figure 7).

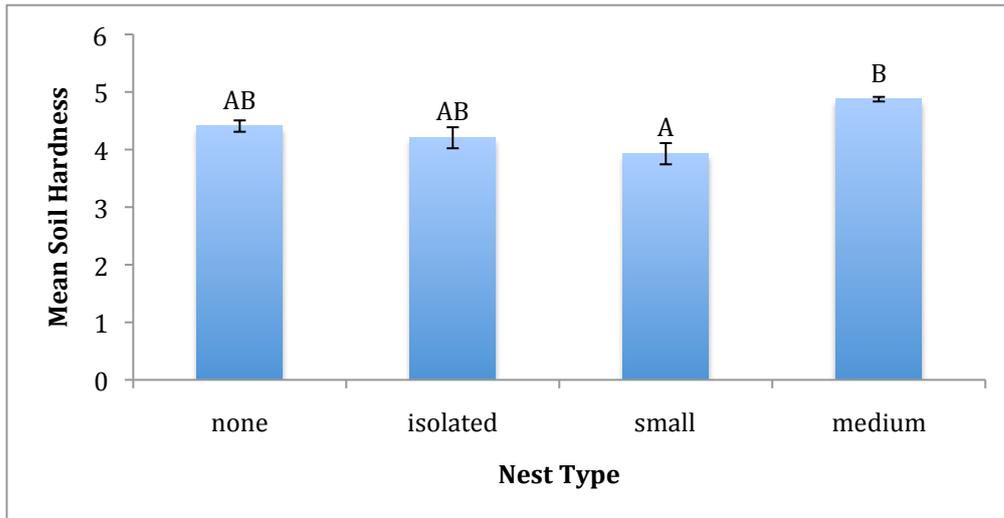


FIGURE 5. Mean soil hardness (± 1 SE) as measured near different nest distribution types within Plot A, one of three 1 x 4 m plots containing *C. luctuosa* nest holes marked on the clay road to Cerro Amigos around 1750 m elevation. ‘None’ indicates no holes present where soil measured, ‘isolated’ indicates a single nest hole with no close neighbors, ‘small’ indicates a clump of 2-3 nest holes close to one another, and ‘medium’ indicates a clump of 4-6 nest holes. Means labeled with different letters are significantly different (Tukey *post-hoc* pairwise comparison, $p < 0.05$).

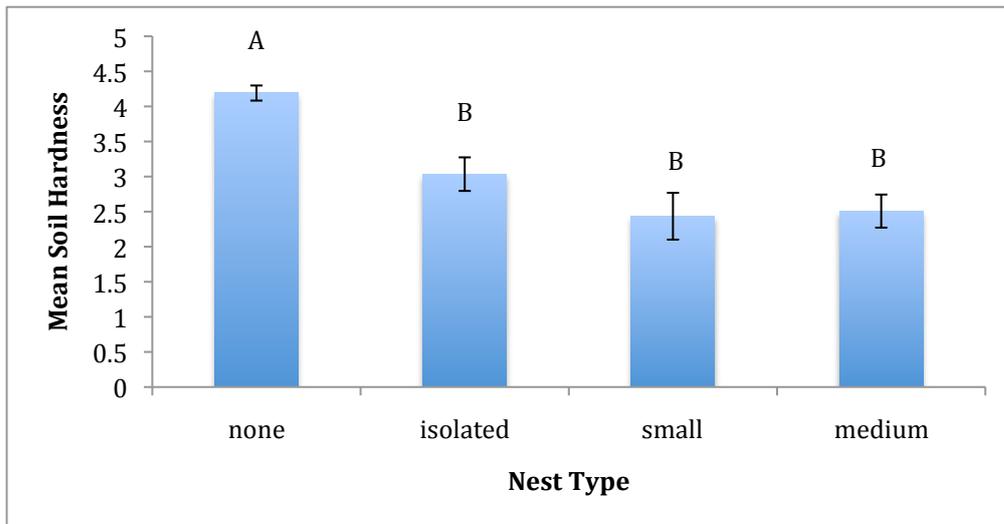


FIGURE 6. Mean soil hardness (± 1 SE) as measured near different nest distribution types within Plot B, one of three 1 x 4 m plots containing *C. luctuosa* nest holes marked on the clay road to Cerro Amigos around 1750 m elevation. ‘None’ indicates no holes present where soil measured, ‘isolated’ indicates a single nest hole with no close neighbors, ‘small’ indicates a clump of 2-3 nest holes close to one another, and ‘medium’ indicates a clump of 4-6 nest holes. Means labeled with different letters are significantly different (Tukey *post-hoc* pairwise comparison, $p < 0.05$).

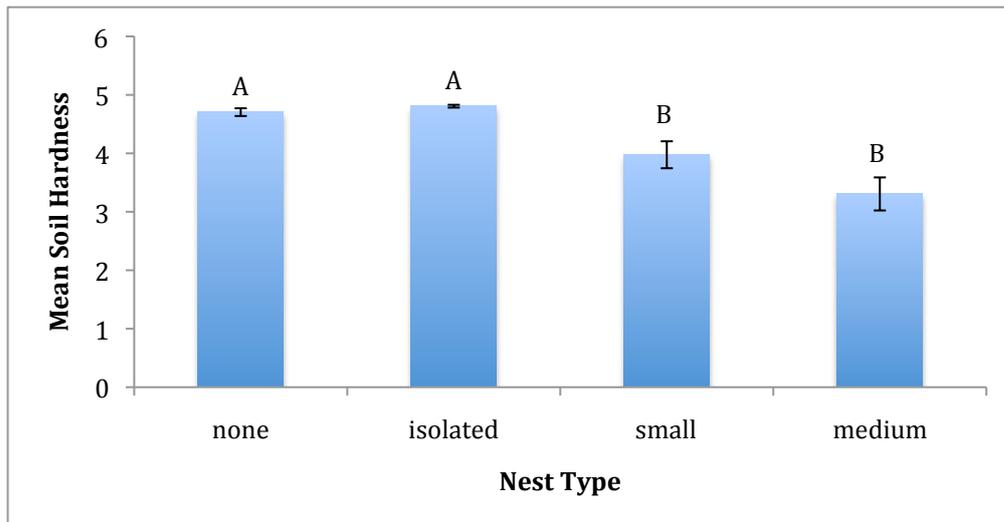


FIGURE 7. Mean soil hardness (± 1 SE) as measured near different nest distribution types within Plot C, one of three 1 x 4 m plots containing *C. luctuosa* nest holes marked on the clay road to Cerro Amigos around 1750 m elevation. ‘None’ indicates no holes present where soil measured, ‘isolated’ indicates a single nest hole with no close neighbors, ‘small’ indicates a clump of 2-3 nest holes close to one another, and ‘medium’ indicates a clump of 4-6 nest holes. Means labeled with different letters are significantly different (Tukey *post-hoc* pairwise comparison, $p < 0.05$).

Comparing the mean percent of vegetation cover between plots sampled with nests and plots sampled without nests (Figure 8), the plots with nests show a significantly higher mean vegetation cover (ANOVA: $F = 102.37$, $df = 1, 473$, $p < 0.0001$).

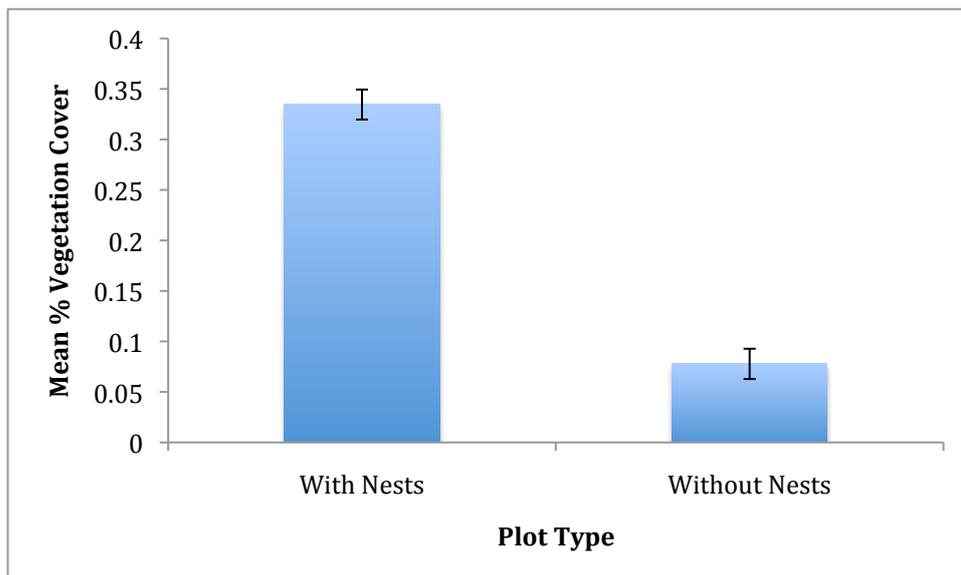


FIGURE 8. Comparison of three 1 x 4 m plots containing *C. luctuosa* nest holes to three 1 x 4 m plots not containing nest holes, in terms of mean percent vegetation cover as an estimate closest to 0, 25, 50, 75 or 100 percent in small (10 x 10 cm) squares throughout the plots. All plots located around 1750 m in elevation on the side of the clay road to Cerro Amigos at a *C. luctuosa* nesting aggregation site—plots without nests were within 50 m of at least one of the plots with nests.

Separated by nest distribution type (Figure 9) within only the plots with nest holes, mean vegetation cover is highest on the ground near the medium sized nest clumps sampled, and significantly lower for areas with no nest holes (ANOVA: $F = 3.8158$, $df = 3, 328$, $p = 0.0104$). All three plots with nest holes roughly follow this general pattern as the interaction between nest type and individual plot compared to mean vegetation cover is not significant (ANOVA: $F = 1.6191$, $df = 6, 328$, $p = 0.141$).

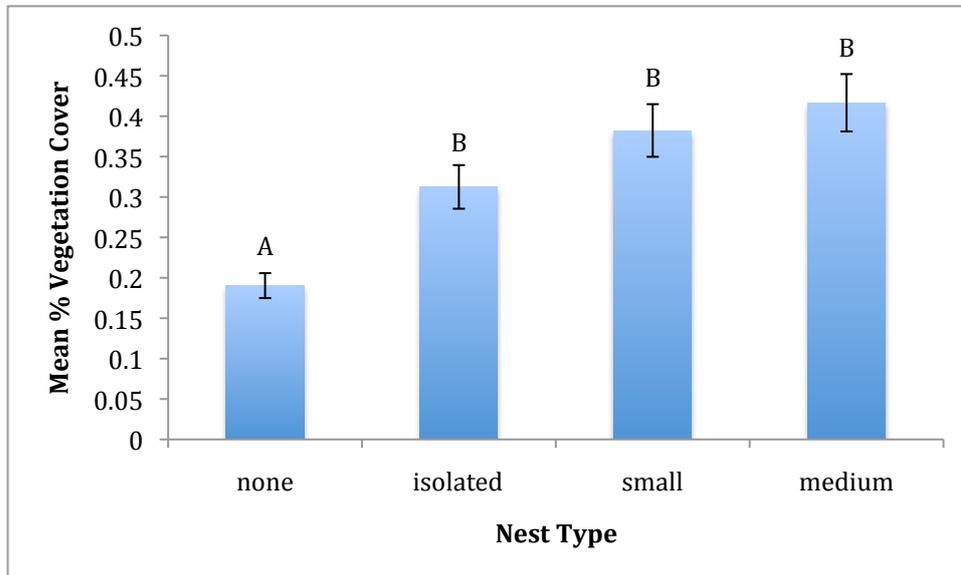


FIGURE 9. Mean percent of vegetation cover (± 1 SE), which was estimated as closest to 0, 25, 50, 75, or 100 percent in each place measured, calculated for each situation found around measuring sites: no nests (none), a single nest hole (isolated), a clump of two to three nest holes (small), or a clump of four to six nest holes (medium). Means labeled with different letters are significantly different (*post-hoc* Tukey pairwise comparison, $p < 0.05$).

DISCUSSION

In the study plots with nest holes, clumps of *C. luctuosa* nests were generally found in areas with softer soils and more vegetation cover, indicating a preference of the bees for these conditions. Though the soils in plots with nests were not any softer than the soils in plots without nests, the plots with nests showed higher vegetation cover, which could indicate that the bees value the vegetation cover as more important than soil hardness in selecting suitable nesting substrate. Vegetation cover may have an influence on nest site selection by making soil itself softer due to presence of roots breaking up the harder soils, or may be preferable as it provides leaf cover over the nest entrances, giving some protection from rainfall on misty days or sun on bright days.

As clumps of nest holes are found in areas of varying soil hardnesses, *C. luctuosa* clearly are capable of digging in a variety of softer and harder soils, so they may choose nest-building sites based upon other aspects such as the vegetation cover, or due to social behaviors. Nest-site selection has also been investigated in halictine bees with a focus on abiotic conditions, showing that *Halictus rubindicus* form aggregations in large part due to limited areas of viable substrate (Potts & Willmer 1997). However, Potts and Willmer (1997) also found that *H. rubindicus* preferred to dig nests near the nests where they were born, and Asís et al. (2014) show that *Bembix* digger wasps nest in clumps due to a tendency for young females to initially

establish nests near one another. Documenting the construction of new nests in *C. luctuosa* aggregations would help to understand whether these social behaviors are a factor to consider in the way that *C. luctuosa* create and structure their nesting aggregations, but in the time I spent collecting data at the nesting aggregation, I did not observe the creation of any new nest holes within my plots and cannot comment on *C. luctuosa*'s nest creation behavior based on personal observation.

Social behavior influencing the bees to deviate from seeking out soft soils would especially help to explain the pattern of soil hardness in Plot A, where medium-sized clumps were found in soil harder than the unused areas of that plot. Plot A had about the same vegetation cover as plots B and C, so the usage of harder soils by the bees of plot A is not likely explained by more vegetation presence, though vegetation presence is clearly favored by the bees overall. In plot A, *C. luctuosa* individuals may be participating in one of the previously discussed social behaviors, and one additional finding by Potts and Willmer (1997) could also offer a potential explanation—they found that in areas with a high density of nests, harder soils were used in order to maintain structural integrity of nests underground.

Overall, the main conclusion of this study is that the abiotic conditions of soil hardness and vegetation cover, aspects of their nest aggregation that had not previously been studied, have an influence on the nesting patterns of *C. luctuosa*. Nest clumps generally occur in areas with softer soils relative to the immediate area, though the bees do not always nest in the softest soils of the overall area, and they tend to choose areas with higher amounts of vegetation cover. The variation in the hardness of soils used by the bees may also indicate that there are social factors at work influencing the bees to clump together in areas that do not necessarily have the softest soils.

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Behavioral Syndrome consistency across metamorphosis in the tropical tree frog *Isthmohyla pseudopuma*

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ABSTRACT

A recently growing field of study because of its importance to ecological and evolutionary processes is behavioral syndromes, which is when individuals in a population have a similar behavior through time or across context. Yet a surprisingly neglected area of research in the field is the effects of ontogenetic niche shift on behavior syndrome and type. There is an obvious importance of a study of this kind because so many different organisms across numerous taxa have complex life cycles or go through metamorphosis and it is not well known how these changes affect the behavior of the individual. In this study the behavior of the tree frog *Isthmohyla pseudopuma* (formerly *Hyla pseudopuma*) was observed in the lab throughout metamorphosis by looking at exploratory behavior and boldness to determine temperament among the population, which is consistent between individual differences in behavior across time. This was done over a four week period while the larva were developing into juvenile frogs. Boldness was measured by the amount of time it took for tadpoles to exit a secure location and enter a novel area and exploratory behavior was measured by the area covered and time spent moving about the novel area. The results indicate a significant correlation between boldness and exploratory behavior and two distinct behavior types; shy and bold. Temperament was first seen after Gosner stage 40 indicating that temperament is not innate but expressed later in metamorphosis. This study does not show how or why temperament was defined but speculates at possible reasons for it.

RESUMEN

Un campo de estudio en reciente crecimiento debido a su importancia hacia procesos ecológicos y evolucionarios es el de síndromes de comportamiento, el cual es cuando individuos en una población tienen comportamiento similar a lo largo del tiempo y entre contextos. Aún así, una sorprendente área poco estudiada en el campo es el desplazamiento en el nicho ontogenético en el síndrome y tipo de comportamiento. Existe una importancia obvia en este tipo de estudio debido a que muchos organismos diferentes a lo largo de taxones diferentes tienen ciclos de vida complejos o pasan a través de metamorfosis y no se conoce muy bien como estos cambios afectan el comportamiento del individuo. En este estudio el comportamiento de la rana arborícola *Isthmohyla pseudopuma* (anteriormente *Hyla pseudopuma*) se observó en el laboratorio a través de la metamorfosis al observar el comportamiento exploratorio y audacia para determinar el temperamento entre la población, en el cual las diferencias individuales son consistentes a lo largo del tiempo. Esto se hizo durante un período de cuatro semanas durante el cual los renacuajos se desarrollaron en ranas juveniles. La audacia fue medida por la cantidad

de tiempo que le tomo a los renacuajos salir un lugar seguro y entrar a un área nueva y el comportamiento exploratorio se midió por el área cubierta y el tiempo en el que se movieron en el área nueva. Los resultados indicant una correlación significativa entre la audacia y el comportamiento exploratorio y dos tipos distintivos de comportamiento; tímido y audaz. El temperamento fue visto por primera vez después del estado 40 de Gosner indicando que el temperamento no es innato si no que se expresa tarde en la metamorfosis. Este estudio no muestra como o por qué el temperamento se define pero especula con razones del por qué.

IT IS WELL KNOWN that individuals that are within the same species, sex, age and are within the same population can have different behaviors (Wilson 1998). Recently there has been a growing interest in behavioral syndromes which according to Bell (2007) “occurs when individuals behave in a consistent way through time or across contexts and is analogous to ‘personality’ or ‘temperament’.” In other words it is a property of a population and is made up of the relative rankings of temperaments between individuals. Behavior type is a property of an individual and signifies a specific arrangement of behaviors that the individual has and repeatability is the consistency of relative behavior across time among these individuals (Lessells & Boag 1987).

There have now been a number of studies done on many different species testing for behavioral syndromes to determine if differences among individuals were indeed held consistent across different tested scenarios. Such studies have included fish, birds, crustaceans, mammals, insects and many others that have each assessed behavioral syndromes by looking at different contexts within a population (Wilson & Godin 2009; Quinn & Cresswell 2005; Briffa *et. al.* 2008; Dochtermann & Jenkins 2007; Brodin 2008). Although it has been studied rarely in amphibians there was a study done on bullfrog tadpoles before metamorphosis started, that measured activity, how much time they spent moving about novel and natural areas, boldness, how much time they spend in open area, and exploration, the number of areas explored. This study showed that activity wasn't different among individuals but boldness was moderately repeatable and exploration highly repeatable between individuals across trials. This study confirmed the presence of typical temperaments in tadpoles making them ideal specimen for behavioral research (Carlson & Langkilde 2013). There has been a lack of study bringing such tadpole research one step further across ontogenetic niche shift. Countless organisms go through such changes, yet it is not well known whether temperament will hold constant while the individual itself changes so drastically (Wilson & Krause 2012b).

The most general question when looking at animal behavior is when personality is first defined and whether individuals change over time. Ontogenetic niche shift is the perfect way to study this because it involves a physiological niche shift as well. Since this is a neglected area of study it cannot conclusively be said whether or not individual's personality behind these behaviors has changed, and if they do change at what point during metamorphosis does this occur. Metamorphosis encompasses a vast change in individuals that includes morphological, and physiological changes (Wilbur 1980), and on top of that there is often a change in habitat which would also result in a very large niche shift. In the case of amphibians this would be a transfer from an aquatic to a terrestrial habitat, but for many insects this would be a terrestrial to aerial change. Along with such niche shifts will come changes in foraging,

mating, and predator avoidance. It is possible that early developmental experiences change the course of ontogenetic development and from that comes a predictable pattern in personality despite other factors such as niche shift and morphological change (Chapman *et. al.* 2010; Wilson & Krause 2012a).

One such study that looked at personality throughout metamorphosis and the consistency of behavior across ontogenetic niche shifts was a study done on the lake frog, *Rana ridibunda* (Wilson & Krause 2012a). The study used activity, exploration and boldness to assess personality and it was not only done for tadpoles and juvenile frogs but across metamorphosis itself. They found that both activity and exploratory behavior were consistent across metamorphosis as well as in adult frogs. This is one of the only study of its kind and further studies will need to be done to determine if this is consistent across the order Anura. In the present study, the behavior syndrome of the tropical tree frog *Isthmohyla pseudopuma* was observed across metamorphosis in a lab setting by measuring boldness and exploratory behavior. This is possibly the first times that temperament is assessed through metamorphosis in a tropical amphibian.

METHODS

STUDY LOCATION - Test subjects were originally collected from a temporary roadside pond at around 1600 m in the Tropical Lower Montane Wet Forest of Monteverde, Costa Rica. Between 60 and 70 *Isthmohyla pseudopuma* (formerly *Hyla pseudopuma*) tadpoles were collected from the pond and brought back to the Monteverde biological station lab where they were kept for the remainder of the experiment. Thirty healthy tadpoles were chosen to be put into individual 12.5 x 12.5 cm plastic containers in order to differentiate between test subjects (Figure 1b). The remaining tadpoles were kept together in a 48x24x30 cm glass tank and were saved in case test subjects died or escaped and needed to be replaced (Figure 1c). Any tadpole that needed to be replaced was recorded and omitted when looking at temperament repeatability in individuals across metamorphosis.

TEST SUBJECT - *I. pseudopuma* larvae were all around the same size and were at the same developmental age because they are explosive breeders, following heavy rains, and would have hatched at the same time regardless of whether or not they were a part of the same egg mass (Crump & Townsend 1990). They were about 4 cm long with their heads being about 1 cm wide. All were a uniform brown/green color when looked at from above. Once they start to go through metamorphosis, all feeding virtually stops because they use the energy from the reabsorption of their tail in order to sustain themselves (Duellman & Trueb 1986). In order to identify stages of development in the tadpoles Gosner's (1960) and Crump's (1984) method of identification was used. They were collected when they were around Gosner stage 34 and there was very limited variation in development among individual throughout the rest of study. Trial two had the greatest variation in development stage with the oldest individual being in Gosner stage 43 and the youngest individuals being in Gosner stage 39. The average age for test two was Gosner stage 41. Test three had less variation however the difference between youngest and oldest was three stages with the average being Gosner stage 43. Test four had an average age of 45 and by test five 80 percent of all juvenile frogs were fully developed (Gosner stage 46).

GENERAL MAINTENANCE - Once tadpoles were separated into their appropriate containers they were fed a small pinch of standard fish food every other day and were never fed the day prior to testing so that they

would be more likely to express temperaments by foraging for food during the test. The individual containers were cleaned every other day, on the same day that they were fed, and the water was always filled up to 4 cm. Once the froglets had climbed out of the water for the first time, the water was decreased so that there was only enough to cover the bottom, in order to avoid drowning. The large tank was fed three pinches of fish food every day and the tank was cleaned and the water changed once a week. Water was filled 10 cm high initially and then was decreased to 1 cm once all froglets had left the water. Fecal matter was removed with a plastic pipet in both the tank and the individual containers every day. Once some of the froglets had all four legs, various rocks and a stick were placed in the tank for the frogs to climb on, to again, avoid drowning. Additionally mosquito netting was placed on the tank and all individual containers and held in place by rubber bands to avoid escaping. Even though tadpoles virtually stop eating after metamorphosis starts (Duellman & Trueb 1986), fish flakes were continuously given to them just in case they still needed to eat, although less often and in a smaller quantities.

BOLDNESS AND EXPLORATORY BEHAVIOR EXPERIMENT - The experiment designed to test the tadpoles was designed in a way to test both boldness and exploratory behavior within the same test. It was based off the cylinder test that was used in Brodin *et. al.* (2013). One at a time tadpoles were taken out of their individual containers and placed in a cylindrical holding container with an opening at one end. The container was dark piece of PVC pipe (11 cm long, 6 cm wide) with a black piece of plastic covering the opening so that it would be darker inside and resemble a more secure location for the tadpoles. The holding container was in a glass aquarium exactly the same size as the tank holding the overflow tadpoles. There was nothing in it except for water filled 6 cm high or to the very top of the cylinder. There was a grid made up of 1 cm x 1 cm squares that was taped underneath the tank so that the distance that the tadpole traveled after leaving the cylinder could be recorded (Figure 1a).

Once the tadpole was placed in the holding vessel it was allowed to sit in there for one minute before the door was lifted up. The time it took the tadpole to leave the cylinder was recorder from the moment the door was lifted. The maximum amount of time allowed to leave the container was ten minutes and if the tadpole took the full allowed time it was forced out of the container in order to assess exploratory behavior. In the event that the tadpole was forced out, video recording would not start until the tadpole came to a full stop for three seconds so that startled movement was not confused with exploratory tendencies. If the tadpole came out of the container of its own accord video recorder would start the moment the front of its head passed outside of the cylinder. Video recording was done with a standard GoPro camera mounted above the tank so that a full view of the grid on the bottom of the tank was possible (Figure 1a). The tadpole was recorded for three minutes and then removed from the tank and the test of the next individual would start. The distance that tadpoles traveled was determined later from the video by the number of grid squares that their head passed through within the three minutes. Time spent moving included when the individual was moving and when there was tail movement but no locomotion.

A number of changes were made in order to compensate for the tadpoles growing legs and being able to walk and live outside of the water, including lowering the water level so that the bottom of the testing tank was only moist, and lining the walls of the tank with the same grid that was used on the bottom of the tank since the juvenile frogs could now climb the walls. Since juvenile frogs jump instead of swim all grid squares that they passed over during jumps was counted. There were also several instances of frogs jumping outside the tank. When this happened it was recorded and the distance that

they traveled outside of the tank was measured. This same test was done five different times over four weeks by testing all 30 tadpoles over a two day time period.

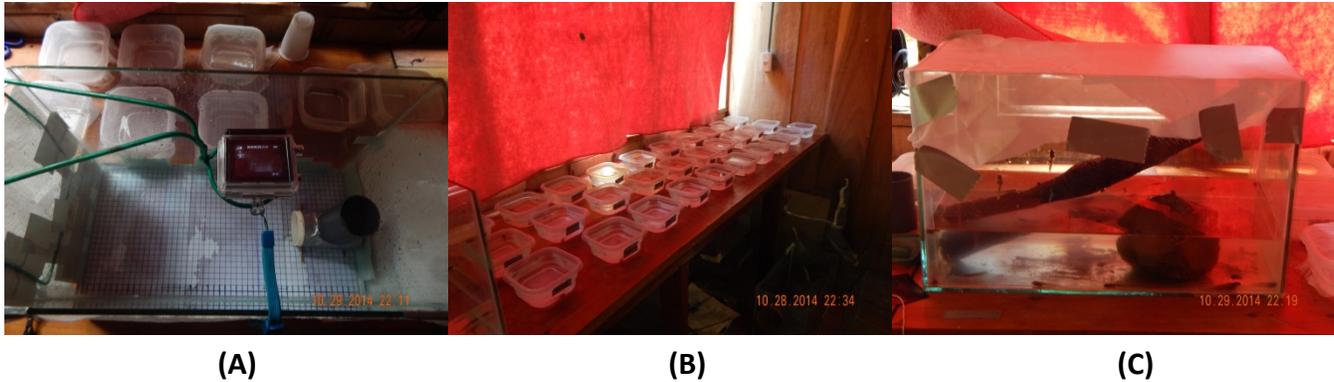


FIGURE 1. The station in the Monteverde Field Station Lab where all tadpoles and juvenile frogs (*Isthmohyla pseudopuma*) were tested for temperament and kept in between tests. (A) The tank that all tadpoles were tested in for all five behavior trials, where boldness and exploratory behavior were observed during the four week testing period. This was done by timing the number of seconds individuals took to exit a secure location and enter a novel area (Boldness evaluation) and the area covered and time spent moving around the novel area (Exploratory behavior evaluation). The GoPro camera was mounted above the test tank to record individuals for exploratory behaviors. (B) The individual 12.5 x 12.5 cm containers that test subject tadpoles were kept in. (C) The overflow tank where all other tadpoles were kept, in case they were needed for the future.

RESULTS

All tadpoles were in Gosner stage 34 during the first stage and as was the case for every other stage, there was a significant amount of variation among individuals for all observations recorded. Tadpoles ranged from exiting the cylinder with in a second to being forced out at 10 minutes. Distance explored (DE) ranged from >80 squares to none. Time spent exploring (TE) ranged from >80 to 0 seconds as well. Using a Pearson correlation test there was no observable trend between exit time (ET) and DE ($r < -0.10$, $df=23$, $p > 0.5$) for Gosner stage 34 (Figure 2a). Gosner stage 41 only had a weak correlation that was only marginally significant between ET and DE ($r < -0.3$, $df=28$, $p=0.05$) (Figure 2b) but all development stages following stage 41 showed a significant correlation between boldness and exploratory behavior (Figure 2). There was an increase in correlation between ET and DE in Gosner stage 43 ($r > -0.44$, $df=28$, $p < 0.018$) (Figure 2c) The correlation increases even more in Gosner stage 45 ($r > -0.5$, $df=28$, $p < 0.0009$) (Figure 2d), but then decreases slightly in Gosner Stage 46 even though the correlation is still statistically significant ($r > -0.43$, $df=28$, $p < 0.2$) (Figure 2e).

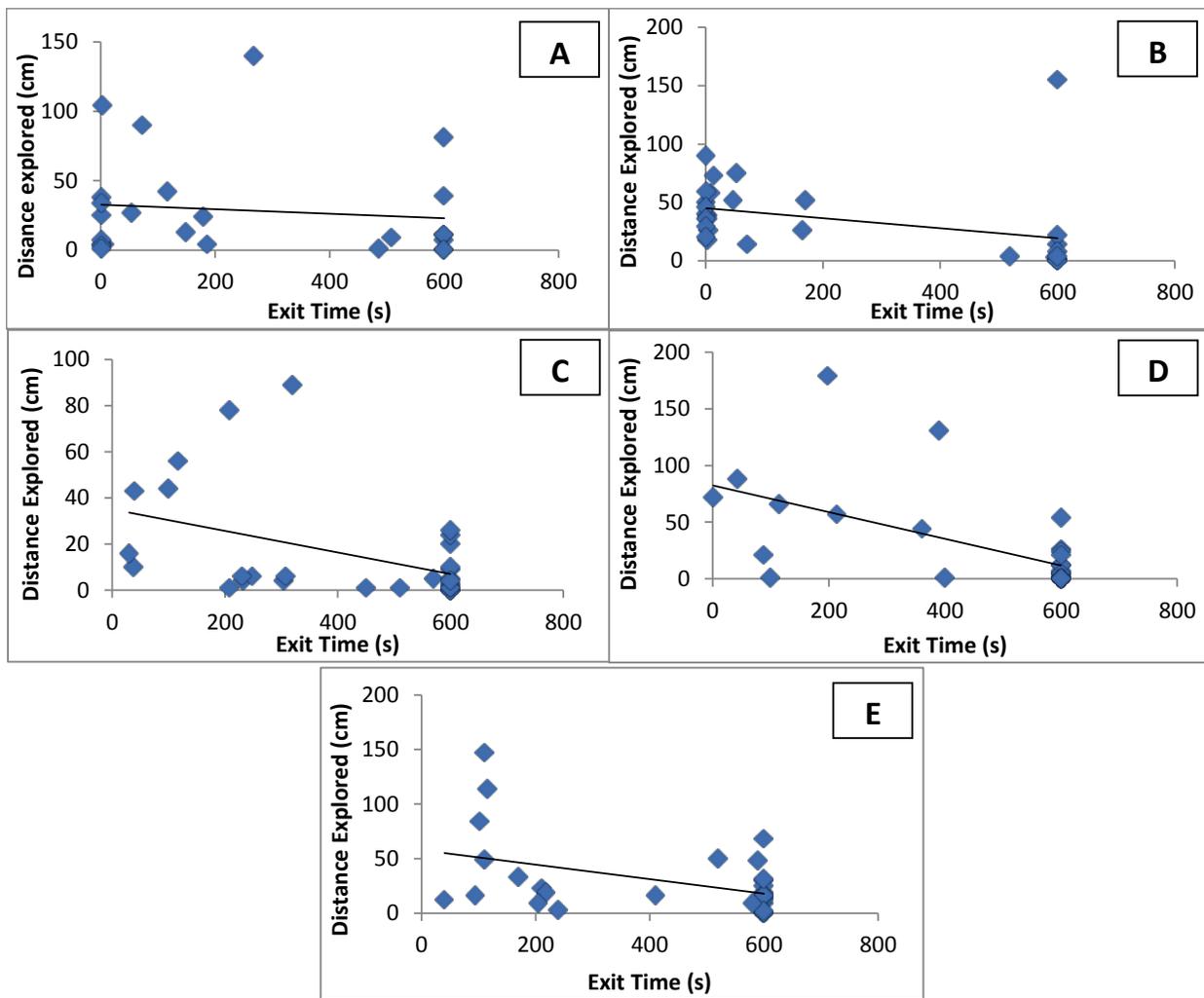


FIGURE 2. The relationship between boldness and exploratory behavior in *I.pseudopuma* tested in a lab setting. Exit time represents boldness and distance explored represents exploratory behavior. It was measured by the amount of time individuals would take to enter a novel area and how much they traveled in that area. The test was repeated five times over four weeks using the same individuals, who are each represented by a separate mark. For test 2-5 there is a correlation between boldness and exploratory behavior and two separate behavior types are present; ‘shy’ and ‘aggressive’ seen on opposite ends of each graph. (A) Gosner stage 34 ($p > .5$), (B) Gosner stage 41 ($p = .05$), (C) Gosner stage 43 ($p < .018$), (D) Gosner stage 45 ($p < .0009$), (E) Gosner stage 46 ($p < .2$).

The only tests that showed a significant correlation between ET and TE were Stages 43, 45, and 46. Gosner stage 34 ($r>0.08$, $df=23$, $p>0.6$) (Figure 3a), and Gosner stage 41 ($r>0.08$, $df=28$, $p>0.6$) (Figure 3b), showed no correlation between boldness and exploratory behavior. Gosner stage 45 again had the most significant correlation ($r>-0.6$, $df=28$, $p<0.0007$) (Figure 3d). The next most significant correlation was seen in Gosner stage 43 ($r>-0.45$, $df=28$, $p<0.02$) (Figure 3c) while Gosner stage 46 again had a decrease in correlation despite the fact that the trend was still statistically significant ($r>-0.4$, $df=28$, $p<0.4$) (Figure 3e).

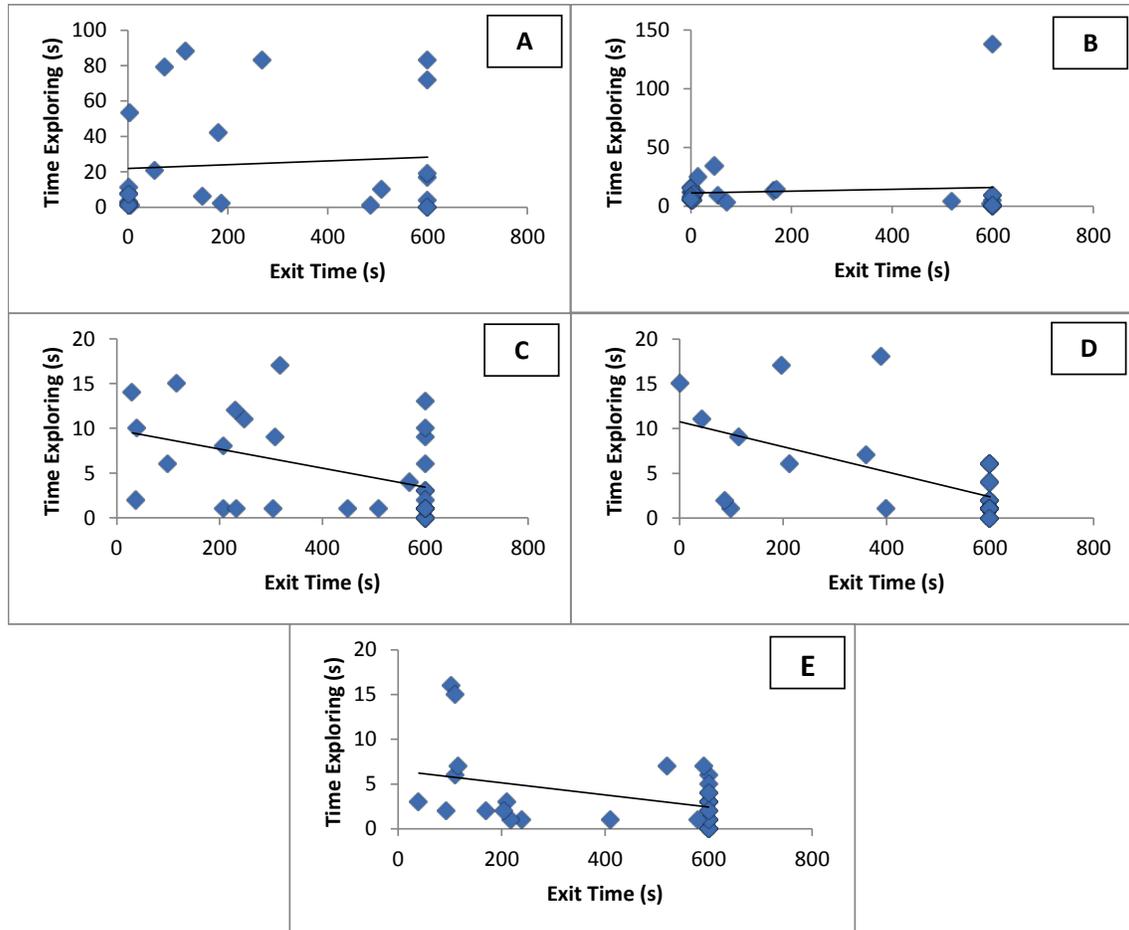


FIGURE 3. The relationship between boldness and exploratory behavior in *I.pseudopuma* tested in a lab setting. Exit time represents boldness and time exploring represents exploratory behavior. It was measured by the amount of time individuals would take to enter a novel area and the time they spent moving in that area. The test was repeated five times over four weeks using the same individuals, who are each represented by a separate mark. For test 3-5 there is a correlation between boldness and exploratory behavior and two separate behavior types are present; ‘shy’ and ‘aggressive’ seen on opposite ends of each graph. (A) Gosner stage 34 ($p>.6$), (B) Gosner stage 41 ($p>.6$), (C) Gosner stage 43 ($p<.02$), (D) Gosner stage 45 ($p<.0007$), (E) Gosner stage 46 ($p<.04$).

The overall trends that were seen here were two distinct behavior types. There was a very prominent group of “shy” individuals that took the full time to exit the cylinder and did not move in the novel area. There was also group of bold individuals, although maybe less distinct, that exited the cylinder immediately and moved about the tank a significant amount. Additionally there were a significant number of individuals that followed neither behavior type (Figure 2 & 3).

Using Kendall’s coefficient of concordance test it was possible to determine that there was only marginal repeatability among individuals. There was an average of 35 percent behavior type consistency among the 23 individuals that were tested throughout the entire experiment. TE was the most consistent with 37.6 percent similarity ($W=0.34, p=0.02, n=23$) (Figure 4a), and ET was the least similar with only 32.3 percent consistency ($W=0.29, p>0.07, n=23$) (Figure 4c). DE was in the middle with 36.5 percent similarity ($W=0.33, p<0.03, n=23$) (Figure 4b).

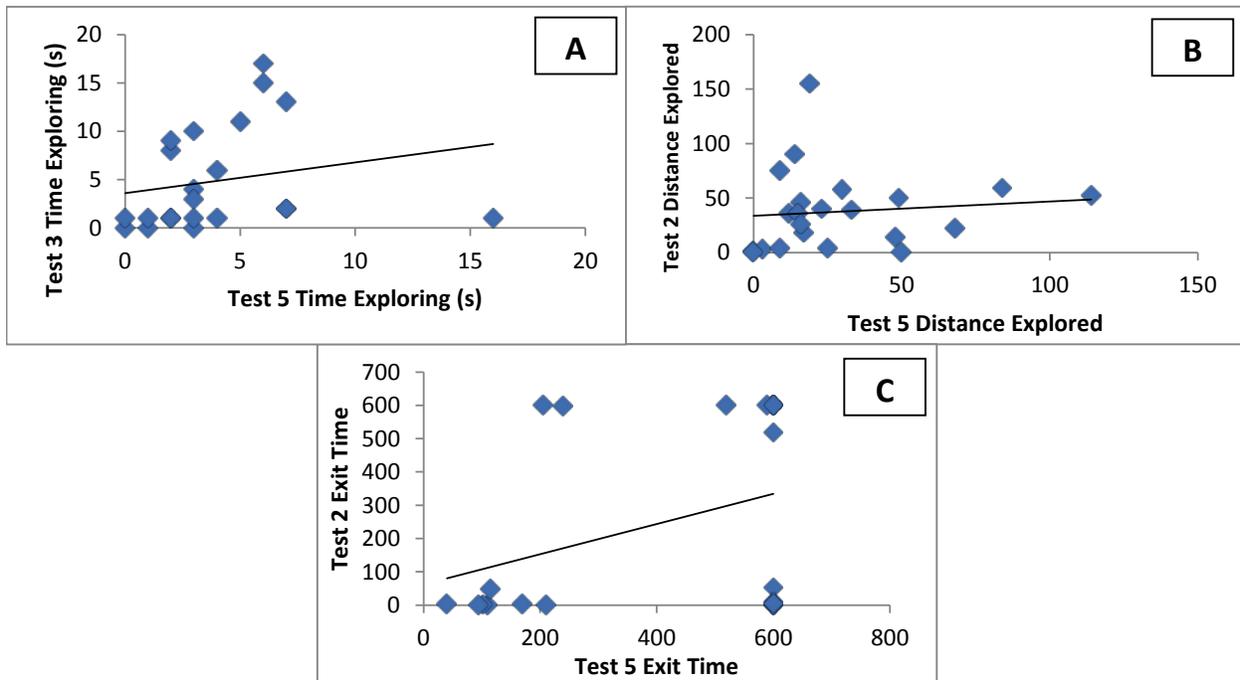


FIGURE 4. There were 23 *I.pseudopuma* individuals that were tested in the lab throughout the entire experiment. With these individuals an analysis of repeatability was done to determine how consistent they were over time. Tests 2 and 5 were compared for distance explored and exit time because test 2 was the first test that showed a trend for both of those variables. Test 3 and test 5 were compared for time exploring because that was the first time a trend was seen in that variable. (A) Time Exploring had the highest percent repeated with 37.6 percent repeatability, (B) the next highest was distance explored with 36.5 percent repeatability, (C) and the least repeatable was exit time with 32.3.

DISCUSSION

In this study *I. pseuopuma* was observed throughout metamorphosis in order to determine the presence of behavioral syndromes and whether this was held consistent throughout ontogenetic niche shift. The main findings of the experimental results were a significant correlation between boldness and exploratory behavior that was first seen in tadpoles somewhere around Gosner stage 40. Although it was still statistically significant, significance of the correlation did decrease slightly during the final test when most individuals were fully developed (Gosner stage 46). This was most likely because all individuals would jump instead of walk or swim during movement which was much harder to record during video analysis. If the full extent of exploration can't accurately be recorded it would decrease the correlation between the two behaviors since ET was still being recorded with accuracy.

For each test where exploratory behavior and boldness were correlated, there were a number of individuals that fell well outside of the expected trend. One possible reason for the surprising number of uncorrelated individuals is the lack of predators. A study done on stickleback fish (*Gasterosteus aculeatus*) showed that there wasn't a correlation between boldness and aggressiveness within the population until a predator was released into the test area (Bell & Sih 2007). The addition of predators had a major effect on the population that would cause the correlation between behaviors to be shown. The correlation was shown because, those individuals that fell outside the trend were most likely to be predated against since certain combinations of traits are more favored and increase fitness. For example individuals that are very shy in some situations but aggressive in other situations may leave them more susceptible to predators. With the absence of uncorrelated individuals after predation the correlation was possible to see among the survivors (Bell & Sih 2007). This suggests that in the presence of real predation the tadpoles would express a more significant correlation between boldness and exploratory behavior.

It was found that temperament was not innate and was only expressed sometime after Gosner stage 40. It is still unclear what caused temperament to be expressed after this stage and what caused the specific differences among individuals themselves. One prediction is that different temperaments could be a result of the environment where these tadpoles did most of their developing. It is known that experiences can have a pronounced effect on behavior (Stamps 2003), however it is thought that early experiences in development have a much greater affect than more recent ones (Chapman *et. al.* 2010). There are also studies suggesting that unpredictable environments influence boldness in individuals (Chapman *et. al.* 2010), because as organisms become more food deprived they will become more likely to take risks to avoid starving (Damsgard and Dill 1998). Since in this case tadpoles were not tested until Gosner stage 34 they spent a significant amount of time developing in the temporary pond. It is possible that tadpoles experienced differing amount of environmental predictability within the same pond leading to different temperaments being expressed later on. However, since tadpoles were collected later in development this cannot be confirmed.

A second possible reason for the expression of behavioral syndromes is differences in growth rate. The growth-mortality hypothesis says that faster growing individuals will tend to have bolder temperaments (Stamps 2007). This is case because individuals that are growing faster have to eat more meaning they are taking more risks in foraging situations. In this study while there was only a slight variation in development rate but there was still some. According to the growth-mortality hypothesis this would mean that some relationship between growth rate and boldness could be expected however, in this

study that was not the case. There was no observable correlation between the two that would suggest that this was the reason for a visible behavioral syndrome. Another factor that needs to be considered is that once metamorphosis starts the tadpoles stop eating (Duellman and Trueb 1986), and since in this hypothesis increased growth is tied directly to bolder foraging behavior there is reason to believe that tadpoles would not follow this model. It is also important to note that metabolic rate is thought to have an impact on consistent individual differences in personality (Careau *et. al.* 2008; Biro & Stamps 2010). The question here is whether or not tadpoles have different metabolic rates and if these differences would be expressed during a time they don't have to eat. It appears as if they are since there were different development rates, no matter how small. In this case there was no correlation during the first trial suggesting that differences are not innate. Therefore whatever it was that caused differences in temperament had to occur later on. There is a possibility that this was due to metabolic differences that were harder to detect (if they exist) when the individuals are not eating. However since metabolism wasn't something that was recorded in this study there is no way of determining if this was indeed what happened.

Repeatability among the individual tadpoles was only marginally significant with only a little more than a third showing a consistent behavior type across time. This is less significant than what was found in an experiment done on damselfly's (*Lestes congener*) which showed that temperaments can be carried across different life stages (Brodin 2008). However, there was slightly less consistency across life stages in another study looking at the common frog (*Rana temporaria*) temperaments. Across metamorphosis behavior was less than a third (boldness $r=0.25$, exploration $r=0.25$), repeatable and non-significant (Brodin 2013). This may suggest that repeatability of temperaments across metamorphosis isn't very defined in amphibians. It's possible that repeatability would increase in adult frogs due to biological processes like predation taking uncorrelated individuals out of the population however this cannot be confirmed because no such studies have been done.

In conclusion, the tropical species *I. pseuopuma* does show a consistent behavioral syndrome throughout metamorphosis with two distinct behavior types. These findings were consistent with the only other studies done across amphibian metamorphosis (Brodin *et. al.* 2013; Wilson & Krause 2012a). However, it is still unclear what is causing these differences in temperament among individuals and why they are defined at a specific time. There was also limited repeatability among individuals in both studies suggesting that this may be typical for amphibians, but in the absence of any other studies it is not possible to make any definitive conclusions.

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Benthic Macroinvertebrate Food Webs and Tropical Montane Stream Contamination

Austen Hilding

Abstract

Many tropical montane streams are becoming increasingly contaminated as human population growth and land transformation add sedimentation and turbidity (Henley *et al.* 2010, Wallace & Webster 1996). In this study, benthic macroinvertebrates (BMIs) were collected from four stream sites in Monteverde, Costa Rica that experience different levels and types of contamination. The pristine forest site had 19 families, the site underneath the Monteverde Cheese Factory had 11 families, the site underneath a culvert and dirt road also had 11 families, and the site in the town of Santa Elena had only 1 family. Water quality was assessed using the BMIs as bioindicators and food webs were created to analyze how contamination affects them. Species richness, total number of links, linkage density, and similarity were reduced with more contamination. Fraction of omnivores (species feeding at multiple trophic levels) was significantly reduced with more contamination as well. Connectivity, fraction of basal, intermediate, and top species, and prey to predator ratio were not influenced by contamination, suggesting that there are “ecological equivalents” to the pristine species that are tolerant to some amounts of contamination which allows for certain ecosystem functions and feeding guilds to be retained. Preservation of pristine streams is vital and greater protection of contaminated streams is important for maintaining functions of stream ecosystems.

Resumen

Muchos ríos tropicales están volviéndose altamente contaminados al aumentar la población humana y debido a la sedimentación y turbidez provocada por el cambio en el uso de suelo (Henley *et al.* 2010, Wallace & Webster 1996). En este estudio, los macroinvertebrados bénticos (BMI's por sus siglas en inglés) se colectaron en cuatro quebradas con diferentes niveles y tipos de contaminación en Monteverde, Costa Rica. El bosque pristino tuvo 19 familias, el sitio bajo la Fabrica de Quesos de Monteverde tuvo 11 familias, el sitio bajo una alcantarilla y camino de lastre tuvo 11 familias, y el sitio en el pueblo de Santa Elena tuvo solo 1 familia. La calidad del agua se estimó usando BMI's como bioindicadores y se crearon cadenas alimenticias para analizar cómo la contaminación los afecta. La riqueza de especies, número total de conexiones, densidad de conexiones y similitud se vieron reducidos con una mayor contaminación. La fracción de omnívoros (especies alimentándose a múltiples niveles tróficos) fue significativamente menor con mayor contaminación también. La conectividad, fracción basal, intermedia, y especies tope en la cadena y la ración de presa a depredador no se vieron significativamente influenciados por la contaminación, sugiriendo así que estos son “equivalentes ecológicos” a las especies pristinas que son tolerantes a alguna cantidad de contaminación lo cual

permite ciertas funciones del ecosistema y cadenas alimenticias de ser conservadas. La conservación de ríos prístinos es vital y una mayor protección de los ríos contaminados es importante para el mantenimiento de las funciones del ecosistema en las quebradas.

Introduction

TROPICAL MONTANE STREAMS are increasingly contaminated by rapid population growth and land transformation for agriculture and urban development (Jordán *et al.* 2012). For example, 90% of wastewater in the developing world still flows untreated into local fresh water sources, and tropical montane streams are no exception (World Water Development Report 2012). Resultant contamination from direct dumping, erosion, and runoff in the form of pollutants and sedimentation can lead to dramatic changes in stream biodiversity through reduced primary production, local extinctions, lower diversity, and changes in relative abundances. (Castillo *et al.* 2006, Brisbois *et al.* 2008, Al-Shami *et al.* 2011, Allan 2004, Jordán *et al.* 2012, and Woodwell 1970). Thus, food webs are likely to change also as there is a reduction in biodiversity and production, which can cause cascading effects to higher trophic levels (Yule *et al.* 2010).

Benthic macroinvertebrates (BMIs) react to the presence of toxins, sedimentation, and the changing substrate as pollution-tolerant taxa proliferate at the expense of those that require pristine conditions, giving direct measurements of water quality as bioindicators (Wallace & Webster 1996, Clements 1994). Biodiversity and productivity are reduced and the food web is altered as filter feeders, grazers, shredders, and many predators are eliminated because of contamination (Jordán *et al.* 2012, Yule *et al.* 2010). Stream food webs are important to study as they greatly impact the function of stream ecosystems, which are incredibly important for biodiversity, the water cycle, nutrient and sediment discharge into oceans and lakes, and human well being.

Exactly how stream food webs are altered due to contamination is still somewhat unknown. Yule *et al.* (2010) found that high water quality stream sites had more complex food webs, with more species, links, linkage density, and linkage complexity than more polluted sites that lacked all filter feeders, grazers, and shredders. Likewise, Henley *et al.* (2010) found that primary production is reduced with increased sedimentation and turbidity, which results in negative cascading effects through depleted food availability for the upper trophic levels. There is little known about how other aspects of food webs are effected by contamination, such as connectance, fraction of omnivores (feed in more than one trophic level), ratio of basal species to intermediate species to top species, prey to predator ratio, as well as simply how much the species richness and evenness are reduced. Also, there is not much known about how these parameters are influenced by the extent of which a stream is contaminated.

Many streams around Monteverde, Costa Rica have become contaminated since the tourism boom in the 1980s that has led to increased runoff and human use

and development. In this study, I collect BMIs from four different stream locations with different types and levels of human pollution: a pristine forested site above anthropogenic development, a site directly below the Monteverde Cheese Factory, a site in Santa Elena right next to a highly used road and buildings, and a site directly below a culvert that gets traffic above as well as next to a few houses with gardens and banana trees. With this, I determine the water quality using the BMIs as bioindicators, and also construct the food webs from each to analyze how different types and levels of contamination impact the food web parameters mentioned above. The objective of this study is to show how and to what extent different types and amounts of contamination affect stream food webs. Learning these aspects of the effects of pollution in streams could be used to better understand the affects of water pollution at a greater scale and as more incentive to change environmentally damaging practices in Monteverde streams.

Materials and Methods

STUDY SITES

Four study sites in Monteverde, Costa Rica area were chosen based on their levels of contact with sources of anthropogenic pollution (fig. 1). The first sample (A) came from Quebrada Máquina in an apparently pristine mountain forest site free from pollution sources. The second sample (B) came from Quebrada Cuecha in a site located just downstream from the Monteverde Cheese Factory and just upstream from the bridge that receives traffic. The third sample (C) came from directly under a culvert and small bridge that receives heavy traffic in the town of Santa Elena from Quebrada Rodriguez. The final sample (D) came from Quebrada Máquina again, directly under a culvert that receives car traffic above and next to houses.



A.



B.



C.



D.

Fig. 1. Stream sites chosen to collect benthic macroinvertebrates (BMIs) with different levels of pollution. A=Forest.) A forested location above any source of pollution. It had clear water and a rocky and sandy substrate. B=CF.) A site just downstream from the Monteverde Cheese Factory and above stream from the bridge. On both sides were trees for a few meters and it also had a rocky and sandy substrate and fairly clear water, but there was a strong scent of cheese whey. C=Town.) A site from the town of Santa Elena in a location directly downstream from a small bridge and culvert that gets a lot of traffic on the concrete road and is next to houses, restaurants, and stores. It was the most turbid water and had a slimy and mucky substrate. D=Culvert.) A site next to a couple houses with small areas for crops and was directly downstream from a culvert that gets car traffic above on the gravel road. It also had a rocky and sandy substrate and the somewhat clear water.

COLLECTING BMIS

Benthic macroinvertebrates were collected from each site using the same sampling method defined by Hauer and Lamberti (1996). Riffle locations are the most biodiverse habitats in streams (Harrington and Born 2000), so I found the nearest or most accessible riffle from each stream location to take my sample. Using a kick-net to collect BMIs, I turned over rocks within a meter-squared area of the riffle and brushed off the attached BMIs. I then dug into the substrate in that area and kicked up any BMIs in the substrate so that they would float into the net. I dumped the sample out on a tray and separated the BMIs from the substrate by searching the

tray and placing all BMIs in a jar with 95% ethanol. The same sampling effort was used for all sites.

IDENTIFYING BMIS SPECIES RICHNESS AND BIOINDICATOR VALUES

BMIs were identified to the family level using a dissecting microscope and information from the Biological Monitoring Working Party modified for Costa Rica (BMWP-CR) and with the help of Monika Springer from the University of Costa Rica. With this, each family is given a Family-level Biotic Index (FBI) number 1 through 10 that represents the water quality that they are found in (higher numbers being from higher quality water). I then summed the FBI number of each family identified from each site to assess water quality based on the level of quality given by BMWP-CR.

CONSTRUCTING FOOD WEBS

By using Internet and journal sources, I was able to find the diet of each specimen collected to construct a food web from each location based on that information. Food webs were compared on the following criteria: species richness, similarity, total links, connectance, linkage density, fraction of omnivores, fraction of basal families, intermediate families, and top families, and prey to predator ratio.

Results

IDENTIFYING BMI SPECIES RICHNESS AND BIOINDICATOR VALUES

From the forested site, 51 BMIs were collected from 19 different families (fig. 2). This site had a BMWP-CR bioindicator of stream quality value of 114, which is in the level of non-contaminated, good quality water. I collected 86 BMIs from the Cheese Factory site that encompassed 11 different families (fig. 3). The BMWP-CR bioindicator value there was 42, which is in the level of poor quality, contaminated water. At the site in town, 204 BMIs were collected, but only from a single family that had a BMWP-CR bioindicator value of 2, is in the level of extremely contaminated, very poor water quality (fig. 4). At the fourth site beneath the culvert and by a few houses, 45 BMIs were collected from 11 different families that gave the site a BMWP-CR bioindicator value of 51, also in the category of contaminated, poor water quality (fig. 5). There were significant differences in number of individuals collected from each site, number of families collected from each site, and bioindicator of stream quality value from each site (Chi-square: individuals: $X^2 = 169.833$, d.f.= 3, $p < 0.001$; families: $X^2 = 23.5$, d.f.= 3, $p < 0.001$; water quality: $X^2 = 123.344$, d.f.=3, $p < 0.001$).

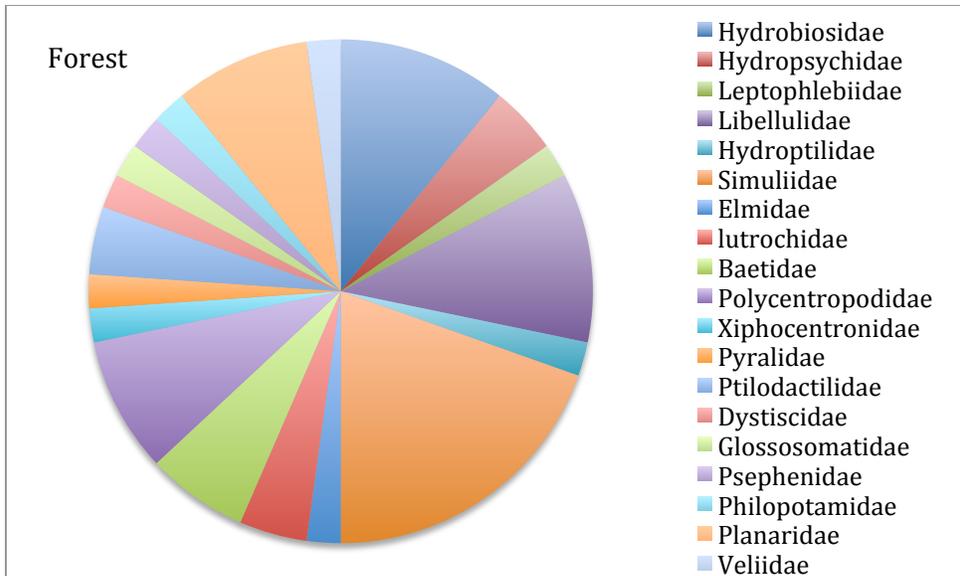


Fig. 2. Family richness and evenness in a montane tropical forest stream ecosystem. A total of 51 BMIs were collected from the site representing 19 families.

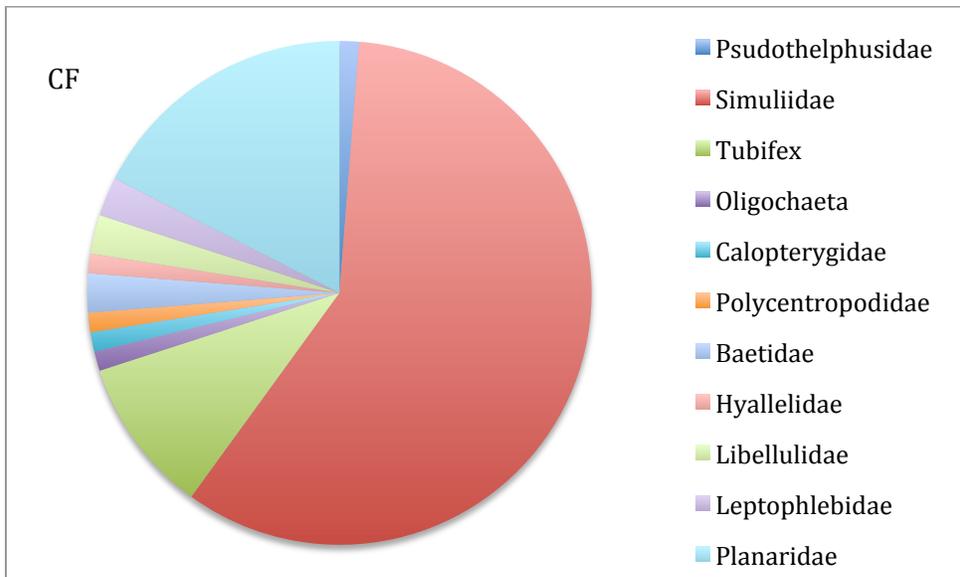


Fig. 3. Family richness and evenness in a montane tropical stream directly underneath the Monteverde Cheese Factory. A total of 86 BMIs were collected from the site from 11 families.

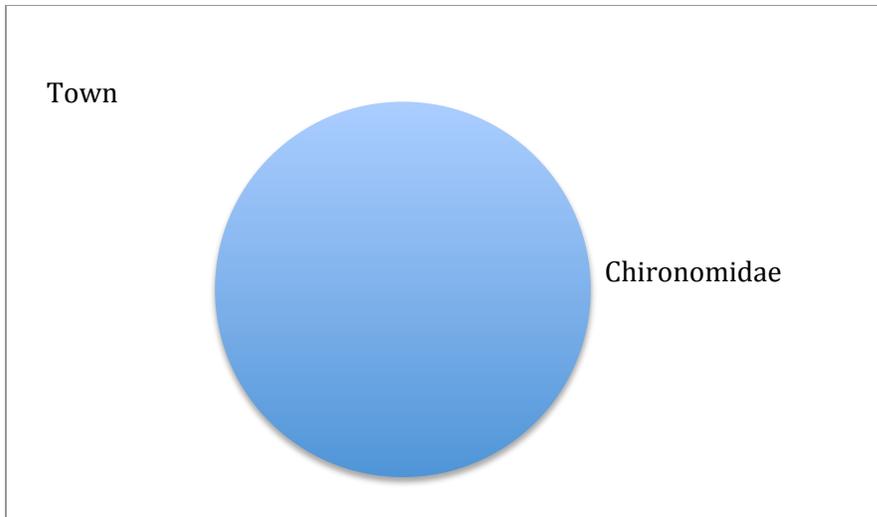


Fig. 4. The family richness and evenness in a montane tropical stream at a site located in the town of Santa Elena, Costa Rica. 204 BMIs were collected from a single family.

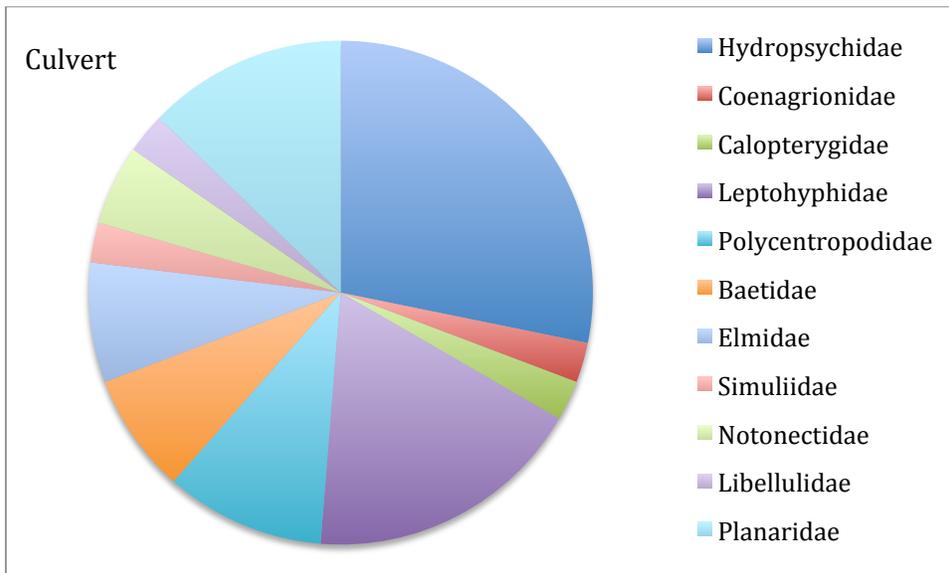


Fig. 5. Family richness and evenness in a tropical montane stream in Monteverde, Costa Rica. 45 BMIs from 11 families were collected from this site located near a road and a few houses.

CONSTRUCTING FOOD WEBS

The difference in diversity was strongest between the forest and town sites (Shannon index $t=20.10$, $d.f.=47$, $p<.0001$), but was significantly different between all sites. The other food web characteristics were analyzed using a jackknife pseudo-value procedure where pseudo-values are taken for each food web by randomly eliminating a family and testing how that affects the parameter of interest. This is done for the number of families for each food web. The pseudo-values are used in place of the original values to estimate the parameter of interest and their standard deviation is used to estimate the standard error of the parameter, which is used for null hypothesis testing for computing confidence intervals. These means

are then compared using ANOVA and post hoc Tukey comparisons, if necessary, to test if they are significantly different with a 95% confidence interval.

The biggest differences in the tested food web characteristics came between the town site and the other three. The forest had significantly more links than the other sites, and town had significantly fewer links than the other sites (fig. 6). Connectivity was highest in the forest, but was not significantly different between the four sites (ANOVA $f = 0.80$, $d.f. = 3$, $p = 0.49$; fig. 7). The food web from town had a significantly lower linkage density than the other three sites and the forest had the highest, although not significantly so over the Cheese Factory or the culvert (fig. 8). The town food web had significantly lower fraction of omnivores than the forest and Cheese Factory sites, but not significantly lower than the culvert site (fig. 9). There was no statistical difference between the ratio of basal to intermediate to top families between the four sites (ANOVA: fraction basal, $F = 1.52$, $d.f. = 3$, $p = 0.221$; fraction intermediate, $F = 0.311$, $d.f. = 3$, $p = 0.82$; fraction top, $F = 1.79$, $d.f. = 3$, $p = 0.156$) (fig. 10). The prey to predator ratio was highest in the town food web, but was not statistically significant between the four sites (ANOVA: $F = 0.11$, $d.f. = 3$, $p = 0.954$) (fig. 11).

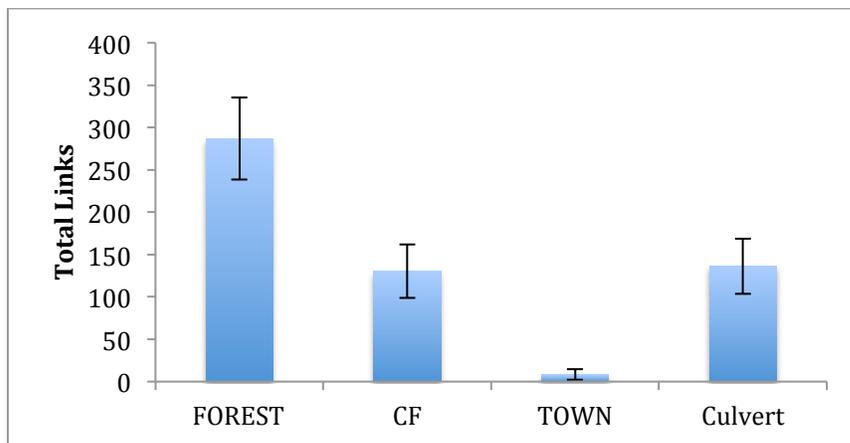


Fig. 6. Total food web links for four different stream sites in Monteverde, Costa Rica. There is significant difference between the forest food web and the others and between the town food web and the others (Tukey contrasts: FOREST-CF: $p < 0.001$; TOWN-CF: $p = 0.004$; Culvert-FOREST: $p < 0.001$; TOWN-FOREST: $p < 0.001$; TOWN-Culvert: $p = 0.003$, the other comparisons had $p > .05$). Samples were taken from each site where they were identified to the family level and a food web was then created. A 95% confidence interval is given.

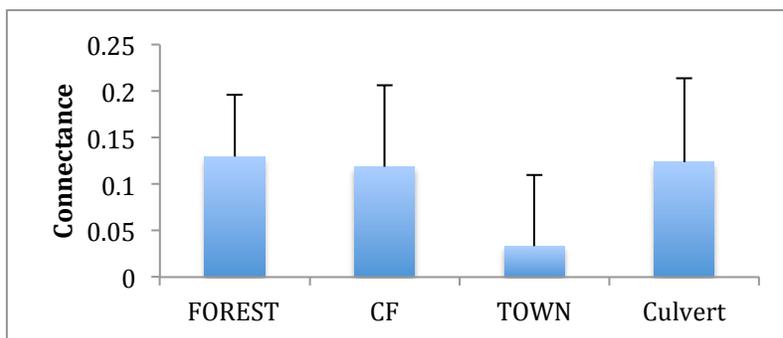


Fig. 7. Food web connectance for four tropical stream locations that experience different levels of pollution. There is no significant difference in connectance (ANOVA: $F= 8.01$, $d.f.= 3$, $p=0.497$). Samples of BMIs were taken from each site, were identified to the family level, and a food web was created. A 95% confidence interval is given.

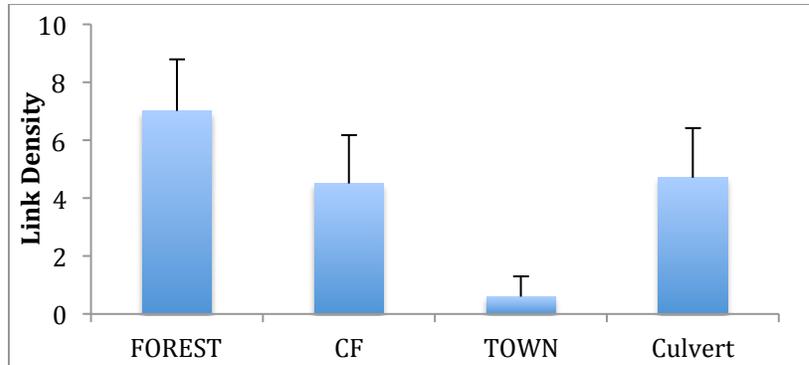


Fig. 8. Linkage density of four food webs from different tropical stream sites that experience different amounts of pollution. The TOWN location has significantly less linkage density than the other three with a 95% confidence interval (Tukey contrasts: TOWN-CF: $p= .049$; TOWN-FOREST: $p<0.001$; TOWN-Culvert: $p= 0.035$, the other comparisons had $p>.05$). BMI samples were taken from each site, identified to the family level, and food webs were created.

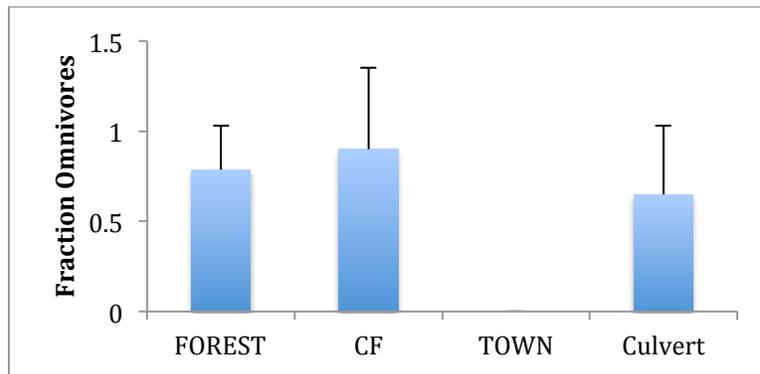


Fig. 9. The fraction of omnivores in the food web at four different tropical stream locations that experience different levels of pollution. TOWN had a significantly lower fraction of omnivores than the FOREST and Cheese Factory with a 95% confidence interval, but not the culvert (Tukey contrasts: TOWN-CF: $p=0.01$; TOWN-FOREST: $p= 0.02$). BMI samples were taken from each site and were identified to the family level so that food webs could be created.

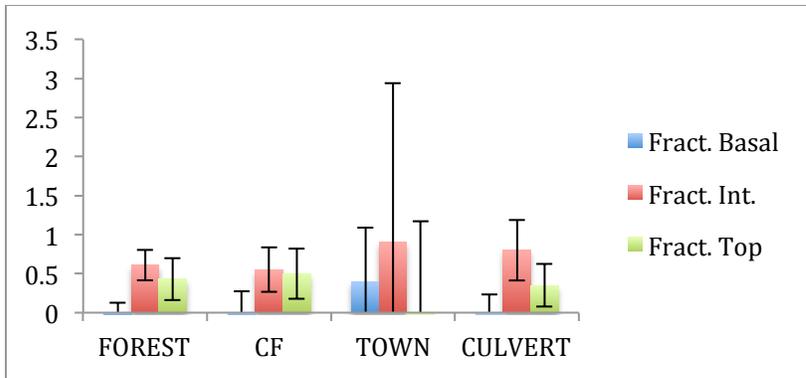


Fig. 10. The fraction of basal, intermediate, and top families in four different tropical stream food webs. There is no statistical difference between the food webs with a 95% confidence interval. BMI samples were taken from each of the four sites that experience different amounts of pollution, they were identified, and a food web was created.

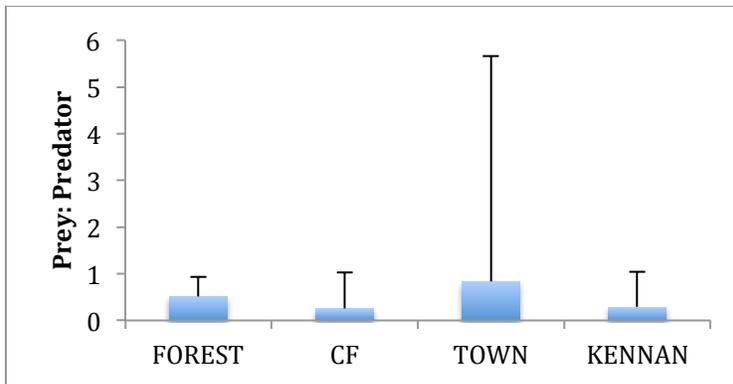


Fig. 11. The ratio of prey families to predator families in four different tropical stream food webs that were from four locations that experience different amounts of pollution. There was no statistical difference between the sites based on a 95% confidence interval. BMI samples were taken from each site where they were identified to family level and food webs were created.

Discussion

These findings support other research that suggests anthropogenic pollution affects stream food webs (Yule *et al.* 2010, Henley *et al.* 2010, and Jordán *et al.* 2012). The stream water quality was affected by the sources of contamination, which lowered species richness, similarity, number of links, and linkage density. Similar trends can be found in streams in Borneo, Indonesia (Yule *et al.* 2010). As the number of species declines, so does family diversity, and the food web becomes simpler as there are less species interacting.

Fraction of omnivores was also affected by contamination, which is a new finding, and suggests that with increased pollution, the percentage of species that can feed at more than one trophic level is greatly reduced. This is likely because there are fewer species present, making it less likely that a species can predate organisms from different trophic levels simply because there are less options. It could also be because there is less species richness but greater individual species

abundances, making it beneficial for species to become more specialized on what is most abundant and ignore other options at different trophic levels. Connectance, basal, intermediate, and top families/species, and prey to predator ratio in the streams were not significantly affected by contamination, but the town site did show trends. These data suggest the certain food web functions that relate to feeding guilds and trophic level interactions are not significantly affected by contamination. This is likely because there are ecological equivalents of pristine species that fill the roles of filters, grazers, shredders, and predators and can tolerate some contamination and maintain the structure of the food web, just with less overall species present.

I believe that there is still a strong trend toward lots of contamination having an affect on prey to predator ratio, which turned out not statistically significant. Contamination in the town site resulted in only one family being found, making them the only predator. With fewer species preying on the basal food web and no predatory species, the prey to predator ratio should increase when contamination is really bad.

My results were definitely strongest for the town location compared to the other three sites in the affects of contamination of stream food webs. This site had by far the murkiest water and slimiest substrate, and being right next to a heavily used paved road means that when it rains, oil and other substances easily runoff into the stream. Increased turbidity and changes in substrate are directly influenced by contamination (Henley *et al.* 2010, Jordán *et al.* 2012, and Crunkilton & Duchrow 1990). Evident oil and other particles make the water more turbid so that less primary production can take place (Henley *et al.* 2010, Crunkilton & Duchrow 1990). This likely had a cascading effect on other trophic levels as fewer and fewer different species could tolerate contamination or survive with less food sources. The oil runoff possibly is what also made the substrate so stringy and slimy instead of sandy and rocky like the other sites because oil can stay in riffle substrate for over 450 days (Crunkilton & Duchrow 1990). However, it was the sample that acquired by far the most individual organisms, and since they were all from one family (Chironomidae), it shows that the food web and species composition was dramatically altered due to stream contamination. Crunkilton and Duchrow (1990) found that Chironomidae was one of three families that dominated an oil-polluted stream in Missouri. Chironomidae is likely the only family in Monteverde that can survive and thus thrive in the town waters that experience consistent oil runoff. Thus, lower productivity leads to lower richness and evenness, which shortens the food chain and reduces the number of links and link density. This means contamination makes a simpler food web with less species interactions, making it more susceptible to further environmental stresses.

The other three sites did not appear to change substrate from contamination. Still, runoff from the road (culvert) and pollution from the Cheese Factory created changes in the food web. These sites do not appear to be contaminated to the point that primary productivity is altered much because they are still relatively complex and have as many trophic levels as the pristine forest site, though with fewer overall species and links. So, they are clearly impacted in a negative way, but to a lesser degree than the town site. It is more likely that they are contaminated to a point

that certain species cannot handle the contamination, but there are still other grazers, shredders, filters, and predators that can survive and function at these levels of contamination for the good of the food web as a whole. However, the data suggests that there is a point at which a stream becomes too contaminated and only a few species can survive, and at this point the food web becomes seriously degraded as feeding guilds and trophic roles are lost.

Contamination affects water quality, but also the species present and the food. Thus, contamination affects the whole ecosystem. Stream food webs are very fragile when it comes to species diversity and composition and the many different ways species interact (Yule *et al.* 2010, and Crunkilton & Duchrow 1990). Local extinctions alter the entire community, and with many different BMIs in tropical streams, contamination in streams likely causes global extinctions. Stream food webs are fragile because the data show that being next to a road with a lot of runoff can degrade the water to a point that hardly any species can survive. Lower diversity makes the system more susceptible to further environmental changes that might occur, such as climate change, loss of canopy cover, or further contamination. Stream food webs also show resilience however in feeding guilds and trophic level interactions being maintained through some contamination. A productive food web can exist in the presence of contamination, which is important because many streams are contaminated because of proximity to human development and land use. Increasingly ecosystems must adapt to anthropogenic stress, so to know that many food web functions can sustain through some contamination is good for the ecological communities nearby streams. This is incentive to conserve still pristine streams because they are important sources of biodiversity and provide food and clean water for people and other animals all over the world. It is also incentive to restore degraded streams to a point that they can still function on all trophic levels. In Monteverde specifically, planting trees as buffers by the stream site in town or having a drainage/filtration system that makes sure not so much road runoff is contaminating the streams are two ways to restore the stream water quality to a point that feeding guilds and upper trophic levels can return. It would also improve the aesthetic beauty of town. Tropical montane streams and fresh water sources worldwide are at risk of decreasing the ecosystem services that they provide because of contamination from human sources. Conservation and restoration are vital for maintaining stream food web functioning, biodiversity, and clean water.

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Climate warming on the altitudinal ranges of tropical montane hummingbirds in open and forested areas

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ABSTRACT

Anthropogenic climate change is already having undeniable effects on the natural world. One of the most pronounced is upward shifting of thermal zones in tropical montane areas, causing range shifts for many species. Forest fragmentation caused by expanding infrastructure is also affecting tropical species, creating edges in otherwise pristine forest. This study examines effects of open vs forest sites on altitudinal range shifts for hummingbirds in tropical montane rainforest near Monteverde, Costa Rica, at 1500-1800m in altitude, a comparison which has not been done before. Hummingbird feeders were hung at 100 vertical m intervals and observed to construct distributions estimates. Comparisons were made between open and forest distributions, then compared to a 2001 data set and predicted relative abundances from 1993. Species identified were: Violet Sabrewing, Green Violet-ear, Green Hermit, Green-crowned Brilliant, Purple-throated Mountain Gem, and Stripe-tailed Hummingbird. The high-mountain endemic Fiery-throated Hummingbird, supposedly common in elevations sampled, was absent. When compared to 2001 data, it was found that only the Stripe-tailed Hummingbird had entirely different mean elevations, which were significantly lower in both open and forested areas. When compared to relative abundances, all species showed either no movement or had distributions skewed towards the lower life zones they were expected to inhabit. However, the Green Violet-ear was found at 1600m in elevation, higher than top elevations identified in previous studies. My average elevation was found to be 1527m, whereas past studies found it to be 1505m. Other species average elevations were between 1541m and 1605m for species in open areas and 1590m and 1640m for forested areas. Trends suggested that forest populations of species identified had slightly higher elevational ranges than their open area counterparts. Overall, it appears that hummingbirds in this area are only experiencing minor changes in habitat, and that forest and open areas may respond differently to anticipated future warming.

RESUMEN

El cambio climático por causas antropogénicas tiene actualmente un efecto innegable en el mundo natural. Uno de los más pronunciados es el desplazamiento hacia arriba de las zonas termales en las áreas tropicales montañosas, causando desplazamientos de rango en muchas especies. La fragmentación del bosque causada por la expansión de la infraestructura está

afectando también las especies tropicales, creando bordes en de otra manera bosques prístinos. Este estudio examina el efecto de sitios con bosque o abiertos en el desplazamiento altitudinal de colibríes en el bosque tropical montano en Monteverde, Costa Rica, a una altitud de 1500-1800 m, una comparación que no se ha hecho con anterioridad. Comederos de colibrí se colgaron a intervalos de 100 m verticals para determinar estimados de distribuciones. Se hicieron comparaciones entre las distribuciones en bosque y áreas abiertas, y luego se compararon con datos del 2001 y abundancias relativas predichas de 1993. Las especies identificadas fueron: el Ala de sable violáceo, el Colibrí orejivioláceo Verde, el Brillante Frentiverde, el Colibrí Montañés Gorgimorado, y el Colibrí Colirrayado. El endémico de montaña Colibrí Garganta de Fuego, supuestamente común en las elevaciones muestreadas, estuvo ausente. Al comparar con los datos del 2001, se encontró que solo el Colibrí Colirrayado tiene elevaciones medias totalmente diferentes, las cuales fueron significativamente menores tanto en el bosque con en el área abierta. Al comparar las abundancias relativas, todas las especies mostraron ningún movimiento o tuvieron distribuciones sesgadas hacia la zona de vida más baja en las que se esperaba se encontrarán. Sin embargo, el Colibrí Orejivioláceo se encontró a 1600 m de elevación, más alto que elevaciones encontradas en estudios previos. Mi elevación media fue de 1527 m, mientras que en estudios anteriores fue de 1505 m. Otras especies tuvieron en promedio elevaciones entre los 1541 m y 1605 m para especies en el área abierta y 1590 m y 1640 m para área boscosa. Las tendencias sugieren que las poblaciones de especies en el bosque tienen rangos levemente mayores que sus contrapartes en el área abierta. En total, parece que los colibríes en esta área están experimentando cambios menores en el hábitat, y que las áreas abierta y boscosa pueden responder de manera diferente para el calentamiento futuro anticipado.

INTRODUCTION

The latest Intergovernmental Panel on Climate Change (IPCC) report has shown that anthropogenic climate change has already begun to affect natural ecosystems. The primary effect of these warming trends is the alteration of geographic ranges for many species, both plant and animal (IPCC 2014). This is particularly apparent in tropical ecosystems, where many species occupy very specific thermal niches (Sekercioglu et al 2008, Sekercioglu et al 2012, Freeman & Class-Freeman 2014). Warming forces these species either into higher latitudes or higher altitudinal ranges (Colwell et al 2008, Feeley et al 2013), patterns that can only go on so long before running out of space (Sekercioglu et al 2012, Freeman & Class-Freeman 2014). Altitudinal range shifts can also be problematic for species that have developed important interspecific relationships, especially those between plants and animals who may depend on each other for food, pollination, or dispersal (Parmesan 2006, Hegland et al 2009). Restricted geographic location has been shown to correlate strongly with extinction risk, making tropical montane species more vulnerable than most (Sekercioglu et al 2008). Recent studies have also shown that climate warming and resulting range shifts are happening much faster than originally thought, adding an element of urgency to understanding the ecosystem and assessing the potential impacts (Freeman & Class-Freeman 2014).

Another process having a major effect on tropical ecosystems is deforestation and the expansion of human infrastructure, creating more open and fragmented areas (Lewis 2009, Tabarelli et al 2012). In many cases, fragmentation compounds the problems created by warming trends by creating geographic barriers that may prevent species migration as a response to temperature

zone shifts (Odpam & Wascher 2004). It also causes increased edge effect, which affects the species composition of the area (Klapwijk & Lewis). Some more cosmopolitan species can adapt easily to living in such fragments, but species that have limited geographic ranges or are endemic, such as those in mountainous areas, are more effected (Tabarelli et al 2012). Feinsinger et al (1987) suggested that communities in disturbed areas may have looser species interactions, which may affect their rates of upward movement when compared to rates of upward movement in forest dominated areas. However, the effects of fragmentation and climate change together have not been well studied (Klapwijk & Lewis). This study will attempt to discern if there is a difference in range shifts between open and forested areas using birds, which are excellent indicators of warming due to being well documented in the past (Sekercoiglu et al 2012). Hummingbirds were used as study subjects because they would visit feeders placed in each area.

The lower-montane wet forest and lower-montane rain forest areas near Monteverde, Costa Rica, provide a mountain environment with sufficient altitudinal gradient to assess the altitudinal migration of bird species, both along an open area and a forested area. Some studies in the area have already found evidence of range shifts both in bird communities overall and specifically in hummingbirds (Pounds et al 1999, Lynn 2001, Connette 2006, Wallace 2013). The most recent of these found that one species in particular, the Purple-throated Mountain Gem, was moving upwards (Wallace 2013), while another found that the Green-crowned Brilliant showed significant migration as well (Lynn 2001). These results were all found in forested areas, with no transect for comparison in a more open area. No studies could be found concerning the comparison in range shifts of hummingbirds in open areas vs forested areas. Past studies of bird responses to fragmentation suggest that hummingbirds in tropical dry forest may be capable of adapting to fragments and secondary forest (Lasky & Keitt 2010), but that closed forest areas have both greater abundance and species richness than open areas (Bozeman 2013, Garcia 2013).

This study will repeat a forest transect in a similar area to previous forest transects in addition to analyzing a more open area along the same altitudinal gradient. This will allow for potential comparisons of the current elevational ranges of species and past ranges, in addition to differences in the rates at which species are migrating upwards based on their habitat.

METHODS

STUDY SITE

This study assessed presence and abundance of hummingbird species along an altitudinal gradient near a small walking trail in comparison to a larger road. The open transect was located on the Cerro Amigos road leading to the TV towers at the continental divide. It was a cleared road that received traffic from both people and four-wheelers that showed an obvious alteration to the landscape (Fig. 1). The forest transect was along small walking trails; the Principal Trail on the Monteverde Biological station from 1500 to 1700m, and a trail along the continental divide leading to the TV towers through Monteverde Cloud Forest Reserve property for the 1800m point. All points were at least 100m from the edge of the forest. Both transects extended from 1500m to 1800m in elevation. Each transect consisted of 4 points: 1500, 1600, 1700, and 1800m. The 1500 point on the open transect was re-located to 1530m after feeders were stolen at the 1500m point. Both transects were located near Monteverde, Costa Rica. Elevations 1500 and

1600 fall in the lower-montane wet forest category, while 1700 and 1800 are lower-montane rain forest according to Holdridge life-zone classification system (Haber 2000). The forest transect was in primary forest, while the open transect included both primary and secondary forest. Sampling occurred in the wet season, from October 24th through November 13th.

STUDY ORGANISMS

Based on previous studies, the six species of hummingbird expected to be found in this study were the Violet Sabrewing, Stripe-tailed Hummingbird, Purple-throated Mountain Gem, Green Hermit, Green Violet-ear, and Green-crowned Brilliant. Of these, the Violet Sabrewing and Green Hermit prefer understory and edge areas, while the Stripe-tailed Hummingbird, Green-crowned Brilliant, and the Purple-throated Mountain Gem prefer forest areas but visit gaps frequently for the purpose of foraging. The Green Violet-ear prefers open areas (Stiles & Skutch 1989). All species have shown migrations to lower habitats after the breeding season, but all species except the Violet Sabrewing were near the beginning of their breeding seasons during the observational period. Therefore, they should have been at the highest limits of their ranges during the study. Sabrewings breed from May or June through October (Stiles & Skutch 1989). Additionally, it was possible that the Magenta-throated Woodstar, Canivet's Emerald, or Rufous-Tailed Hummingbird, all of which prefer edge or scrub habitat, would move upward and be detected in the lower points on the open transect (Stiles & Skutch 1989, Fogden 1993).

GENERAL APPROACH

Sampling procedure for this study was similar to that of Lynn (2001) and Wallace (2013). At each point, a string was hung 1-2m above the ground with 3 hummingbird feeders arranged along it (Fig. 1). Feeders were approximately 20-30 cm apart on the line. At 1700 and 1800m points on the open transect, feeders were staked out between two aluminum poles to discourage coatis. When feeders were hung in trees, coatis tended to take them down in order to drink the sugar water. Additionally, only one feeder was left between observations and one more was added during observation periods. At 1500m on the tower road, feeders were tied directly to a slanted tree (Fig. 1). The lines of feeders were not more than 8 meters off the trail or road. Each feeder was filled with a 20% mass by volume sugar-water solution, which was refilled every 4-5 days. Feeders were set out 2 days before sampling began so that hummingbirds had time to find them.

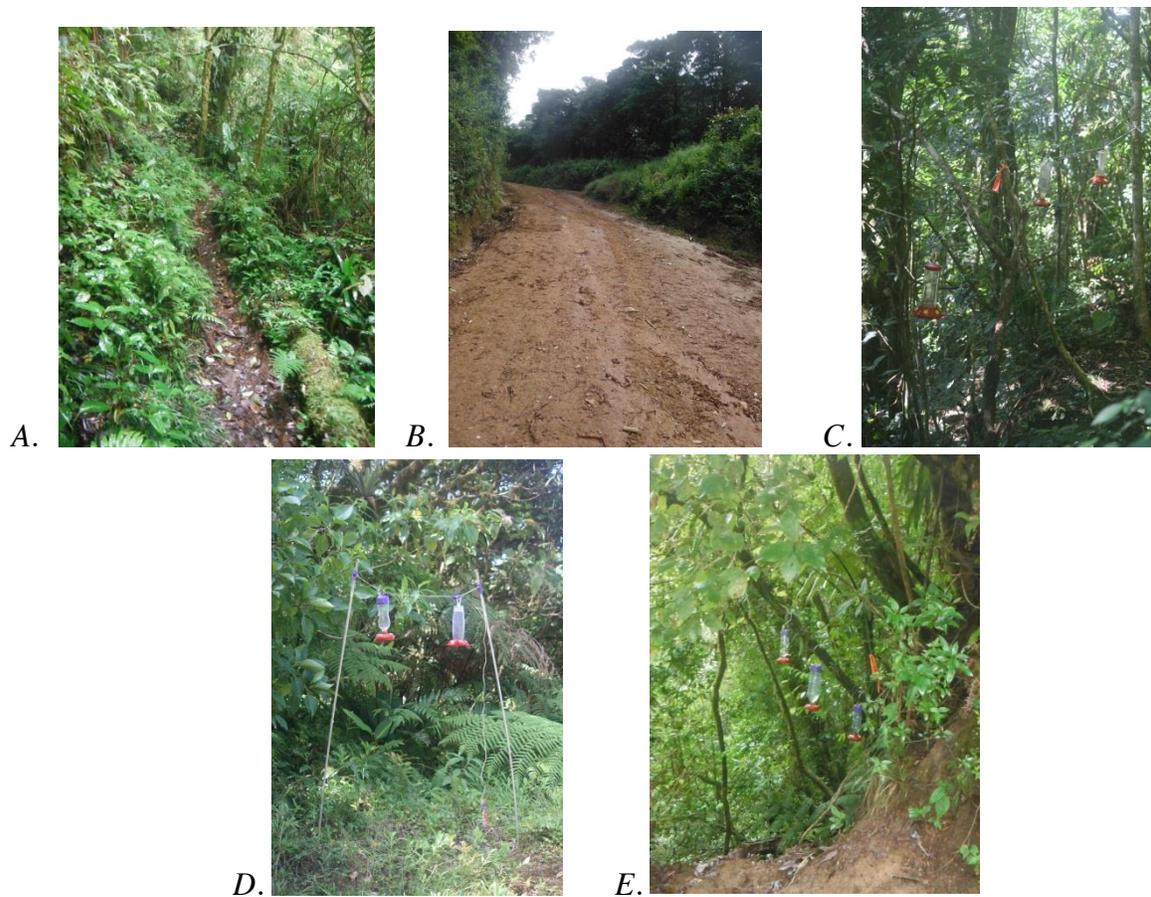


FIGURE 1. *Images of study sites used to compare hummingbird populations in forested vs open areas in tropical montane wet and rain forest near Monteverde, Costa Rica. A) Trail along which forested transect was run. B) Road used for open transect. C) Example of forest transect with feeders hung on string between trees. D) Example of feeders hung on aluminum poles to avoid coati damage. E) Example of feeders hung directly on tree.*

Data collection began between 0630 and 0720. The first point sampled was rotated on a day to day basis to eliminate potential bias due to time of day. Observations were 30 minutes long and occurred from a distance of 4-5 meters. Sampling was occasionally conducted in light rain or misty conditions, especially towards the upper reaches of both transects. In most cases, one transect was sampled per day. Each transect was observed 6 times. At the 1700 and 1800 plots of the open transect, two thirty minute observations were made in one hour-long period to make up for missed observations due to coati damage. Hummingbirds were identified as they approached the feeders and the number of visits by each species was recorded. If a hummingbird perched within sight after feeding, then returned to the feeder, it was not double-counted.

RESULTS

A total of 420 hummingbirds were identified, 156 on the forested transect and 264 on the open transect. No significant difference in mean elevation was found for Green-crowned Brilliants (Kruskal-Wallis Test= 3.321, df=2, p=0.19) and Violet Sabrewings (Kruskal-Wallis Test=4.486, df=2, p=0.11). Green Hermits and Purple-throated Mountain Gems showed differing mean

elevations between data sets (Kruskal-Wallis Test=8.411, df=2, p=0.015 and Kruskal-Wallis Test=6.844, df=2, p=0.033 respectively) The main pairwise difference for both, calculated using a randomization procedure to find p-value, was that open transect was significantly lower than 2001 forest observations (Green Hermit p=0.007, Purple-throated Mountain Gem p=0.021, Fig. 2). The Stripe-tailed Hummingbirds showed significantly different mean elevations for all three sets of observations; open being less than forest 2014, which was less than forest 2001 (Kruskal-Wallis Test=17.838, df=2, p<0.0001, p<0.02 for all pairwise comparisons, Fig. 2). No upward trend was identified for recent observations when compared to past data. Although not significant, there was a trend toward populations in forested areas having slightly higher mean elevations than their open area counterparts.

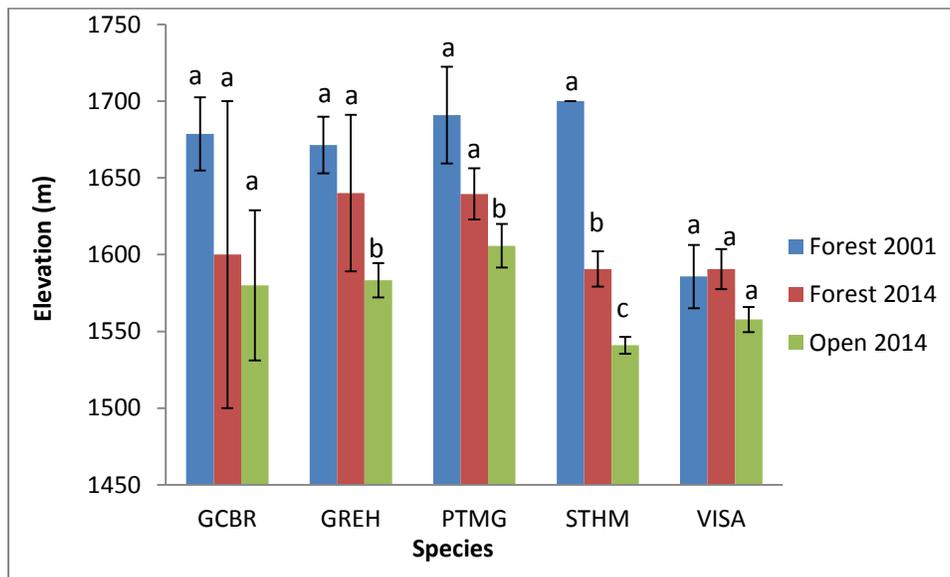


FIGURE 2. Mean elevation with standard error for 5 species of tropical montane hummingbird detected near Monteverde, Costa Rica in forested and open areas. Significant differences in mean elevation are marked by differing letters. Abbreviations are as follows: Green Hermit (GREH), Purple-throated Mountain Gem (PTMG), Stripe-tailed Hummingbird (STHM), Green-crowned Brilliant (GCBR), and Violet Sabrewing (VISA). Pairwise post-hoc comparisons using randomization to calculate p-value were used to find significant differences, which are marked by differing letters. Significance was indicated by a p-value of less than 0.05. Observations were made at hummingbird feeders hung at 100m elevational intervals and took place between October 24th and November 13th, 2014.

Frequency distributions along elevations were compared with Fisher exact tests (contingency tables) for each species for the two recent transects and the forest transect from 2001 (Fig. 3). The distribution of the Stripe-tailed Hummingbird in open habitats was found to be proportionally different from 2001 distribution due to the higher numbers of birds found, especially lower elevation (Fisher test, p<0.001). All other distributions were found to have no significant difference from those found in 2001 (Fisher test, p>0.12 for all).

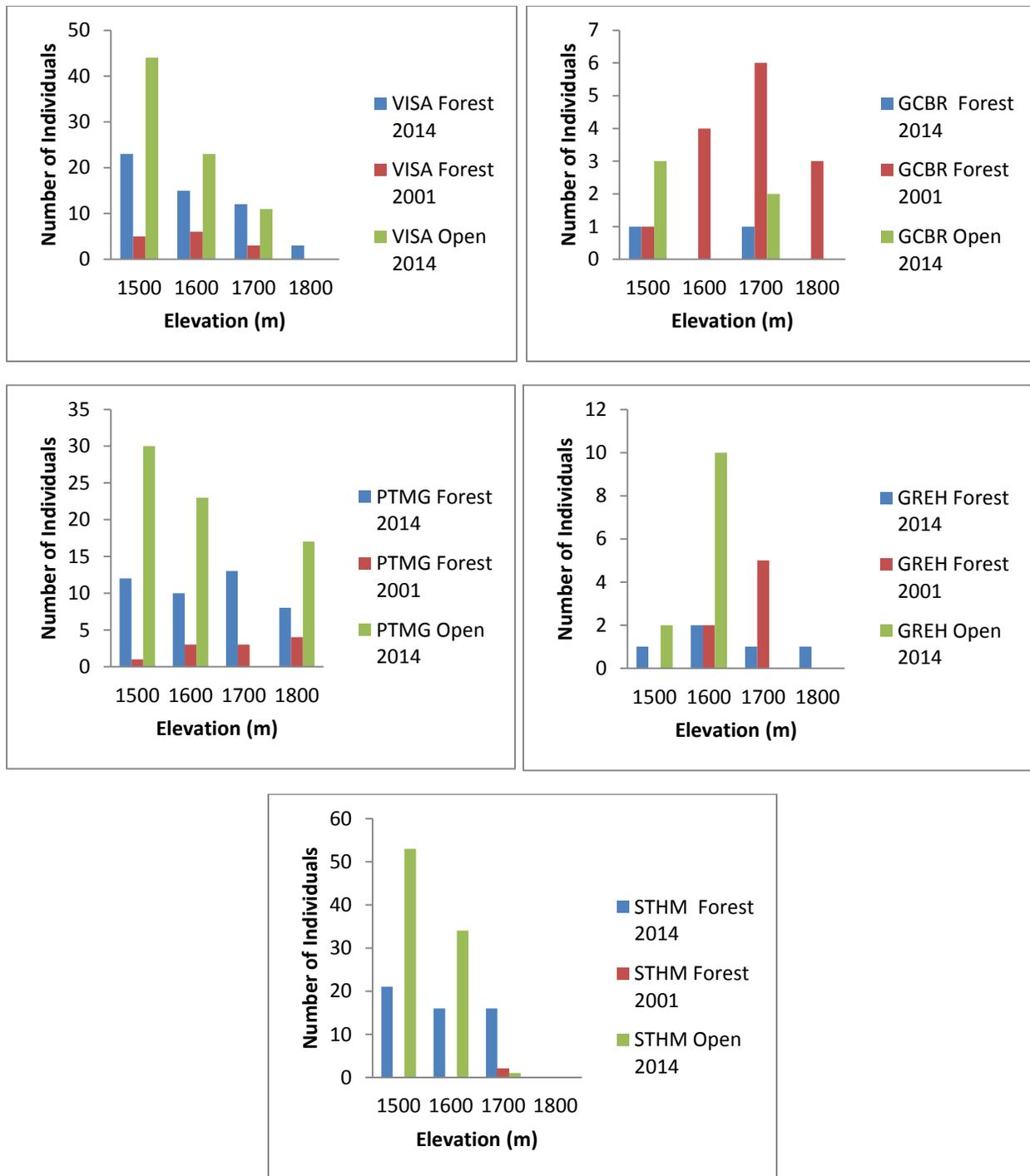


FIGURE 3. *Individuals of 5 hummingbird species observed at hummingbird feeders hung at differing elevations in 2014 forest and open transects, in addition to a 2001 forest transect. Sampling for 2014 occurred near Monteverde, Costa Rica between October 24th and November 13th. Abbreviations as in Figure 2.*

Relative abundances were also compared with those predicted by Fogden (1993), which assigned a commonality score for each species in both pre-montane wet and pre-montane rain forest. To calculate Chi-square values for the predicted descriptions, each relative abundance was assigned

a number: 5 for Common, 4 for Fairly Common, 3 for Uncommon, 2 for Rare, and 1 for Absent. Observed values were compared to expected values in terms of proportion of total birds identified in each life zone (Table 1). All significant differences were due to observed relative abundances being more heavily skewed towards lower zones than predicted relative abundances. Green Hermits, Purple-throated Mountain Gems, Stripe-tailed Hummingbirds, and Violet Sabrewings in open areas were all significantly different then the predicted relative abundances (Table 2). Stripe-tailed Hummingbirds and Violet Sabrewings in forested areas also had different relative abundances than those predicted (Table 2).

TABLE 1. *Example of how Chi-square values were calculated for comparison to Fogden (1993) relative abundances. Expected values were based on the proportion of observed birds that would be present in each life zone should the relative abundances be correct. All observed data was collected near Monteverde, Costa Rica between October 24th and November 13th, 2014.*

Violet Sabrewing (Forest)		
	Lower Montane Wet Forest	Lower Montane Rain Forest
Fogden Relative Abundance	C	C
Observed	38	15
Expected	53*(5/10)=26.5	26.5
Chi Square = 9.981, df=2, p=0.0016		

TABLE 2. *Results of Chi-square tests comparing observed relative abundances of 6 species of montane hummingbird in two life zones to those predicted. Predicted distributions were created by assigning a number to relative abundances for lower montane wet forest and lower montane rain forest given by Fogden (1993) and compared to observed proportions. Observations were made between October 24th and November 13th, 2014 near Monteverde, Costa Rica. For site, F=forest and O=open.*

Species	Site	Chi-square	df	p-value
GCBR	F	0	1	1
GCBR	O	0.2	1	0.6547
GREH	F	0.2	1	0.6547
GREH	O	12	1	0.0005
GREV	O	2.195	1	0.1384
PTMG	F	0.023	1	0.8788
PTMG	O	18.514	1	0.0001
STHM	F	8.321	1	0.0039
STHM	O	84.045	1	0.0001
VISA	F	9.981	1	0.0016
VISA	O	40.025	1	0.0001

Green Violet-ears were found in this study, at 1500 and 1600m on the open area transect. This was higher than they had been found in previous studies (Fig. 4) but was not significantly different from the predicted relative abundance (Table 1).

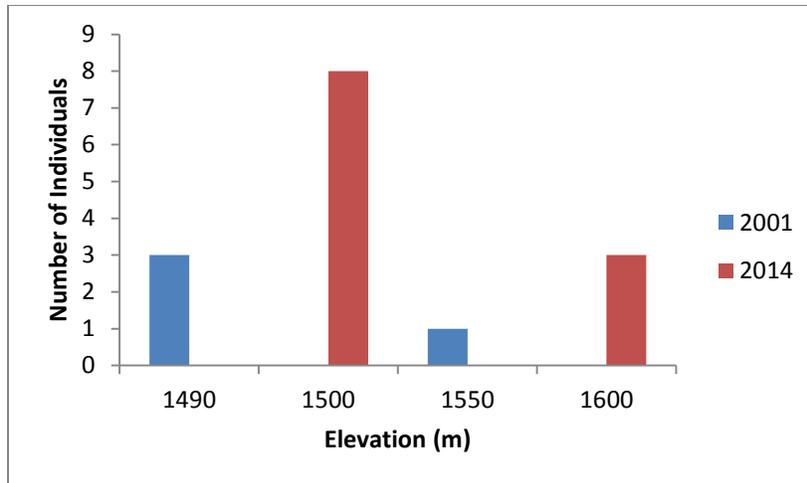


FIGURE 4. Elevations at which Green Violet-ears were found in tropical montane wet forest near Monteverde, Costa Rica. Both sets of observations were completed using hummingbird feeders. 2001 observations were conducted in a forested area and 2014 observations are from an open area.

ADDITIONAL OBSERVATIONS

One species that was notably absent was the Fiery-throated Hummingbird, which is high-mountain dwelling and endemic to southern Central American mountains. The Canivet's Emerald, Rufous-tailed Hummingbird, and Magenta-throated Woodstar were also absent. Results at 1700m on the open transect were difficult to obtain because the feeders were torn down by coatis three times during sampling. Feeders at 1800m on the open transect were stolen once and taken down by coatis twice. Weather at 1800m points for both transects was usually somewhat poor, with mist or rain. Both 1800 and 1700m points on the open transect were typically windy. At some sites, especially at 1500 and 1600m on the open transect and 1500m on the forested transect, Violet Sabrewings exhibited territorial behavior and drove other species away from feeders.

DISCUSSION

Overall results show no upward movement in the altitudinal ranges of the hummingbirds found in this study when compared with data from 2001 and relative abundances from 1993. The primary significant difference is for the distribution of the Stripe-tailed Hummingbird, which was found at higher elevations in 2001 than for both forested and open plots in 2014. However, only 2 Stripe-tailed Hummingbirds were found on the forested transect in the 2001 study, whereas this study found 88 along the open transect and 53 in forested areas. One possible explanation for this is that Lynn's study in 2001 occurred during mid to late April, which is at the end of the breeding season for Stripe-tailed Hummingbirds. After breeding, some migrate to lower elevations, which may have caused them to be less common. Results of this study are more consistent with those of Barry (2012) and Wallace (2013), who both found the mean elevation to be less than 1600m.

This study also shows a trend towards species in forest areas having slightly higher mean elevations than their open area-dwelling counterparts. This could be explained by the increased wind in the open areas which is channeled by the road, especially at higher elevations (personal observation). Wind may have the effect of both making it difficult for hummingbirds to feed and making it colder overall, which may push cold-sensitive species further down in elevation. Data could also have been influenced by the presence of coatis near feeder locations on the higher plots of the open road. When coatis dragged feeders down, hummingbirds were unable to locate them, for possibly as much as one to two days. This may have caused hummingbirds to check these feeders less often. Despite the fact that feeders were observed for an equal amount of time, it is possible that they received less visits than feeders that were not affected by coatis because they were not as well known by hummingbirds in the area.

Comparisons between predicted relative abundances also did not yield any indication of upward altitudinal shifts. Significant differences in relative abundances (Violet Sabrewings and Stripe-Tailed Hummingbirds in open and forested areas, Purple-throated Mountain Gems and Green Hermits in open areas) were all lower than the predicted distribution, primarily due to higher numbers of individuals counted at 1500 and 1600m (lower montane wet forest) than at 1700 and 1800m (lower montane rainforest). Fogden (1993) predicted even relative abundances.

Another species to note from this study was the Green Violet-ear, which was found at 1600 and 1500m on the open transect. Previous studies have found Violet-ears at 1490, 1500, and 1550m, but never at 1600m (Lynn 2001, Kieleczama 2013, Wallace 2013), where it was observed three times in this study. Both 1500 and 1600m are considered lower montane wet forest, with 1600 being the uppermost edge of the transition range to lower montane rain forest. Fogden (1993) states that Green Violet-ears should be common in lower montane wet forest, but absent in lower montane rain forest. The difference in relative abundances is not significant, potentially due to small sample size, but it may show an upward trend, especially when compared with previous studies.

Absence of the high-mountain endemic Fiery-throated Hummingbird is also worth noting. It was once considered common to elevations above 1550m in the Cordillera de Talamanca (Monteverde area) (Stiles & Hespeneheide 1972), however past studies did not detect it in forested areas (Lynn 2001, Kieleczama 2013, Wallace 2013). This data showed it to not inhabit the higher open areas either, despite having an abundance rank of 'fairly common' according to Fogden (1993). This suggests that it may be nearing local extinction (Wallace 2013). Three species that could have moved upwards into the sample area were the Magenta-throated Woodstar, Canivet's Emerald, and the Rufous-tailed Hummingbird, all of which are described as preferring either edge or more open areas (Stiles & Skutch 1989). The Magenta-throated Woodstar was found in two previous studies at 1490 and near 1500 (Lynn 2001, Garcia 2013), but were not detected in open area plots at lower levels, suggesting that they are not experiencing pronounced range shifts either.

Based on the results of this study, it is reasonable to conclude that most montane hummingbirds in the Monteverde area are not currently experiencing significant shifts in their altitudinal ranges in response to climate warming. However, there may still be some upward movement that was not well identified in this study. The upward shift of the Green Violet-ear would be in agreement with this conclusion, in addition to the absence of the Fiery-throated Hummingbird. Further studies may be more successful in identifying trends if they placed all feeders on aluminum poles

to keep them out of reach of coatis, allowing hummingbirds an equal chance of finding all feeders. It could also encompass a larger elevational range. Despite the lack of consistent trends, this study does not necessarily rule out the possibility that tropical montane hummingbirds are being affected by warming. It also suggests that birds in open and forested areas may respond differently to shifting thermal ranges, and provides a strong data set that can be used as a base point for further research.

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