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Species Interactions

Plant – Animal Interactions

Human Impacts on Tropical Animals

Morphological changes in *Begonia involucrata* with elevation: leaf area, drip tips, and stomatal density

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ABSTRACT

Climate may impact a species by changing its range and phenology, and in extreme cases may lead to extinction. However, phenotypic plasticity may allow many species to acclimate. Such morphological plasticity is especially important for sessile organisms such as plants. Climate varies predictably along an elevational gradient, so elevational plasticity of morphological traits is one way to assess likely outcomes for climate change. This study examines drip tip number, stomatal density, and drip tip area in Neotropical understory plant *Begonia involucrata* along an elevational gradient. *B. involucrata* is unusual in having from 1 – 4 apices or drip tips per leaf that vary in relative area. Here "drip tip area ratio" was calculated for each leaf as drip tip area over non-drip tip area. Stomatal density and drip tip area ratio were found to vary by location but not with elevation, as well as to be greater in leaves with more drip tips. Drip tip number had no relationship with elevation (linear regression, $R^2 = 0.02$, $p = 0.14$), but it was found that drip tip area ratio was higher in bigger leaves (linear regression, $R = 0.08$, $p < 0.01$). As *B. involucrata* did not demonstrate measurable morphological plasticity with elevation, it is likely this plant will have to move up with changing conditions or will eventually be faced with local extinction.

RESUMEN

El clima puede impactar las especies cambiando su rango de distribución y fenología, y en casos extremos puede llevar a la extinción. Sin embargo, la plasticidad fenotípica puede llevar a varias especies a aclimatarse. Esta plasticidad morfológica es especialmente importante para organismos sésiles como plantas. El clima cambia de manera predictiva a lo largo de un gradiente altitudinal, así la plasticidad elevacional de rasgos morfológicos es una manera de medir resultados probables del cambio climático. Este estudio examiná el numero de puntas de goteo, densidad de estomas, y área de puntas de goteo en la planta de sotobosque *Begonia involucrata* a lo largo de un gradiente altitudinal. *B. involucrata* es inusual ya que posee entre 1 – 4 ápices o puntas de goteo por hoja que varían en área relative. Aquí la proporción del área de la punta de goteo se calculó para cada hoja como área de la punta de goteo sobre el área de no punta de goteo. La densidad de estomas y la proporción de la punta de goteo variaron por sitio pero no por elevación, así como al ser mayor en hojas con más puntas de goteo. El número de las puntas de goteo no tiene relación con elevación (regresión lineal, $R = 0.02$, $p = 0.14$), pero se encontró que la proporción del área de la punta de goteo es mayor en hojas más grandes (regresión liner, $R = 0.08$, $p < 0.01$). Así como *B. involucrata* no muestra una plasticidad morfológica medible con la elevación, es probable que está planta se tenga que mover hacia arriba con las condiciones cambiantes o se enfrentará eventualmente con la extinción local.

INTRODUCTION

With climate change, species have three options: acclimate, relocate, or die. On tropical mountains, as the climate warms, habitat ranges of specialized organisms may be pushed farther up the mountain in a phenomenon dubbed 'the escalator effect' (Marris 2007). Higher elevations are generally cooler and mistier, but resemble conditions previously found at lower elevations (Pounds 1999). However, uphill relocation is not always an option, as for mountaintop species and species in the lowlands that have nowhere to go. Likewise, sessile organisms cannot easily move uphill, like plants that grow slowly, favor vegetative reproduction, or no longer co-occur with their dispersers. For these species, acclimation through phenotypic plasticity may be the only means to avoid local extinction.

Because abiotic factors change with elevation, an organism's ability to acclimate can be observed through differential phenotypic expression at different elevations. Plants at different elevations vary in stomatal density, leaf size, and leaf shape (Hovenden and Vander Schoor 2004). This variation has followed different patterns on temperate and tropical slopes (Cavelier 1996), as well in different species. In Monteverde, Costa Rica, there is a steep moisture gradient, making increasing mist frequency an important condition for plants to acclimate to at high elevations (Haber 2000). Stomatal pores allow gas exchange for photosynthesis, so more stomata might increase photosynthetic potential but also increase water loss (Hopkins 1995), making a higher density less advantageous in drier conditions. Larger leaf size may be advantageous for sun capture at higher elevations because misty conditions lead to a higher abundance of epiphytes (Heitler-Klevans 2016), which reduces the amount of sunlight that reaches the understory. More prominent drip tips may be advantageous at wetter elevations because they facilitate drying of the leaf, making sun capture more direct and inhibiting growth of epiphylls and pathogens (Ivey and DeSilva 2001).

B. involucrata is a wind-dispersed understory plant that is found in Costa Rica and Panama. In Costa Rica, it occurs at an elevation of between 1000 and 2500 meters (Zuchowski 2007). The lack of an animal disperser for this species may make it difficult for this plant to relocate in response to warming. Leaves of *B. involucrata* can form up to four drip tips, whereas most leaves form no more than one (Fig. 1). They have been shown to increase in drip tip number (Taylor 1997), stomatal density, and size with increasing elevation (Otis 2003).

This study examines whether *B. involucrata* demonstrates the morphological plasticity necessary to acclimate to climatic changes in its range via three experiments. First, I replicated the methods of Taylor (1997) to measure the average number of drip tips along an elevational gradient and to determine if that gradient has changed in the last twenty years. The second experiment measured stomatal density along an elevational gradient. Finally, I examined the relationship between leaf area, drip tip area, and non-drip tip area. Other studies have assumed that the number of drip tips or the degree to which they are divided may influence their effectiveness (Massa 1993), but to my knowledge, no other study has considered that the proportion of area dedicated to drip tips might also influence a leaf's drying rate. Although drip tip number (Taylor 1997) and stomatal density (Otis 2003) have both been shown to differ with elevation, here I examine a suite of traits related to moisture to determine how plastic leaves of *B. involucrata* are with elevation and whether there are tradeoffs between them.

Figure 1. *Begonia involucrata* leaves with one, two, three, and four drip tips.

Table 1. Nine large patches of *Begonia involucrata* along the main trails behind the Estación Biológica in Monteverde used to study changes in leaf morphology with elevation.

Site Number	*Elevation (m)*
1	1540
2	1567
3	1578
4	1646
5	1720
6	1740
7	1746
8	1744
9	1778

MATERIALS AND METHODS

Study Sites

This study was performed at the Estación Biológica in Monteverde, Puntarenas, Costa Rica. The forest behind the station is Tropical Lower Montane Wet Forest and Tropical Lower Montane Rain Forest. This study occurred between October 19 and November 15, fall 2017, which is near the end of the wet season. Following Sendero Principal and Sendero Mirador behind the Biological Station in Monteverde, I located and marked nine sites of *B. involucrata* patches (Table 1). I marked a 20m long section of trail. The end of each site was at least 20m away from the start of the next. Sites were numbered 1-9 in order of ascending elevation. The elevation of each site was estimated using an average of barometric altimeter readings taken on four different days at the start- and end-points of every site. Each day the altimeter was set to the known altitude of the Estación Biológica at 1535 m.

Replication of Taylor (1997): Drip Tip Number Vs. Elevation

I followed the methods of Taylor (1997) to compare possible changes in drip tip number with elevation since 1997. Sites were defined in the same way that Taylor defined “clumps” in his experiment. Only Sites 2-9 were used for this experiment because they fell within the elevational range that Taylor used: 1550m – 1790m. For each site, I counted the number of drip tips on the leaves of 20 plants. A drip tip was defined as at least 1 cm long. For each plant, I counted the drip tips of every leaf, excluding the youngest leaf and any leaves that were too damaged by herbivory for their drip tip number to be certain. I only used healthy plants that had at least three countable leaves. Average drip tip number was calculated for each plant.

Stomatal Density Vs. Elevation

Sites 1-7 and Site 9 were used for this experiment. At each site, I selected 10 *B. involucrata* leaves of each drip tip number for a total of 40 leaves per site. Each group of 10 leaves contained a wide range of the available leaf sizes. If 10 leaves of a certain drip tip number could not be found at a site, as many as possible were selected. All selected leaves had a clear number of drip tips and were fully expanded.

Selected leaves were labeled with circular stickers measuring 1.5 cm in diameter. Each leaf was photographed with an iPhone 6. I used a hole punch to cut out a circular piece of leaf from an area clear of primary veins near the thickest part of the leaf. The resulting leaf fragment had a diameter of 6 mm. It was taped to a glass slide with clear tape and the label of the corresponding leaf was transferred to the slide. Each slide was placed under 75x magnification on a compound light microscope and the underside of each leaf fragment was photographed using a Celestron Digital Microscope Imager, Model # 44421. The microscope was focused to obtain the clearest view of stomata on the surface of the leaf and the tape on each slide was removed if necessary to obtain a clear picture. Stomata in each photo were counted and used as a measure of relative stomatal density.

Analyzing Leaf Area

The photos taken of each leaf were uploaded and opened in ImageJ. Each leaf was traced to measure the total leaf area. For the first 174 leaves, which were selected from Sites 1-5, the non-drip tip area was also measured. While tracing this area, I drew straight lines connecting the points where the angle of the leaf outline changed and the points along the edge between drip tips. This area was subtracted from the total area to get the drip tip area. “Drip tip area ratio” was calculated as drip tip area over non-drip tip area (the remaining leaf blade).

For the regression between drip tip area and non-drip tip area (Fig. 5A), I photographed ten additional leaves with two drip tips from Site 1 so that I had a total of 20 leaves for that subgroup. Leaf area and non-drip tip area were measured for these leaves and drip tip area was calculated.

RESULTS

Drip Tip Number

Regression analysis showed no statistical relationship between drip tip number and elevation ($R^2 = 0.02$, $n = 160$, $p = 0.14$; Fig. 2). Average drip tip number ranged from 1 to 4.5. The lowest average was found at Site 6 and the highest average was found at Site 7. Leaves with five drip tips were only found at Site 7.

Stomatal Density

Different sites differed significantly in average stomatal density (ANOVA, $F = 7.71$, 7 df, $p < 0.01$), but these differences did not follow an elevational trend (Fig. 4A). Site 5 at 1720 m had the lowest average stomatal density (26.8 +/- 0.8 se) and Site 3 at 1578 m had the highest average stomatal density (35.2 +/- 1.8 se). Stomatal density had no statistically significant relationship with elevation ($R^2 = 0.008$, $n = 286$, $p = 0.12$; Fig. 3). Stomatal density did differ significantly between leaves with different numbers of drip tips (ANOVA, $F = 12.54$, 3 df, $p < 0.01$). Leaves with more drip tips had higher stomatal densities (Fig. 4B). Relative stomatal density for leaves of one and four drip tips differed by 6.04 stomata.

Leaf Area

Sites 1-5 differed significantly in average drip tip area ratio (ANOVA, F = 3.19, 4 df, $p = 0.015$, n = 174 leaves), but these differences did not follow an elevational trend (Fig. 6A). Site 3 and Site 5 had the lowest average ratio (0.25 +/- 0.02 se), and Site 2 had the highest average ratio (0.32 +/- 0.02 se). Drip tip area ratio increased significantly with increasing number of drip tips (ANOVA, F = 27.79, 3 df, $p < 0.01$). The average ratios for leaves of one and four drip tips differed by 0.19 (Fig. 6B). Regression analysis for the same subgroup of leaves showed that drip tip area ratio increased as leaves increased in total area (Fig. 5B), i.e., larger leaves had proportionately more area dedicated to drip tips. Generally, eight percent of the variation in drip tip area ratio per leaf was attributable to change in total leaf size ($R^2 = 0.08$, n = 174, $p < 0.01$). Another regression analysis was performed for the 20 leaves selected from Site 1 with two drip tips. As before, drip tip area increased with an increasing non-drip tip area ($R^2 = 0.84$, n = 20, $p < 0.01$; Fig. 5A), even though drip tip number here did not change.

Additional Observations

I observed that leaves with more drip tips tend to be larger. Leaves with one drip tip are particularly small compared to other *B. involucrata* leaves. Leaves with two drip tips are by far the most common. Leaves with four drip tips were less common at mid-elevation sites; I found fewer than ten at Sites 3, 4, 5, and 6. Leaves with one drip tip were less common at high-elevation sites; I found only five at Site 7, and none at Site 9.

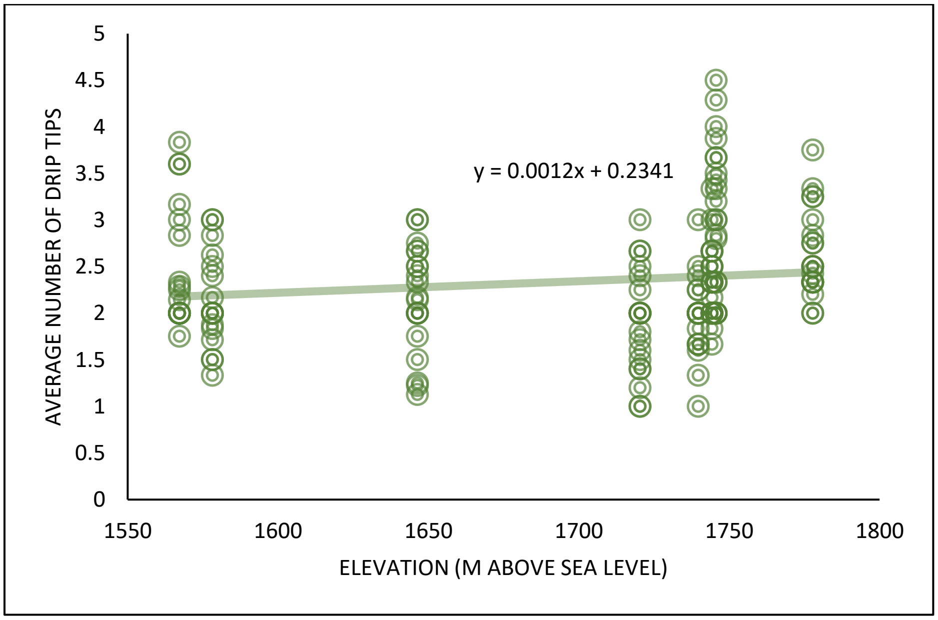

Figure 2. Mean number of drip tips per leaf for individual *Begonia involucrata* plants along an elevational gradient. Drip tips per leaf for each of twenty plants per site were counted at eight sites of varying elevations in Monteverde, Costa Rica. Methods of the Taylor (1997) study were followed. Each point on the graph represents average number of drip tips per leaf in one plant.

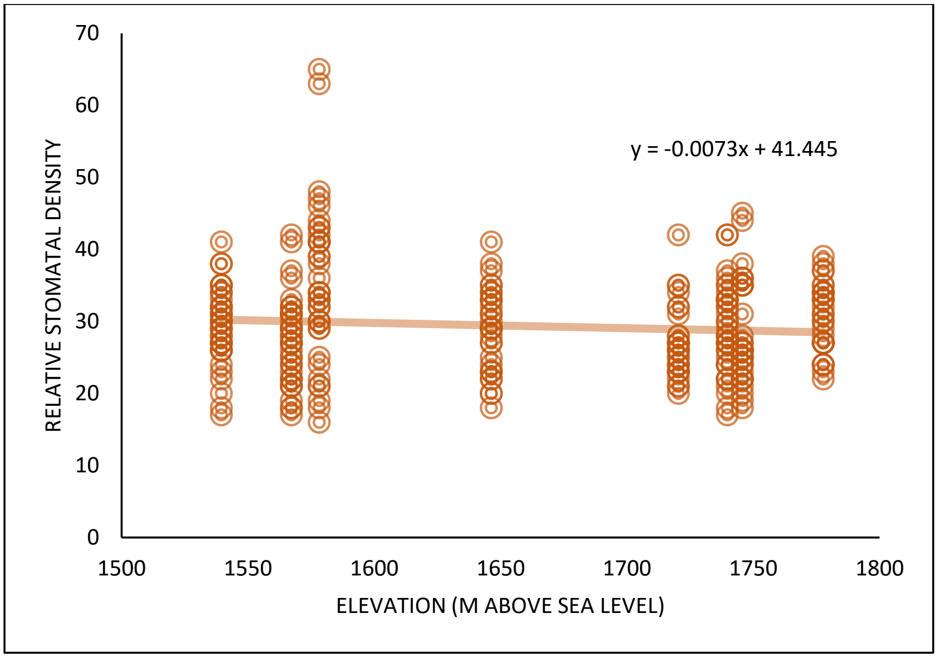

Figure 3. Relative stomatal density in *Begonia involucrata* along an elevational gradient. Leaf fragments were collected from plants along a trail in Monteverde, Costa Rica, and the underside of each was examined under a microscope. Relative stomatal density was defined as the number of stomata visible in a photo taken under 75x magnification of each fragment. Each point on the graph represents one leaf.

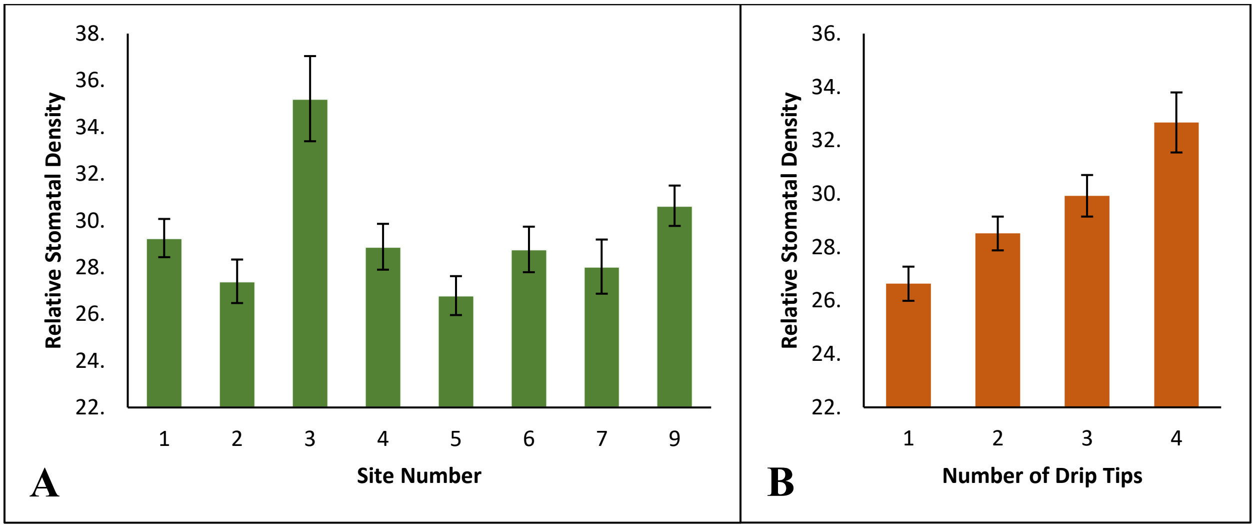

Figure 4. Effects of site and drip tip number on stomatal density in *Begonia involucrata* leaves in Monteverde, Costa Rica. Relative stomatal density was defined as the number of stomata visible in a photo taken under 75x magnification of the underside of a leaf fragment. Error bars represent standard error. (A) Sites differed from each other significantly, but not consistently ($p < 0.01$). Relative stomatal density is represented by averages of leaves grouped by site. (B) Leaves grouped by drip tip number differed significantly from each other ($p < 0.01$). Leaves with more drip tips had higher stomatal densities. Relative stomatal density is represented by averages of leaves grouped by drip tip number.

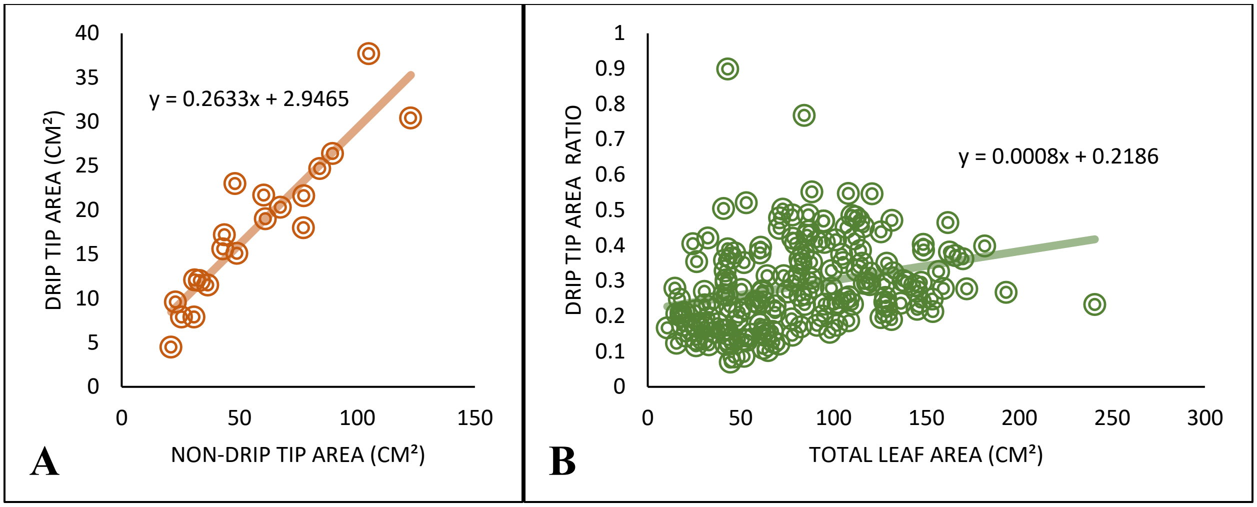

Figure 5. (A) Drip trip area increases with non-drip tip leaf area for leaves of *Begonia involucrata* ($p < 0.01$). Sampled leaves were selected at an elevation of 1540 m and all had two drip tips. (B) Leaves with larger total leaf area have higher drip tip area ratios ($p < 0.01$). Sampled leaves were selected between an elevation of 1540 m and 1720 m in Monteverde, Costa Rica. Drip tip area ratio was defined as drip tip area over non-drip tip area.

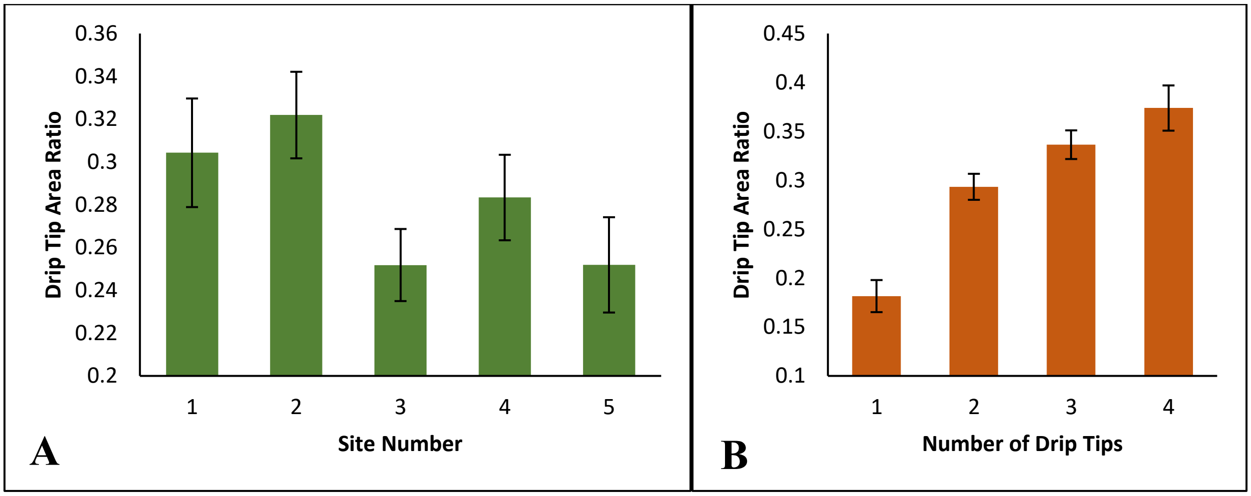

Figure 6. Effects of site and drip tip number on drip tip area ratio in *Begonia involucrata* leaves in Monteverde, Costa Rica. Drip tip area ratio was defined as drip tip area over non-drip tip area. Leaf areas were measured using ImageJ. Error bars represent standard error. (A) Sites differed significantly in drip tip area ratio, but not consistently ($p = 0.015$). Drip tip area ratio is represented by averages of leaves grouped by site. (B) Leaves grouped by drip tip number differed significantly in drip tip area ratio from each other ($p < 0.01$). Leaves with more drip tips had higher drip tip area ratios. Drip tip area ratio is represented by averages of leaves grouped by drip tip number.

DISCUSSION

This study examined morphological plasticity in leaves of *Begonia involucrata*. Drip tip number and stomatal density varied by site but not with elevation, and stomatal density was higher in leaves with more drip tips. Drip tip area increased with leaf size. Drip tip: non-drip tip area was greater in bigger leaves. This drip tip area ratio also varied by site but not with elevation, and was greater in leaves with more drip tips. Therefore, this study provides no evidence for phenotypic plasticity or acclimation in *B. involucrata* leaves with elevation, but does show that changes in leaf size area are accompanied by disproportionate changes in drip tip area and increased stomatal density.

Drip tip area and stomatal density both varied significantly between sites, but not with elevation. Differences in leaf morphology between sites could be caused by differences in microhabitat, genetic variation, or both. Individual plants within a patch are likely to be related, due to dispersal limitation and vegetative growth. Low rates of gene flow between populations have been documented in two other *Begonia* species (Matolweni et al. 2000). Therefore, different genotypes of *B. involucrata* could have characteristic drip tip area and stomatal density that reflects their unique allelotypes. If stomatal density and proportion drip tip area are plastic and respond to the environment, they may respond to microhabitat differences, like nutrient or water availability. For example, Site 3 appeared consistently sunnier than Site 9 or Site 1, as it was situated next to a landslide. Higher sun exposure has been associated with higher stomatal densities (Cavelier 1996).

Leaves with more drip tips were found to have higher stomatal density and a higher drip tip area ratio. In *B. involucrata*, leaves with more drip tips tend to be bigger (Misako Azuma 1997), so drip tip number may be used as a proxy for leaf size. Bigger leaves are generally favored in the understory because they capture more sunlight, but they require more structural investment to be held parallel to the ground. After *B. involucrata* leaves have reached a certain size, it may be less worthwhile for plants to invest in leaf size and more worthwhile to invest in leaf quality. An increase in stomatal density increases photosynthetic efficiency. Investing more in drip tip area may also increase efficiency by drying the leaf more quickly. Increasing the separation of a leaf blade also allows a leaf to reach out to more sun flecks with the same amount of leaf area, providing it with a more stable supply of energy (Muir 2013).

The data suggests that *B. involucrata* does not exhibit morphological plasticity along an elevational gradient. This could mean either that *B. involucrata* is unable to change its leaf morphology in response to moisture availability, or that there is not much variation in climate along the elevational gradient observed. Taylor (1997) and Otis (2003) did observe elevational changes in the leaf morphology of this species in the same location over the same elevational range. It is possible that *B. involucrata* leaves are plastic and that conditions have changed between these studies and the present. Climate at high and low elevations may be more similar now. Landslides are frequent in the area and have altered canopy cover along many of the trails, affecting plant populations randomly. A decrease in the decline in temperature with increasing elevation in recent years is also possible. According to the rising cloud bank hypothesis, rising sea temperatures lead to more evaporation and a greater release of heat when condensation occurs, which accelerates warming at high elevations relative to low elevations (Pounds et al. 1999). This makes the elevational temperature gradient less steep. Another possibility is that leaf morphology in *B. involucrata* is so plastic that it adjusts to seasonal changes. The Taylor (1997) and Otis (2003) studies were both conducted in summer rather than fall. The elevational moisture gradient may be less steep in fall, especially if this year was a particularly wet one. However, this explanation would require *B. involucrata* to both produce new leaves and adjust to its environment very quickly.

Although my results do not rule out the possibility of morphological plasticity in *B. involucrata*, they do not support it. Given the natural history of this plant, the variation between sites was most likely due to genetic differences between populations. As a wind-dispersed understory plant, *B. involucrata* is unlikely to be able to move uphill as its habitat warms. Genetic variability may provide some resilience, as some populations may be better suited to new conditions than others. However, if the conditions of its current habitat change significantly, *B. involucrata* is likely to face local extinction.

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Cloud Forest Epiphyte Diversity, Elevation and Climate Change

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ABSTRACT

Climate change is lifting the cloudbank on tropical mountains, raising the elevational gradient of mist there. The purpose of this study is to analyze the diversity, abundance, and richness of epiphytic plants along an elevational mist gradient from 1540 m to 1800 m in elevation in the Monteverde Cloud Forest. A tropical storm created hundreds of recently fallen branches that were sampled for epiphytes. Fallen branches of 20 cm to 40 cm circumference and 0.5 m in length were collected and epiphytes were inventoried. Photographs of each branch were used to analyze percent bryophyte and lichen cover. Epiphyte abundance ($R^2 = 0.0089$), richness ($R^2 = 0.105$), and diversity ($R^2 = 0.083$), showing a significant linear increase with elevation. Likewise, bryophytes increased ($R^2 = 0.17$) while lichens decreased ($R^2 = 0.077$). A 17% increase in bryophyte cover was coupled with a 15% increase in epiphyte richness, 17.7% in abundance, and 10.0% in diversity, all linked to elevation. This increase along an altitudinal gradient shows that epiphytes at lower elevations with lower mist frequency are simpler and less diverse. With global warming, mist frequency will continue to rise with the lifting cloudbank, causing declines in moss and epiphyte abundance, richness, and diversity.

RESUMEN

El calentamiento global está elevando el banco nuboso en las montañas tropicales, elevanado el gradiente altitudinal de la neblina ahí. El propósito de este estudio es analizar la diversidad, abundancia, y riqueza de plantas epífitas a lo largo de un gradiente altitudinal de neblina de los 1540 a los 1800 m de elevación en el Bosque Nuboso de Monteverde. Una tormenta tropical creo cientos de ramas recien caídas que se muestrearon por las epífitas. Ramas caídas de entre 20 y 40 cm de circunferencia y una longitud de 0.5 m se colectaron y se inventariaron las epífitas. Fotografías de cada rama se usaron para analizar el percentaje de cobertura de briófitas y líquenes. La abundancia ($R^2 = 0.0089$), riqueza ($R^2 = 0.105$), y diversidad de epífitas ($R^2 = 0.083$) muestra un incremento linear significativo con la elevación. También, las briófitas aumentan ($R^2 = 0.17$) mientras que los líquenes disminuyen ($R^2 = 0.077$). Un aumento del 17% en la cobertura de briófitos se combina con un aumento del 15% en la riqueza de epífitas, 17.7% en abundancia, y 10% en la diversidad, todos ligados con la elevación. Este aumento a lo largo de un gradiente altitudinal muestra que las epífitas en las elevaciones más bajas con menor frecuencia de neblina son más simples y menos diversas. Con el calentamiento global, la frecuencia de la neblina se elevara con el banco nuboso, causando disminución en la abundancia, riqueza, y diversidad de musgo y epífitas.

INTRODUCTION

Climate change can alter species ranges, phenology, composition and community dynamics (Walther et al. 2002). On tropical mountains, rising sea surface temperatures result in a lifting-cloud-base (Still 1999), raising the natural gradient of mist frequency with elevation (Pounds et al. 1999; Colwell et al. 2008; Karmalkar et al. 2008). Many species move up mountains to remain at their preferred mist levels (Pounds et al 1999) changing community composition (Frei et al. 2010). Based on these assumptions, species at the top of the mountain may face local extinction, as upward migration is no longer possible. Therefore, tropical montane species that respond strongly to elevation are likely to change with climate-induced elevational increases in the mist gradient there.

Tropical Cloud Forest epiphytes are highly dependent on mist (Foster 2001, Clark et al. 1998). The great diversity of epiphytes there is largely due to frequent, prolonged periods of cloud cover (Sugden and Robins 1979). Monteverde, Costa Rica, a prime example of the delicate systems found in a Cloud Forest, has very high epiphytic diversity and specifically the highest diversity of orchids worldwide (Nieder et al. 1999). Because epiphytes have no contact with the ground, they have morphological and ecophysiological adaptations to collect and hold onto rainwater (Rundel and Gibson 1996). Epiphytes have many different methods for dealing with water stress including specialized means of water uptake, structural adaptations, and ecophysiological adaptations to increase efficiency of water use (Rundel and Gibson 1996). Many epiphytes also utilize crassulacean acid metabolism (CAM), a photosynthetic system that increases water efficiency use and thus drought resistance (Nobel 1991).

Specific examples of how epiphytes are adapted to water stress are as follows: nest ferns grow in rosette form to trap debris and water. All subfamilies of bromeliads use CAM; some have tanks for water storage based on structure and others have large surface areas covered with specialized trichomes for water and nutrient absorption (Rundel and Gibson 1996). Epiphytic orchids have CAM leaf succulents; many have a characteristic referred to as “all-cell succulence” as succulence appears in chlorenchymatous mesophyll (von Willert et al. 1992). In the seasonal Tropics, dry seasons are too severe for many epiphytes (Benzing 1998), except in mountains with high inputs of mist caused by cloud formation (Pounds et al. 1999, Nadkarni and Solano 2002). Here, mist bathes mountaintops throughout most of the dry season, allowing abundant and diverse epiphyte communities to form (Gotsch et al. 2015, Nadkarni and Solano 2002). As mist frequency declines on tropical mountains, epiphytes are likely to decline there, as well.

One past study, *Potential Effects of Climate Change on Canopy Communities in a Tropical Cloud Forest: an Experimental Approach*, by Nadkarni and Solano tested the effects of reduced mist frequency on vascular epiphytes by transplanting mats of epiphytes and arboreal soil from trees at higher elevations in the Cloud Forest to trees at lower elevations where they would naturally be exposed to less mist. They monitored leaf production and mortality rates over time with the results from this study showing a significant decrease in fitness in the vascular epiphytes in response to decreased mist frequency. Similarly, a study by Gotsch et al., *Life in the Treetops: Ecophysiological Strategies of Canopy Epiphytes in a Tropical Montane Cloud Forest,* looked at variation in functional strategies of canopy plants to assess drought avoidance or drought tolerance; this was done by quantifying variations in functional traits in different species of epiphytes and hemiepiphytes. Another study by Gotsch, investigated seasonal microclimate on whole plant water balance in epiphytes using both an observational & manipulative experiment.

Tropical Storm Nate hit the Monteverde Cloud Forest in October 2017, with strong winds resulting in many fallen branches containing epiphytes. This provided an opportunity to sample canopy epiphyte communities. Monteverde, Costa Rica is ideally located in the zone of cloud formation and has an exceptionally high diversity of epiphytes (Schuettpelz and Trapnell 2006). It is on the seasonal Pacific side of Costa Rica but has historically been bathed in mist for most of its 4-month dry season (Clark et al. 2000). With climate change, Monteverde is experiencing more consecutive, mist-free days (Pounds et al. 1999). Here, as on other tropical mountains, mist frequency is a function of altitude, providing a sharp gradient in mist frequency that is moving up in elevation with the lifting cloudbank. Because no past studies have looked at vascular epiphytes diversity along an altitudinal gradient in Monteverde, Costa Rica before, the purpose of this study is to see if there is a change in the richness, abundance and diversity of epiphytes with increasing elevation. This will help predict likely responses of Cloud Forest epiphytes to climate change.

MATERIALS AND METHODS

Study Site

This study was conducted above the Estación Biológica in Monteverde, Costa Rica. The altitude of the area ranges from 1540 m to 1800 m. There are two different Holdridge Life Zones present in the range data were collected: Tropical Lower Montane Wet Forest and Tropical Lower Montane Rain Forest, with the division between the two currently at 1700 m in elevation. This is a private reserve of mostly old growth forest. The Lower Montane Wet Forest is found along a restricted area on the upper Pacific slope (Wright 2000). This life zone has a less pronounced dry season due to the mist that comes in over the Continental Divide; the mist in this area fosters a diverse and abundant epiphyte community, characteristic of the forest (Wright 2000). It has closed canopy of approximately 30 to 40 m, but tree stature declines rapidly with altitude so that forest at 1800 m is called Elfin Forest, with trees no taller than 15 m. Elfin Forest's structural, wind-blown mist and cloud cover are integral components of the climate; this zone typifies the Cloud Forest: dense vegetation, broken canopy, and epiphytes that form dense mats on the trunks and branches of the trees (Wright 2000). This life zone is restricted to the high peaks and ridges of the Continental Divide.

Branch Selection

All data were collected by sampling fallen branches. The branches used were all of a similar circumference between 20 cm to 40 cm and 0.5 m meter in length. One hundred branches were selected as evenly as possible throughout the 260-meter elevational gradient. The exact altitude of each branch was recorded using an altimeter; the circumference was measures and recorded for each branch. All branches collected were originally from canopy of closed, mostly primary forest. Only recently fallen limbs were collect rather than old and/or rotting branches.

Epiphyte Inventories

Each branch was inventoried for its species of vascular epiphytes. Abundance of each species was recorded per branch. A photo was taken of each branch, and ImageJ was used to determine percent cover by both bryophytes and lichens. Species were initially identified to morphospecies and family. Subsequent taxonomic information was added by field guides and the help of local biologists.

RESULTS

Vascular Epiphytes

Richness, abundance, and diversity of vascular epiphytic plants increase with elevation. Approximately 10% of the increase in vascular epiphyte species richness is explained by changes elevation ($R^2 = 0.105$; N = 100; P = 0.001; Fig. 1). Mean species richness at 1540m was about 6 species, but ranged from 2-8 species per branch, while species richness at 1800m was closer to 8 species per branch. The greatest species richness occurred at 1750m, where several branches had over 12 species of vascular epiphytes. The branch with most vascular epiphytes had 19 species of vascular epiphytes and was also found at 1750m. When looking at abundance there were two outliers; both were heavily covered by small plants. The first outlier was at 1625 m with 41 individuals, but this only had 7 different species present, which was average for this elevation; the second outlier present was at 1750 m with 43 individuals. The second was found in a tree fall gap, and had a single individual for more than 12 species and approximately 10 small individuals of Elaphoglossum peltatum. In terms of abundance, an 8.3% increase in the number of individuals is explained by elevation ($R^2 = 0.083$; N = 100; P = 0.0036; Fig. 2); and 8.8% of the increase in diversity is explained by the same factor ($R^2 = 0.088$; N = 100; P = 0.0026; Fig. 3). For diversity, the Shannon-Weinner diversity index had an average of 1.77. The lowest value was 0.41 at 1689 m in elevation and the highest was 2.64 at 1740 m. Diversity of epiphytes is randomly distributed along the elevational gradient, which is why both the lowest and highest diversity values are found within approximately 50 m of each other along the altitudinal gradient. An increase of 100 m in elevation results in an increase in 1.37 species. Similarly, the same change in distance would result in an increase of 2.77 individuals and increase of diversity index by 0.171 (Fig. 1- 3).

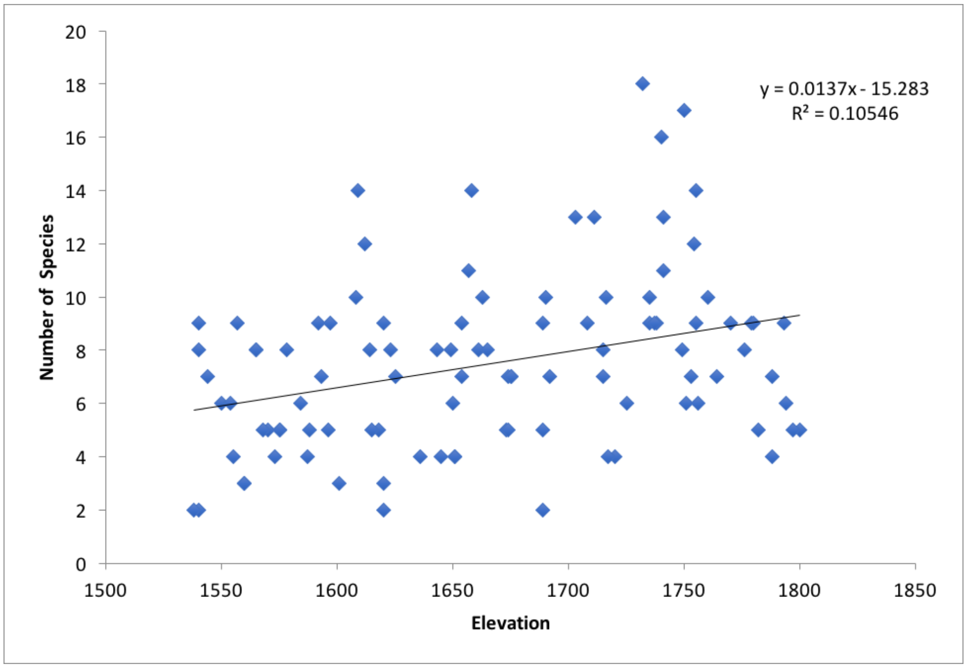

Figure 1. Vascular epiphyte species richness along an altitudinal gradient from 1540 m to 1800 m used to examine the effects of mist frequency and climate change on vascular epiphyte diversity. Samples collected from fallen branches with in a size range from 20 cm to 40 cm in circumference; each 0.5 m in length. N = 100; P = 0.001.

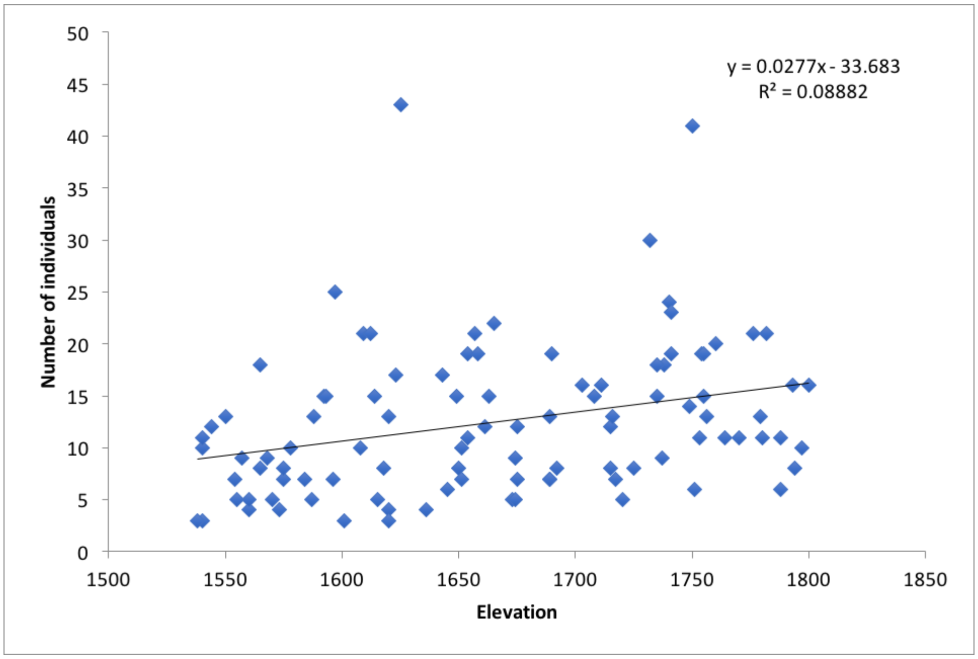

Figure 2. Abundance of vascular epiphytes along an altitudinal gradient from 1540 m to 1800 m used to examine the effects of mist frequency and climate change on vascular epiphyte diversity. Samples collected from fallen branches with in a size range from 20 cm to 40 cm in circumference; each 0.5 m in length. N = 100; P = 0.0036.

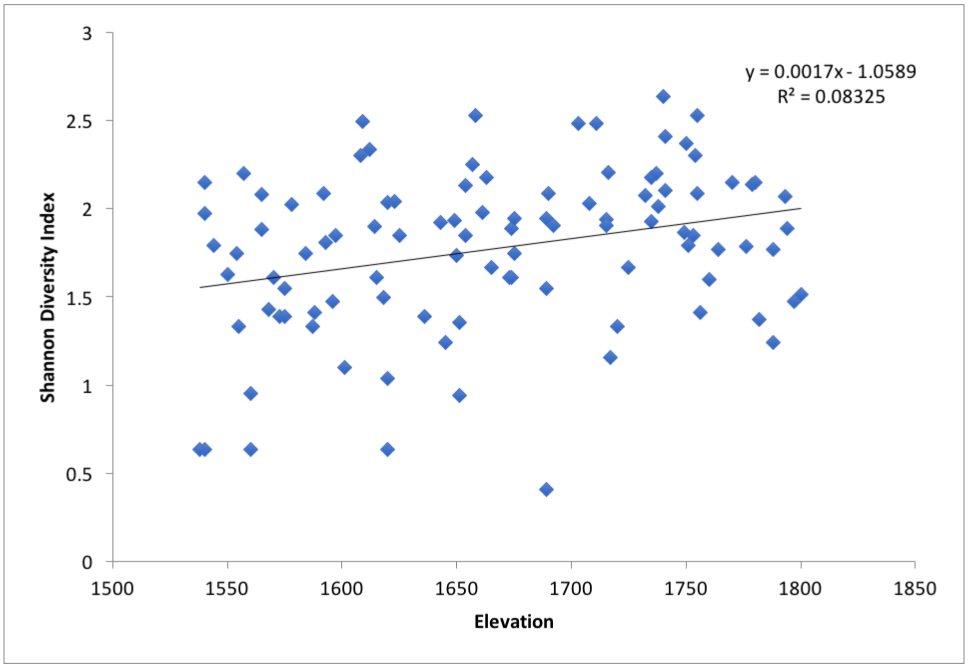

Figure 3. Vascular epiphyte diversity along altitudinal gradient from 1540 m to 1800 m used to examine the effects of mist frequency and climate change on vascular epiphyte diversity. Samples collected from fallen branches with in a size range from 20 cm to 40 cm in circumference; each 0.5 m in length. Abundance and richness was recorded for each branch then diversity was determine for each of the branches using Shannon-Weinner index. The diversity was plotted along the elevational gradient. N = 100; P = 0.0026.

Nonvascular Species

Bryophytes covering branches consisted of both moss and liverworts. Percent bryophyte cover responded to elevation. The lowest percent cover of bryophytes (0%) was found at the lowest recorded elevation, 1540 m; the highest recorded bryophyte cover (100%) was found at the highest recorded elevation, 1793 m. The average percent coverage of bryophytes per branch was 58.8%, found around 1656 m in elevation. In percent cover of bryophytes, 19.4% of the increase along the altitudinal gradient is explained by elevation ($R^2 = 0.194$; N = 88; $P < 0.001$; Fig. 4). Percent cover of lichen showed the opposite trend, with a 5% decrease in lichen cover is explained by increased elevation ($R^2 = 0.047$; N = 88; P = 0.043; Fig. 5). The number of branches with lichen coverage was significantly less than those observed with bryophyte cover; only 12 branches were recorded with lichens present. Those with lichen cover ranged between 4.6% cover and 29.8% cover with one outlier measuring 95.2% lichen cover. The branch with 4.6% cover was found at 1596 m in elevation and the highest cover was found at 1592 m. The outlier of 95.2% was found at 1588 m in a more open area. All branches with lichen cover were found at the bottom of the range with lower mist frequency. With increased bryophyte cover, there was observed decrease in lichen cover ($R^2 = 0.0865$; N = 89; P = 0.005; Fig. 6). Of the branches observered with lichen cover there was an average of only 20.85% bryophyte cover, which was significantly less than the observered average of bryophyte cover for all branches (58.8%). An increase in elevation by 100 m results in an increase of bryophyte cover by 18.34% and a decrease in lichen cover by 1.84%.

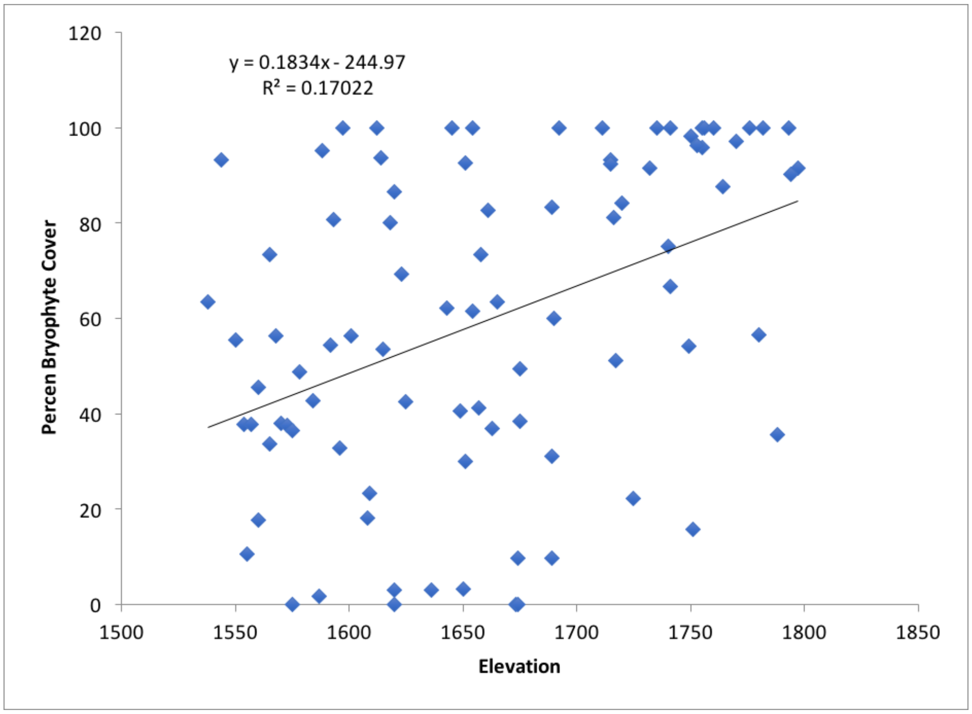

Figure 4. Bryophyte Coverage Along Altitudinal Gradient from 1540 m to 1800 m in Elevation Samples collected from fallen branches with in a size range from 20 cm to 40 cm in circumference; each 0.5 m in length. *N = 88; P < 0.001*

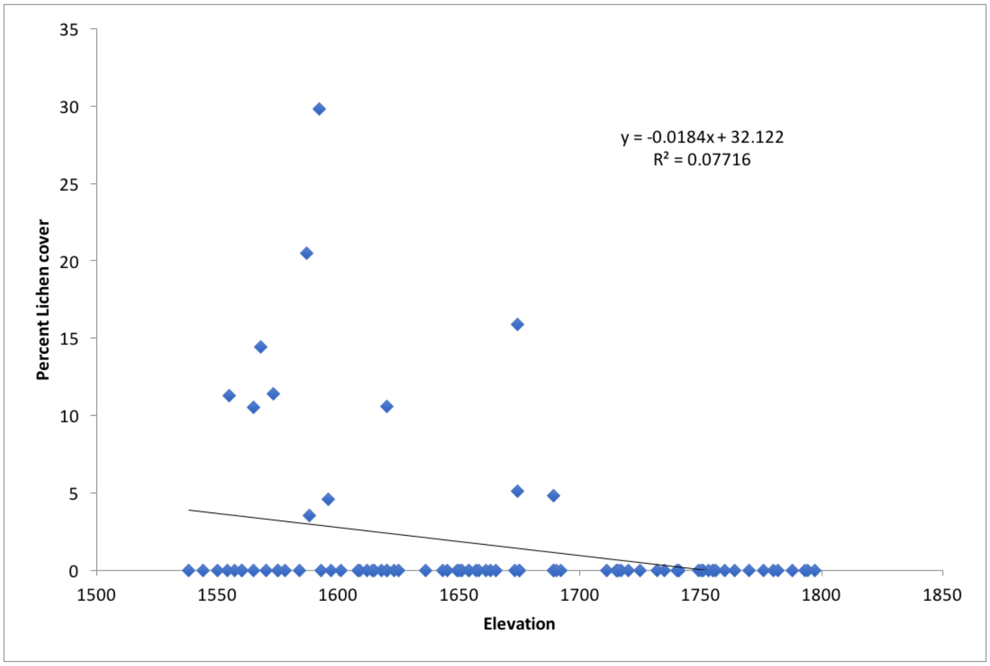

Figure 5. Percent Bryophyte Cover Along Altitudinal Gradient from 1540 m to 1800 m in Elevation. Samples collected from fallen branches with in a size range from 20 cm to 40 cm in circumference; each 0.5 m in length. N = 88; P = 0.043

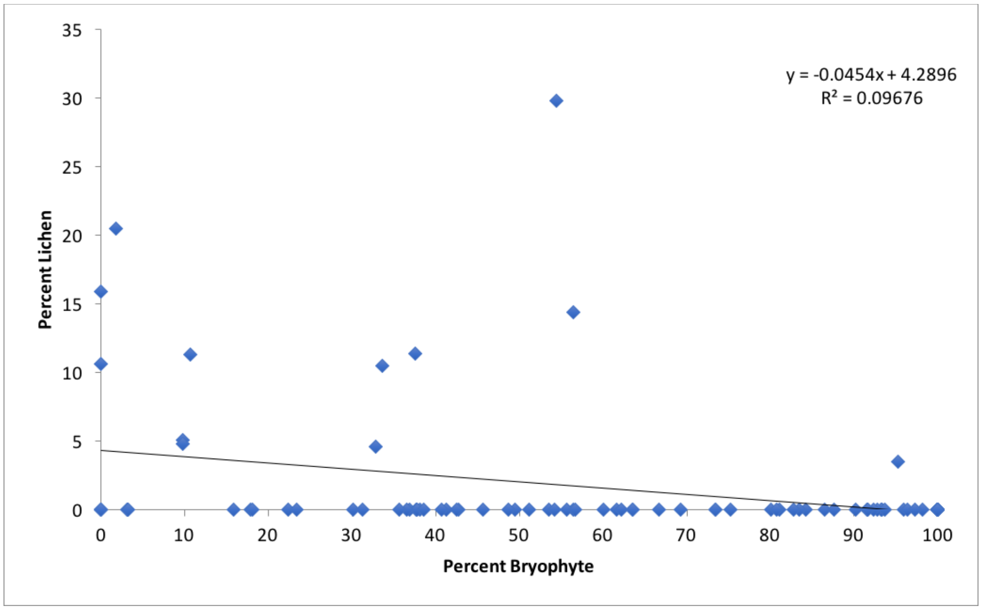

Figure 6. Percent of Bryophyte Coverage in relation the Percent of Lichen Coverage. Samples collected from fallen branches with in a size range from 20 cm to 40 cm in circumference; each 0.5 m in length. N = 89; P = 0.005

Interactions Between Vascular and Nonvascular:

With an increased percent cover of bryophytes there is an increase in the richness, abundance, and diversity of vascular epiphytes. A 15% increase in the number of species are explained the increase in bryophyte cover ($R^2 = 0.15$; N = 88; $P < 0.001$; Fig. 7). Bryophyte cover accounts for a 17.7% increase in the number of ($R^2 = 0.177$; N = 88; $P < 0.001$; Fig. 2). Diversity of epiphytes also have a 10.0% increase explain by the increase in percent bryophyte cover ($R^2 = 0.10$; N = 88; P = 0.002; Fig. 8). Other factors that may influence the increase in both variables include mist frequency, elevation, and precipitation. Both bryophytes and vascular epiphytes may be responding to the same hidden variables and therefore both show the same changes due to other external factors. Lichen cover leads to a decrease in epiphyte growth for richness, abundance and diversity; for richness of epiphytes in relation to lichen cover, the equation is expressed as, $y = -0.0386x + 7.414$ (P = 0.0.23; N = 89; $R^2 = 0.016$). Abundance of epiphytes based on lichen cover is expressed with the equation $y = -0.046x + 12.481$ (P = 0.53; N = 89; $R^2 = 0.004$); diversity of epiphytes in relation to lichen cover is expressed with the equation: $y = -0.0046x + 1.7529$ (P = 0.307; N = 89; $R^2 = 0.011$). Graphs are not presented as they are the inverse of the bryophyte cover graphs. A change in bryophyte cover by 50% results in an increase of vascular epiphyte species by 2.0; similarly, a 50% increase in bryophyte cover would result in an increase in the number of individuals per branch by 4.74 and an increase in the diversity index by 0.22.

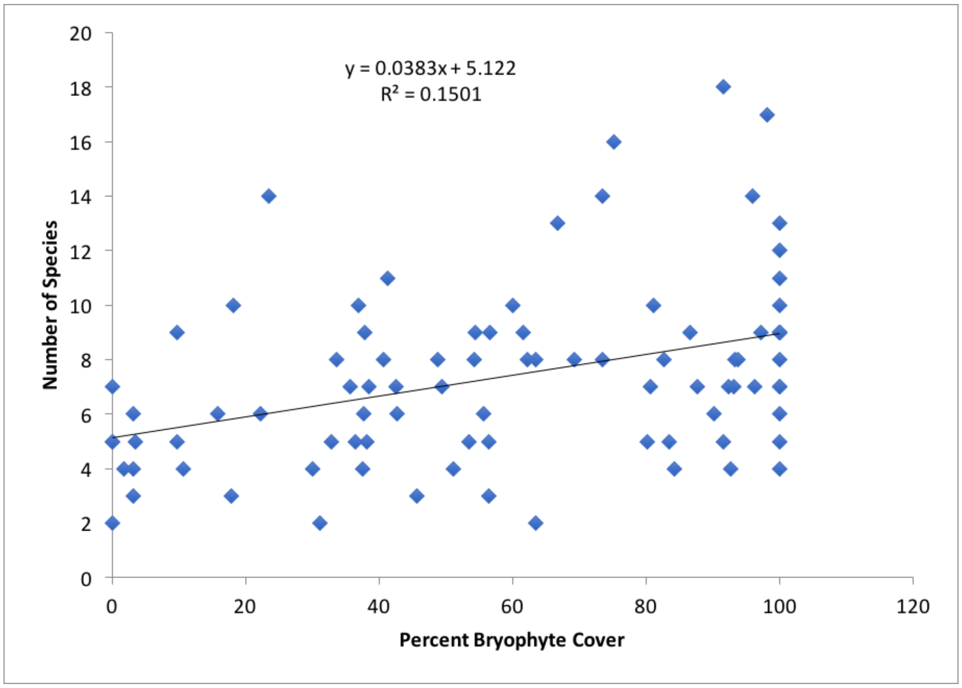

Figure 7. Species Richness of Vascular Epiphytes Based on Percentage of Bryophyte Coverage. Samples collected from fallen branches with in a size range from 20 cm to 40 cm in circumference; each 0.5 m in length. N = 88; $P < 0.001$

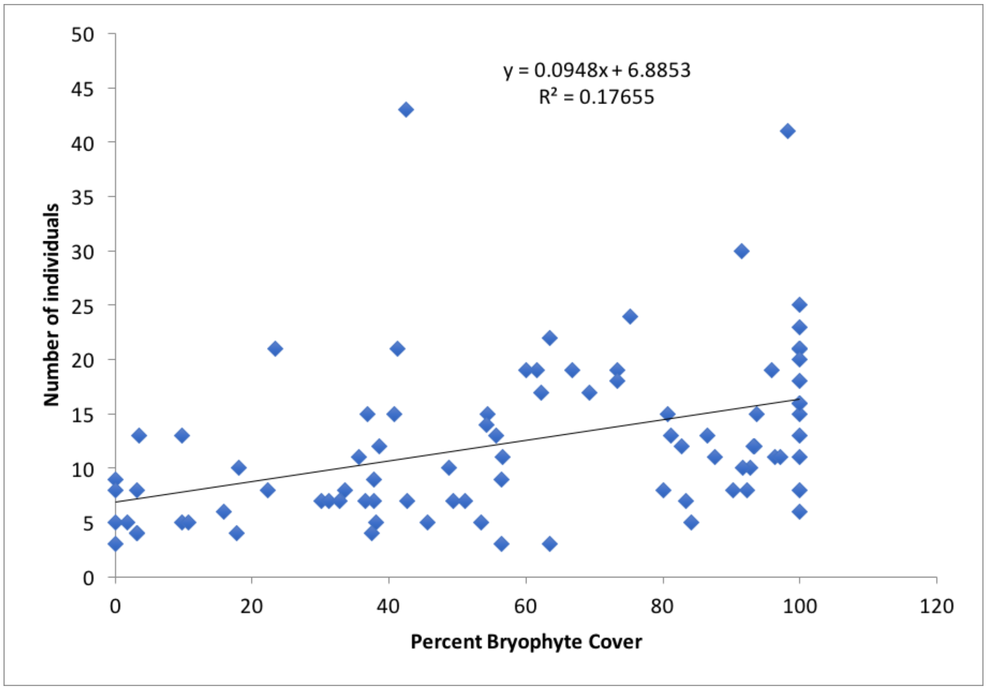

Figure 8. Abundance of Vascular Epiphytes Based on Bryophyte Coverage. Samples collected from fallen branches with in a size range from 20 cm to 40 cm in circumference; each 0.5 m in length. N = 88; $P < 0.001$

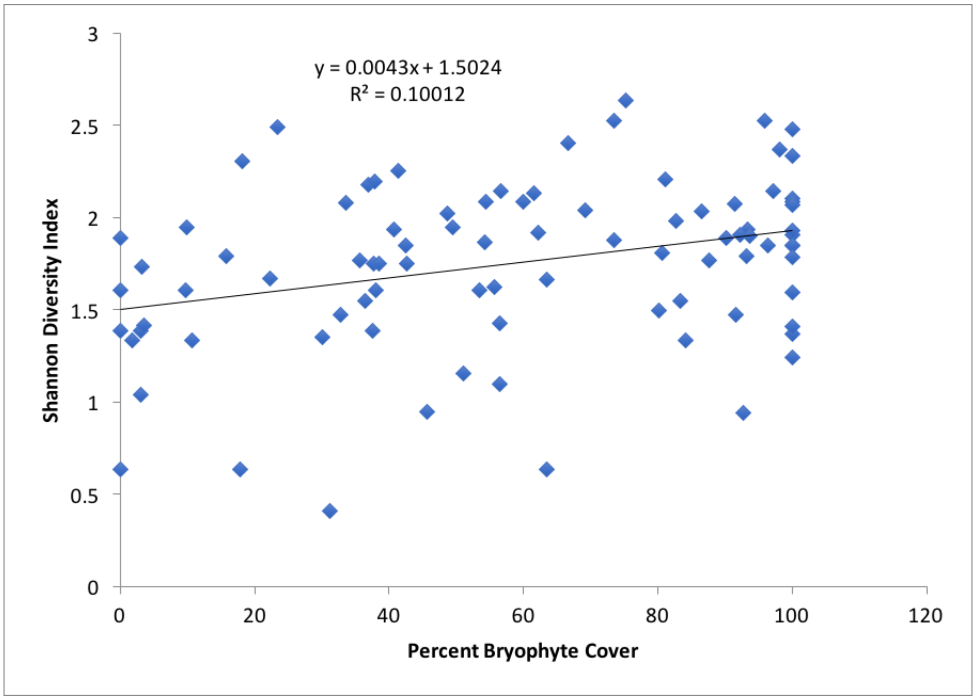

Figure 9. Diversity of Vascular Epiphytes Based on Percentage of Bryophyte Coverage. Samples collected from fallen branches with in a size range from 20 cm to 40 cm in circumference; each 0.5 m in length. *N = 88; P = 0.002*

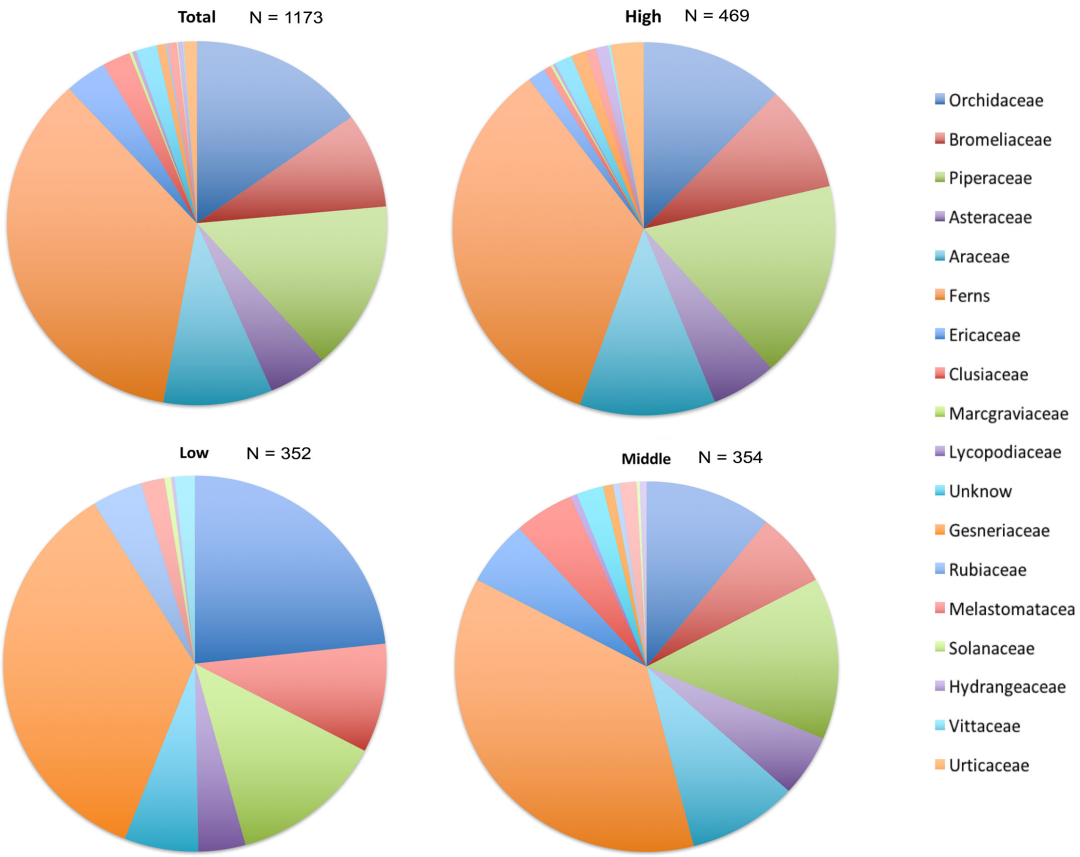

Figure 10. Richness of epiphytes by families found along an altitudinal gradient between 1540 m and 1800 m in elevation throughout the Tropical Lower Montane Wet Forest and Tropical Lower Montane Rain Forest in Monteverde, Costa Rica The lowest elevational range of the data collection was between 1540 m and 1625 m gradient in elevation throughout the Lower Montane Wet Forest. The middle range is between 1625 m and 1710 m in elevation marking the transitional point between the Lower Montane Wet Forest and Lower Montane Rain Forest. The highest range is between 1710 m and 1800 m in elevation in the Lower Montane Rain Forest, is above the divide between these two life zones, serving as the upper elevational range from the data samples.

Some of the families of epiphytes facing this challenge based on elevational range include Gesneriaceae, Hydrangeaceae, Urticaceae, Solanaceae, Lycopidiaceae, Vitaceaeae, all of which were only observed above 1650 m in elevation (Appendices 7-18). The Orchidaceae family was much more prevalent at the lowest range, with 82 individuals found; there were about the same number in the middle and high ranges combined as there were in the lower elevational range alone (Appendix 12). The highest number of orchids are found at the lower elevational range (Fig. 10). Piperaceae, Asteraceae, Araceae, and Ferns all increase across the entire elevational range. Gesneriaceae, Rubiaceae, Melastomataceae, Solanaceae & Hydrangeaceae families were only obsereved at middle and high, whileVittaceae & Urticaceae were only found in the high elevational range (Fig. 10).

DISCUSSION

Results of this study indicated a greater abundance, richness, and diversity of vascular epiphytes and bryophytes with elevation between 1540 m and 1800 m in Pacific Slope Cloud Forest of Monteverde, Costa Rica. Likewise, bryophyte cover increased, while lichen cover decreased along the gradient. This increase with elevation shows a positive response to increased mist frequency; also higher elevations are experiencing more days with mist and a greater quantity of mist than at lower elevations (Nadkarni and Solano 2002). In addition, these data support previous studies showing that bryophytes act as foundation species (Heitler-Klevans 2016) and contribute to increased epiphytic diversity as they are able to hold water and nutrients that vascular epiphytes can draw from. Other studies have also showed when elevation is held constant, there is still an increase in vascular epiphyte abundance, richness, and diversity with increased bryophyte cover (King 2016). My findings also support that the increase in abundance between epiphytes along altitudinal gradient ($R^2 = 0.088$) is less than the increase in abundance observed between that of epiphytes and bryophyte cover alone ($R^2 = 0.178$). This would indicate that the bryophyte cover aids in the accumulation of epiphytes more than just increased elevation alone. These results support the idea that a loss of bryophyte cover over time would lead to inhospitable areas for epiphytes to accumulate on tree branches in the canopy.

In line with the lifting-cloud-bank hypothesis, the actual amount of precipitation has not changed, only the percent of mist frequency, changing the intensity and rate at which plants are able to obtain water (Pounds et al. 1999). Because the average temperature does not change significantly in the area (Pounds et al. 1999), this indicates that the fitness of vascular epiphytes and percent bryophyte cover is directly related to the frequency of mist, suggesting epiphytes will likely become less abundant and less diverse at lower elevations if the forest continues to dry out due to these changes in abiotic factors that are being observed.

These findings are supported by studies regarding change in local climate, showing implications for tropical montane forest ecosystems in Costa Rica; these point to a future with significantly more moisture stress due to higher temperatures and a reduction in precipitation from both horizontal and vertical processes (Karmalkar et al 2008). Epiphytes at lower elevations are suffering due to climate change; if this trend continues, epiphytic plants further up the mountain will continue to see these same declines. The data from this study further supports experimental approaches showing declined fitness when epiphytes from higher altitudes were transplanted to lower altitudes with decreased mist frequency (Nadkarni and Solano 2002).

Previous studies support the notion that these changing abiotic factors will heavily impact the Montane Cloud Forest leading to decreased fitness due to changes in these abiotic factors. Effects of climate change in Central America, and Costa Rica in particular, will be more pronounced over time at higher elevations than in the lowlands, particularly on the Pacific side (Karmalkar et al 2008). It is significant to predict future changes in the composition of epiphytic growth in the forest; because of the dependency of epiphytes on mist, they are sensitive to abiotic changes and act as a good indicator of environmental changes in the area. As these trends further develop, epiphytic growth and bryophytes will decline with the more commonly occurring dry conditions. With these trends and decreased fitness for epiphytic plants, the only true winners will be lichens.

Based on how sensitive species are to the abiotic changes, species will continue with an upwards shift at different rates. Species found in more restricted ranges along the higher altitudinal gradient may experience a local extinctions, as they can no longer shift further uphill to meet their previous abiotic conditions (Nadkarni and Solano 2002). Based on observed elevational ranges, species only found in the Lower Montane Rain Forest may be more at risk as they will not have room for any further upward movement (Fig. 13).

As shown in the results, there are small differences in the families of epiphytes observed along the altitudinal gradient (Fig. 10). Some families of epiphytes may face more of a challenge with increasing impacts of climate change and the observed shift in the mist frequency following the lifting cloud bank. Based on elevational range, Gesneriaceae, Hydrangeaceae, Urticaceae, Solanaceae, Lycopidiaceae and Vitaceaeae, all of which were only observed above 1650 m in elevation (Appendices 7-18), may have some species that face local extinction if trends continue and there is no means of adapting to the changing abiotic factors, as there is little room for further uphill migration for some of the species in these families. Another observation noted in the results was the high abundance of orchids found in the lowest elevational range (Fig. 10; Appendix 12). Orchids have many different adaptations for drought resistance not observed in other families; these adaptations include pseudobulbs, velamen, succulent leaves, CAM photosynthesis as well as other unique adaptations (Rundel and Gibson 1996). This would suggest because of these modifications for water stress that orchids may fair better than other plant families that do not have as sufficient adaptations.

In one experimental study, *Variations in the Resilience of Cloud Forest Vascular Epiphytes to Severe Drought*, from Gotsch et al., the response to induced drought symptoms were compared among different epiphyte species. They found that in these conditions, recovery occurred more quickly for plants in the lower and drier sites; epiphytes in the Cloud Forest experienced the slowest recovery, showing these plants are particularly sensitive to severe drought (Gotsch et al. 2017). The data in this study support the findings Gotsch et al. and further emphasis the sensitivity of this ecosystem. The sensitivity of these Cloud Forest epiphytes could pose a serious problem if changes in the environment continue at such a rapid pace, resulting in upward shifts in mist frequency faster than the species are able to follow an upward shift; at the very least, there will be observed changes in the community composition as some species will experience a more rapid elevational shift. Overall, all epiphytic plant growth will become less abundant and diverse in the Cloud Forest.

Even small disruptions such as the changes in community composition can have a significant impact on the ecosystem as a whole. Defloration is not a commonly considered change, as most studies focus on the significance of defaunation; the significant role of plants, specifically epiphytic plants is frequently underestimated. Some of the services provided by epiphytes include epiphytes ability to regulate the release of precipitation seasonally, providing control against flood and erosion in the rainy season as well as water storage during the dry season (Still et al. 1999). Another significant role is the interactions between epiphytes and other forest flora. Vascular epiphytes serve to intercepts water and nutrients from the atmosphere and aid to significant inputs of these resources to the forest floor (Gotsch et al 2015). It has also been noted that epiphytes and their associated decomposing litter and arboreal soils are significant to many animals as well. Biomass, leaf litter, as well as decomposing epiphytes all are important to the overall ecosystem; used as habitat and food for birds and mammals, over 200 species of birds have been documented as using this source in the Neotropics (Gotsch et al 2015).

This study demonstrates the noteworthy dependence of vascular epiphytes on the fleeting mist frequency as it shifts higher in elevation in response to the effects of climate change. The great diversity of the tropics is due to the high connectivity found in these forests. When critical foundational components of this ecosystem are lost, it is difficult to believe that there will not be a domino effect observed throughout other levels of the ecosystem. The results of this study, in tandem with prior studies, points towards a grim future for vascular epiphytes in the Cloud Forest faces further defloration. The degree of influence humans have had on the planet and changes that will come about due to the stresses put on the ecosystems are dependent on the forest's ability to recover. The more species lost and ecological processes disrupted or altered, the more difficult it will be and the longer it will take to regain diversity (Malhi et al. 2014). Cloud Forest epiphytes are currently going through a bottleneck due to the changing abiotic factors; what will result as the future composition of epiphytes will be those that make it through the induced stresses currently implemented by climate change (Malhi et al 2014).

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APPENDIX

Appendix 1. Araceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Anthurium flexile	5	1704	1609	1751
Anthurium caperatum	1	1755	1755	1755
Anthurium microspadix	1	1740	1740	1740
Anthurium pallens	3	1747	1732	1755
Anthurium pittieri	5	1685	1654	1703
Anthurium scandens (m35)	3	1660	1565	1749
Anthurium sp	2	1723	1720	1725
Anthurium sp 3	6	1723	1597	1760
Anthurium sp 6	4	1638	1609	1674
Anthurium sp 7	2	1745	1740	1749
Anthurium uteyorum	6	1685	1597	1755
Anthurium utleyorum	4	1694	1587	1776
Araceae sp 1 (m21)	12	1701	1609	1755
Philidendron sp 2	4	1706	1608	1793
Philidendron sp 3	1	1703	1703	1703
Philidendron sp 5	4	1737	1661	1793
Philidendron sp 6	3	1742	1716	1760
Philidendron sp.	10	1671	1575	1755
Philodendron aurantiifolium	11	1668	1550	1755
Philodendron brenesii	1	1675	1675	1675
Philodendron crassipathum	3	1666	1587	1737
Philodendron wilburii	3	1695	1654	1755

Appendix 2. Asteraceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Neomirandea sp 1 (m20)	23	1694	1565	1782

Neomirandea sp 2 (m15)	12	1696	1575	1794
Neomirandea sp 3 (m15)	2	1641	1557	1725
Senecio sp (m2)	5	1603	1540	1657

Appendix 3. Bromelideae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Bromeliad sp 1	9	1685	1540	1754
Bromeliad sp 10	4	1711	1625	1770
Bromeliad sp 2	31	1671	1540	1800
Bromeliad sp 4	4	1635	1588	1689
Bromeliad sp 9	1	1612	1612	1612
Guzmania angustifolia	1	1741	1741	1741
Guzmania glomerata	1	1755	1755	1755
Guzmania nicaraegensis	2	1725	1715	1735
Guzmania scherzeriana	4	1624	1565	1716
Guzmania sp 1	1	1554	1554	1554
Racinea spiculosa	4	1728	1690	1776
Tillandsia bulbosa	1	1612	1612	1612
Tillandsia punctulata	6	1592	1540	1650
Vriesea sp 2	2	1682	1570	1794
Vriesea sp.	4	1776	1754	1800

Appendix 4. Clusiaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Clusia sp 1 (m40)	15	1660	1540	1779
Clusia sp 2 (m30)	3	1729	1658	1780

Appendix 5. Ericaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Disterigma humboldtii (m3)	12	1617	1540	1732
Ericaceae sp 1	1	1708	1708	1708

Ericaceae sp 1 (m1)	1	1540	1540	1540
Gonocalyx pterocarus (m23)	6	1701	1658	1737
Satyria sp (m28)	5	1660	1612	1716
Sphyrospermum sp (m24)	5	1647	1555	1750
Disterigma humboldtii (m3)	12	1617	1540	1732

Appendix 6. Fern

Genius & Species	Genius & Species	Genius & Species	Genius & Species	Genius & Species
Anetium sp	1	1544	1544	1544
Asplenium sp.	23	1683	1560	1788
Blechnum sp	5	1696	1596	1760
Campylonerum sp. (f4)	15	1680	1573	1797
Elaphoglossum caricifolium (e4)	14	1649	1544	1788
Elaphoglossum erinaceum	2	1767	1754	1779
Elaphoglossum eximium	5	1712	1584	1788
Elaphoglossum glabellum	6	1623	1592	1658
Elaphoglossum nigrescens (e3)	8	1653	1544	1755
Elaphoglossum peltatum	47	1682	1544	1797
Elaphoglossum sp 1	12	1679	1540	1750
Elaphoglossum sp 2	29	1675	1538	1793
Elaphoglossum sp 5	3	1742	1716	1770
Fern 3	6	1749	1618	1800
Fern 5	1	1615	1615	1615
Elaphoglossum sp. 4 (o14)	7	1702	1550	1780
Pecluma sp 2	6	1740	1665	1776
Pecluma sp.	18	1683	1560	1797
Polypodiaceae spp. (f6)	5	1672	1620	1741
Polypodium sp 1 (f8)	4	1604	1550	1675
Elaphoglossum sp. 3	1	1612	1612	1612
Terpsichore sp.	16	1637	1540	1800

Fern 4	11	1696	1554	1793
Elaphoglossum sp. 6 (o11)	1	1620	1620	1620
Fern 8 (o8)	12	1654	1544	1750

Appendix 7. Gasneriaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Capanea sp (m37)	1	1665	1665	1665
Columnea magnifica (g1)	2	1774	1754	1794
Columnea microcalyx (g2)	2	1735	1732	1738

Appendix 8. Hydrangeaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Hydrangea sp (m18)	4	1721	1651	1770

Appendix 9. Lycopodiaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Huperzia sp	4	1661	1555	1735

Appendix 10. Macgraviaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Marcgravia brownei (g4)	4	1667	1568	1760

Appendix 11. Melastomataceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Blakea gracilis	7	1671	1609	1750

(m17)				
Blakea sp 1 (m16)	2	1713	1711	1715

Appendix 12. Orchidaceae Family

Genius & Species	Genius & Species	Genius & Species	Genius & Species	Genius & Species
Ada chlorops sp.	1	1725	1725	1725
Dichaea sp (c1)	1	1540	1540	1540
Elleanthus sp 1	1	1612	1612	1612
Elleanthus sp.	1	1620	1620	1620
Epidendrum centropetalum (o30)	1	1609	1609	1609
Epidendrum sp 3 (X)	1	1614	1614	1614
Epidendrun sp 1 (m34)	2	1708	1665	1750
Epidendrun sp 2 (25)	2	1612	1575	1649
Lepanthes sp.	7	1706	1661	1750
Malaxis sp (m11)	3	1716	1615	1794
Masdevallia sp 1	4	1693	1650	1794
Maxillaria sp 1	7	1658	1540	1780
Maxillaria sp 2	2	1751	1708	1793
Oerstedella sp 1	5	1670	1565	1779
Oncidium or Encyclia (10)	4	1586	1544	1620
Oncidium or Encyclia (17)	3	1629	1550	1715
Orchid sp 24	3	1744	1690	1793
Orchid sp 26	1	1557	1557	1557
Orchid sp 27	3	1622	1557	1716
Orchid sp 29	2	1688	1596	1780
Orchid sp 5	2	1649	1609	1689
Pleurothallis cardiochila	2	1698	1658	1737
Pleurothallis cardiothallis	3	1670	1608	1741
Pleurothallis sanchoi	1	1614	1614	1614
Pleurothallis sp 1	3	1597	1550	1620
Pleurothallis sp 2	12	1654	1565	1793
Pleurothallis uncinata	1	1750	1750	1750
Prosthecia Encyclia	1	1570	1570	1570
Scaphyglotis sp (m34)	1	1597	1597	1597

Stelis sp 1	3	1648	1557	1780

Appendix 13. Piperaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Peperomia sp 7 (b2)	1	1735	1735	1735
clusia sp (p1)	23	1673	1540	1800
Peperomia "common alternate"	13	1724	1609	1788
Peperomia hernandifolia	1	1755	1755	1755
Peperomia maculosa (p5)	7	1679	1584	1741
Peperomia palmana (m22)	1	1675	1675	1675
Peperomia pittieri	2	1723	1711	1735
Peperomia rotundifola (m4)	2	1660	1540	1779
Peperomia sp (m25)	2	1675	1661	1689
Peperomia sp (m31)	3	1729	1690	1764
Peperomia sp 2	9	1630	1540	1793
peperomia sp 4	11	1628	1538	1779
Peperomia sp 6	12	1659	1565	1753
Peperomia tetraphylla	1	1612	1612	1612

Appendix 14. Rubiaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Hillia sp (m14)	2	1653	1651	1654

Appendix 15. Solanaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Schultesianthus leucanthus (m26)	1	1703	1703	1703

Appendix 16. Urticaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Pilea sp 1 (m38)	1	1782	1782	1782
Pilea sp 2 (m39)	1	1782	1782	1782

Appendix 17. Vitaceae Family

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Cissus sp. (m33)	1	1753	1753	1753

Appendix 18. Morphospecies

Genius & Species	Total Abundance	Mean of Altitude (m)	Minimum (m)	Maximum (m)
Morphospecies 12	3	1676	1645	1725
Morphospecies 29	3	1669	1609	1741
Morphospecies 32	2	1744	1732	1756
Morphospecies 36	2	1600	1557	1643
Morphospecies 5	6	1729	1689	1779
Morphospecies 6	3	1751	1738	1760
Morphospecies 6.5	4	1687	1584	1782

Establishment and habitat preferences of the invasive pink banana (*Musa velutina)* in Neotropical cloud forest

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ABSTRACT

Humans increasingly introduce exotic species into native ecosystems. Without natural controls, these invasive species can threaten native biodiversity and ecosystem function. Here, I study habitat preferences, dispersal systems, and germination success of the invasive *Musa velutina* (Musaceae) in Monteverde Costa Rica. Seeds were placed into germination trays and placed in closed vs. open canopy, in swampy and drained conditions, with and without scarification. After 18 days, no seeds germinated nor showed consistent signs of doing so, suggesting *M. velutina* has long germination times and difficulty germinating, which is borne out by hobbyist growers. Despite this, almost four times more plants in study plots started from seed rather than through vegetative reproduction (82 from seed, 20 from asexual reproduction).The density of *M. velutina* was measured for different habitat conditions to show there is a higher density of plants in open, swampy conditions (closed non-swampy mean of 0.931, closed swampy mean of 4.45, open non-swampy mean of 6.83, open swampy mean of 19). Observational data confirmed the existence of generalized avian omnivores and frugivores in the area, including data from camera traps of a brown jay attempting to eat the fruit. While *M. velutina* prefers open swampy habitats, they have moved into closed habitats in Monteverde, as elsewhere in Costa Rica, possibly using rivers and avian seed dispersers. Local attempts to control *M. velutina* are not only ineffective, they may increase its abundance and eventual spread.

RESUMEN

Los humanos constantemente introducen especies exóticas en ecosistemas nativos. Son controladores naturales, estas especies invasivas pueden amenazar la biodiversidad nativa y las funciones del ecosistema. Estudié las preferencias del hábitat, sistemas de dispersión, y éxito de germinación de la invasiva *Musa velutina*(Musacea) en Monteverde Costa Rica. Las semillas fueron colocadas en bandejas de germinación y ubicadas en dosel abierto versus cerrado, en condiciones de charco o secas, con y sin escarificación. Después de 18 días, no hubo germinación de semillas o señal alguna de posible germinación, sugiriendo que *M.veluntina* posee largos tiempos de germinación y dificultad para germinar, lo cual ha ocurrido en cultivos por agricultores aficionados. A pesar de esto, casi cuatro veces mas las plantas en las parcelas de estudio empezaron de semilla más que de reproducción vegetativa(82 de semilla, 20 por reproducción asexual). La densidad de *M.velutina* fue medida en diferentes condiciones de habitat para mostrar que existe mayor densidad de plantas en abierto, las condiciones de charco(cerrado no-charco promedio de 0.931, cerrado charco promedio fue 4.45, abierto no charco promedio de 6.83, abierto charco promedio de 19). Datos de observaciones confirmaron la presencia de aves generalistas omnívoras y frugívoras en el área, incluyendo datos de cámaras trampa de una Urraca café(*Psilorhinus morio*) tratando de comer fruta. Mientras que *M.velutina* prefiere condiciones abiertas encharcadas, se han movido en condiciones de habitat cerradas en Monteverde, y otras partes de Costa Rica, posiblemente usando ríos y aves dispersoras de semillas. Intentos locales para controlar *M.velutina* son inefectivos, si no además podrían incrementar su abundancia y eventual propagación.

INTRODUCTION

Humans now have global environmental impact (Vitousek et al. 1997). This is increasing and now characterizes the current epoch as the Anthropocene (Malhi et al. 2014). Humans increasingly introduce exotic species that can become invasive, lower biodiversity and disrupt ecological processes (Malhi et al. 2014). Invasive exotic species threaten ecosystems, habitats, or other species, are the second greatest agent in the endangerment and extinction of native species (Pejchar & Mooney 2009). It has been proven that invasive species lower the diversity and abundance of native species (Didham et al. 2005). Invasive species have been shown to have a cascading effect on ecosystems in terms of pests and pathogens (Malhi et al.). Without their natural predators and herbivores, invasive plants are enabled to outcompete native plants, which causes shifts in the ecosystem and potentially the function of the ecosystem. Alien plants have been shown to affect upper trophic levels directly, as well as indirectly by increasing homogeneity in ecosystems and therefore altering overall ecosystem functioning (Vilà et al. 2011). Invasive plants affect community structure by significantly reducing the fitness and growth of resident plant species, while simultaneously decreasing native species diversity and abundance (Vilà et al. 2011).

Ornamental species are cultivated for aesthetic purposes yet can be a sources of problematic invasives (Foxcroft et al. 2008). The pink banana *Musa velutina* (Musaceae), native to Southeast Asia but grown in gardens throughout Costa Rica, creates bunches of small dehiscent and seeded pink bananas. *M. velutina* has recently moved into secondary and primary forest at La Selva Biological Station in the Costa Rica's Sarapiquí province (Balderama et al. 2012). The invasive pink banana is following a similar trajectory in Monteverde: it has escaped gardens and is moving into areas where humans have not planted it (Maier 2012). *M. velutina* as an invasive threatens the biodiversity of secondary and primary forests. Its quick growth, and resilience make it a tough competitor for native species.

Global positioning satellite (GPS) data exists for the pink bananas both in La Selva and Monteverde. Past studies have looked at the locations of the aliens in Monteverde, and found them to be localized and densest along a river behind two hotels, Los Pinos and Hotel Belmar (Maier 2012). Microhabitat of GPS coordinates is not reported, leaving a partial understanding of what *M. velutina* is looking for when establishing and growing.

Here, I attempt to describe current conditions conducive to the establishment and spread of *M. velutina*. Specifically, I attempt to determine germination requirements, current habitat use in Monteverde and ecological factors that favor its spread. This may offer information for more effective management to minimize the impact of *M. velutina* on Neotropical Cloud Forest biodiversity and health.

MATERIALS AND METHODS

Site Descriptions

The study site is located in disturbed Premontane Moist Forest (Holdridge 1967) on the properties of the Hotel Belmar and Los Pinos in the Monteverde area in Costa Rica. A river divides the two properties and the area that surrounds the river on both properties is open and swampy, quickly giving way to drier and more closed forest canopy conditions.

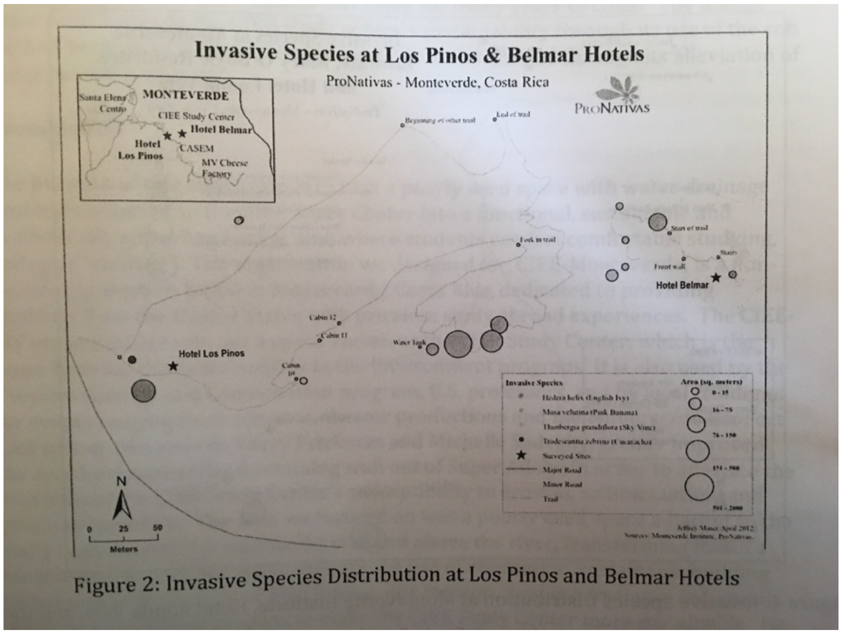

Figure 1. A map of the study area (Maier 2012) Bigger circles indicate larger areas of *M. velutina* plants. The farthest circles closest to the hotels should be ignored since the plants there have been removed. The study site it largely just the large circles in the center of the map near to the water wheel.

Study Organism

Musa velutina is a banana plant that is native to Southeast Asia, and exotic to Costa Rica. It produces small pink bananas that measure 10 centimeters on average and that contain 70-90 hard seeds that vary in color from light tan to dark black. *M. velutina* grows quickly, and can reproduce vegetatively from shoots. Flowers and fruits appear rapidly within one year of germination. *M velutina* grows 1-2 meters tall. Leaves are smaller than those of commercial bananas measuring 50 to 75 centimeters, with a waxy texture. The petiole on the underside is pink, and frequently the edges of the leaves have a pink tinge. The psudostem of *M. velutina* is pink as well. *M. velutina* is significantly smaller than the commercial and common banana *Musa acuminata*, and the pink fruits grow erect rather than upside down. When fully ripe, the fruits open to expose sweet white pulp surrounding the seeds.

Figure 2 illustrates some of the physical characteristics that can be used to identity South Asian *M. velutina*, a recent invasive that has escaped gardens in Costa Rica. The photo on the left shows the small, erect, pink fruits on a mature plant. The middle photo shows mature fruits that have opened on their own to expose the sweet fruits. The photo on the right displays a pink banana plant after being cut. The pseudostem is obvious on this plant since it has no leaves and the pink pigmentation is clear.

Germination

Data were collected using blogs, websites for plant enthusiasts, and gardeners to examine common germination practices for *M. velutina* seeds. The first 20 websites that described *Musa velutina* germination treatment was recorded to see if there were specific conditions required.

In addition, seeds were harvested from fresh, ripe bananas that had not yet opened. Seven different treatments were applied to 700 seeds. 300 seeds were scarified using medium grade sandpaper while 400 seeds were left unaltered. Can you describe the process you used and what the seeds looked like after? Scarified seeds there were placed in trays and put in open, edge, and closed forest habitat. Similarly, unaltered seeds were place in trays with soil in open canopy, open canopy with only water, under closed canopy with soil, and closed canopy with just water.

Soil was collected on site from the same location. Trays with holes in the bottom were filled with soil and 10 seeds were placed onto the surface of the dirt and pushed down 2-3 centimeters into the soil. The seeds were not covered with soil in an attempt to mimic the conditions that would occur for the seeds had they been dropped by a disperser. For trays with only water, water was collected from the nearby swampy site with existing *M. velutina* plants. Ten germination trays per treatment contained 10 seeds each for a total of 100 seeds per treatment. The trays were placed into different conditions and watered. The seeds were only watered immediately after planting but were regularly rained on so never dried out. Observations were made daily to asses if any growth or change to seeds in trays had occurred.

After 18 days the seeds were collected from the field all of the seeds from each treatment were placed into an appropriately labeled bag and brought to the lab. In the lab each seed was individually analyzed to assess signs of development. Seeds were assigned to one of eight categories; no change, gelatinous goo, algae, with worms, larger, smaller, dry, or sprouting.

Mode of reproduction

Visual observations were made to quantify the number of plants that were from seed versus vegetative reproduction. To determine the mode of reproduction it was assessed as to whether or not there was a larger mother plant connected to a much smaller plant, commonly called a sucker. For young or recently cut plants, the sucker can be under the surface of the substrate, so I dug up to 10 centimeters into the ground to correctly determine the mode.

Habitat Preference

The density of *M. velutina* was counted using a one-meter square quadrat, for the following conditions: open and swampy, open and non-swampy, closed and swampy, and closed and non swampy. 29 quadrats were recorded for each condition. Plants the result of both vegetative reproduction and sexual reproduction were counted. Shoots coming off of parent plants in the case of vegetative reproduction were counted as separate individuals.

Potential Dispersers

Four circular feeders of 25-35 centimeters were constructed using wire. Four ripe but unopened bananas were placed in each feeder. Two feeders were placed in trees in open canopy habitat and two feeders were placed in trees in closed canopy habitat. All of the feeders were 2-3 meters off the ground, which is only slightly taller than some *M. velutina* plants. Camera traps recorded visitors to the feeders and visual sightings of frugivorous or omnivorous potential seed dispersers were collected over six days. Six hours were spent at the study site over a three-day period and both closed and open canopy were sampled at the same time.

Figure 3. displays a feeder used to offer fruits from potentially invasive *M. velutina* to potential dispersers.

RESULTS

Germination

Out of the 20 web results that mention *M. velutina* germination, 18 mention soaking the seeds in water as a vital part of germination. Thirteen also mention physical scarification and four mention treating the seeds in acid. According to this information, *M. velutina* seeds will not germinate when just placed in soil and watered, even with seeds from commercial sources. Germination time was reported to be just under a month from most online sources. Additionally, 76.7% of seeds planted germinated after 14 days in a recent study conducted (Nagano et al. 2016).

All of the literature that was looked at for the germination of *M. velutina* lists soaking or physical or chemical scarification as a step that is mandatory for germination success (Nagano et al. 2016 and Pancholi et al.1995), occasionally it is said that both scarification and soaking are required. Additionally, it has been proven that the seed coat for *Musa* as a family, is incredibly complex (Graven et al. 1996).

For the seeds that were physically scarified and then placed in dirt and left in an open habitat, 67 exhibited no change, 19 had a gelatinous goo around the seeds, three had algae and 10 had worms after 18 days (Table 1). These results are fairly similar to those of the seeds that were not scarified but that were also placed in dirt and the open habitat. Scarified seeds in a closed habitat expanded the most, with 29 seeds exhibiting a larger size after 18 days. The majority (52.9%) of seeds showed no change, regardless of treatment. Only one seed showed any evidence of germination, and that seed was not scarified and was in dirt under closed canopy conditions.

Twenty seeds of 683 had worms that had eaten the endosperm. Many seeds had a gooey layer surrounding them when they were analyzed after collecting them from the field after 18 days particularly those that had been in trays with only water. The seeds in the trays without dirt and with only water were often conglomerated, with many seeds being fully covered by a layer of gooey, dirty, gel. The seeds that were recovered from the edge treatment were obviously more dried out and were smaller than when the fresh seeds were planted. None of the seeds in the edge treatment looked like germination was in their future. The soil in the edge pots was incredibly dry.

There was no biological difference between treatments for the seed sizes even though a statistically significant difference was found ($p = 0$, df 6) This is due to a very small variance between treatments (Figure 4).

Table 1. Effect of different treatments on the germination of *M. velutina* seeds after 18 days. As displayed, only one seed successfully germinated. Open, closed and edge all refer to the canopy cover of the habitat the seeds were placed in. Scratched indicates that the seeds in that condition were scarified. Oppositely, un refers to seeds that were left unaltered. Dirt and water both indicate what the substrate the seeds were placed into.

Habitat	No Change	Goo	Algae	Worms	Larger	Smaller	Dry	Sprouting
Open Scratched	67	19	3	10	0	0	0	0
Edge Scratched	0	0	0	0	0	29	70	0
Closed Scratched	70	0	0	0	29	0	0	0
Open Dirt un	70	14	3	6	6	0	0	0
Open Water	48	26	13	0	2	0	0	0
Closed Dirt un	83	2	0	4	9	0	0	1
Closed Water	23	68	8	0	0	0	0	0

Habitat

M. velutina were denser in swampy conditions than nearby dry for both open and closed canopy. There is also a larger overall density of *M. velutina* in open habitats rather than closed (Figure 2. ANOVA F 30.215, df 1, p >0.001). Swampy conditions were characterized by the presence of standing water. The plants were densest in the center of the swampiest conditions and generally became sparser as conditions became drier. A similar trend was observed for canopy cover as well. Plant density decreased as canopy cover increased. Closed conditions had lower banana density.

Mode of reproduction

There are many more plants reproducing by seed rather than vegetative reproduction (Figure 3. Chi-square 37.67, df 1, p = 0.0001). Almost four times more plants started from seed rather than vegetative reproduction.

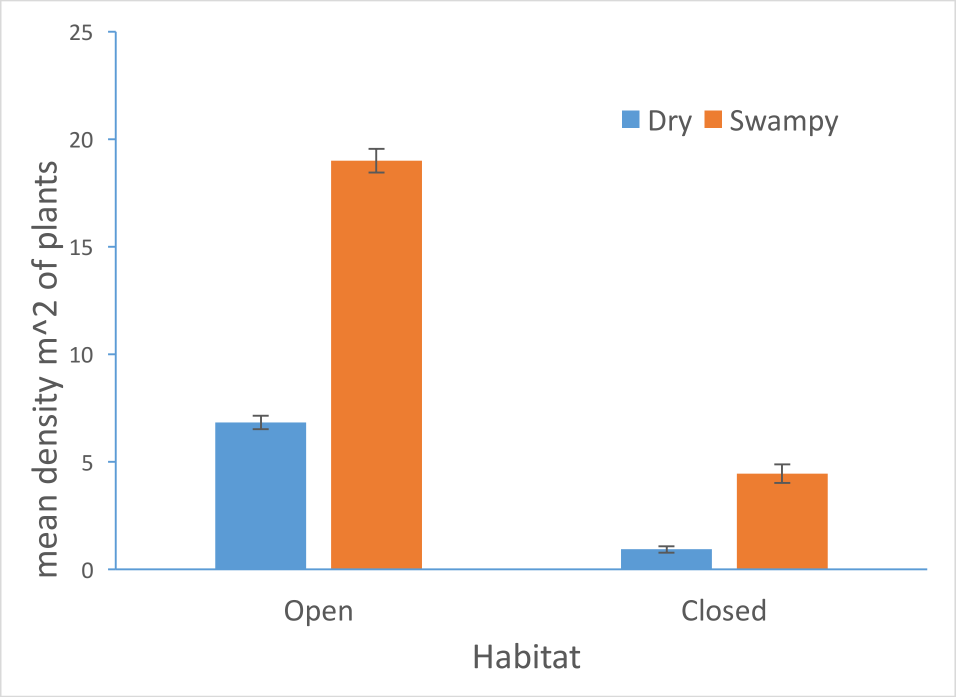

Figure 2. Habitat and mean density of potentially invasive *M. velutina*. Differences in canopy cover in both swampy versus non-swampy were examined. Bars are standard error. Different letters represent statistical difference between treatments (Fisher LSD at $p < 0.05$).

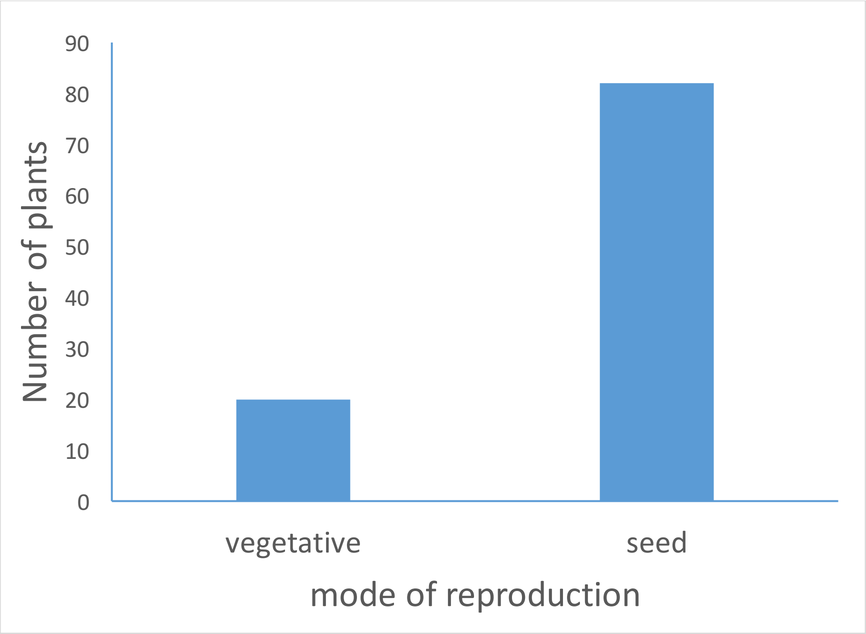

Figure 3. Visualizes the numbers of potentially invasive *M*. velutina plants in relation to their mode of reproduction, either sexual or asexual. Significantly more plants started by seed. Data were collected for this graph on private property in Monteverde, Costa Rica.

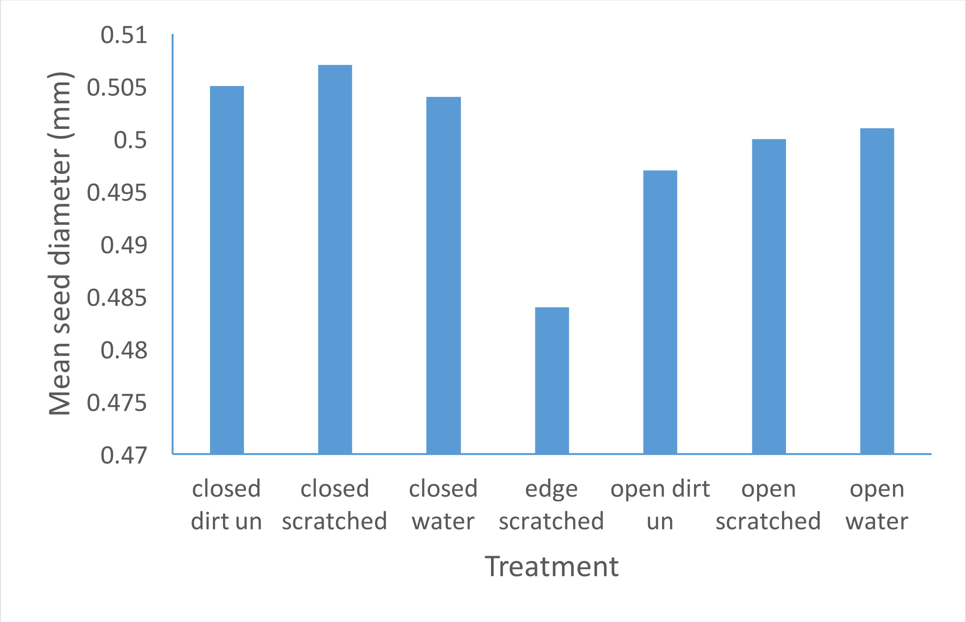

Figure 4. Treatment and mean seed diameter of potentially invasive *M. velutina* in Monteverde, Costa Rica. Bars are SE = 0. Open, closed and edge all refer to the canopy cover of the habitat the seeds were placed in. Scratched indicates that the seeds in that condition were scarified. Oppositely, un refers to seeds that were left unaltered. Dirt and water both indicate what the substrate the seeds were placed into. (ANOVA F 53.75, df 6, p = 0). Very low variance explains small difference between means but statistically significant p-value.

Potential Dispersers

Out of one of the bananas in one of the feeders, a single bite had been taken, most likely from a squirrel. This bite was taken a day before camera traps were set up. Over three days, 89 birds of nine different species were seen around the site flying, nesting, or sitting in trees near the banana patch. Camera traps showed footage of only one bird, a Brown Jay, attempting to eat a banana from one of the feeders in the open area (Table 2). The most commonly seen birds were the Great-tailed grackle and the Brown Jay, both of which are known to be omnivorous.

Table 2. Frequency and species of birds seen over a three-day period in a dense patch of introduced and potentially invasive *M. velutina* in Monteverde, Costa Rica. In bold are any species known to be frugivorous or omnivorous.

Species	Viewing frequency
Great-tailed grackle	**26**
Brown Jay	**24**
House Wren	12
Golden Winged Warbler	10
Blue –grey Tanager	**9**
Variable Seedeater	8
Clay colored Thrush	**7**
Great Kiskadee	**6**
Tennessee Warbler	**4**

Other Observations

The plants that were being observed during this period of time were cut about half way through. It was observed that the plants grew new leaves incredibly quickly, about three days after being cut. The bananas that had been on the stalks were simply dropped into the water, therefore ending up in the water. It was not observed after the fruits were dropped.

GPS Data

GPS data points were taken along the river. A data point was recorded for the banana plant found farthest up the river nearer to Hotel Belmar. A second data point was recorded for the banana plant found farthest downstream near to Los Pinos. In comparison to GPS data from a past study (Maier) in the area, the range does not appear to be expanding very noticeably if at all.

DISCUSSION

Online gardeners and banana-enthuiasts suggest *M. velutina* seeds need to be soaked in water for days to weeks in order to germinate. This may explain why more plants in Monteverde are found in swampy conditions and that most *M. velutina* in swamp come from seed. *M. velutina* is known to establish well after floods in Tropical Lowland Wet Forest of La Selva, Costa Rica (Balderama et al. 2012). It appears that *M. velutina*, along with other noncommercial bananas (commercial bananas having no seeds), depend on the removal, softening or chipping of the outer seed coat (Chin 1996). This necessity to soften or scarify the seed coat hints that the digestion by an animal disperser or soaking in swampy conditions might be important for *M. velutina* germination. Despite that the majority of plants in Monteverde originated from seed, seed germination is thwarted by natural predators and pathogens, as many seeds had worms and gelatinous growth around them. Others dried out.

It was found that more plants originated by seed rather than by vegetative reproduction, which means that seed germination is occurring and frequently in select swampy areas of Monteverde. It is this successful dispersion and quick growth by *M. velutina* that makes it such a potential threat to native species and ecosystems (Balderama et al. 2012). Do these authors explore the threats? Specifically: what are they? Water could also be a highly influential factor when looking at how *M. velutina* is increasing its range. Water, particularly streams or flooding, can facilitate alien species invasions in other plant species (Pyšek & Prach 1993).

Likely seed dispersers in Monteverde include not only water but frugivorous birds and mammals. Generalist avian dispersers facilitate the spread of other invasive species (Gosper et al. 2005). The Brown Jay is not the only frugivorous or omnivorous bird that has the potential to be a disperser for *M. velutina* based on observational data. It is possible that other generalist species are eating and effectively dispersing the seeds. It is also possible that there is a mammal disperser, as evidenced by the bite into a fruit likely from a squirrel. It has been proven that bats are the main disperser for and disperse the largest percentage of wild *Musa acuminata* seeds (Meng et al. 2012). This fact suggests that bats could have a large influence on the dispersal of *M. velutina*.

A higher density of banana plants in open areas rather than closed indicates that land transformation allowed the initial establishment of *M. velutina*. That a few plants were found in closed canopy does not bode well. Current management is periodic chopping of plants, but not always before fruiting. Cut plants grow back incredibly quickly and allowing fruits allows recruitment through seeds. This is at best a short term solution to limiting fruits produced, but does not kill the plants and does not stop them from reaching reproductive maturity. Also, the fruits on cut plants simply fall into the water or nearby land, potentially worsening the problem.

Future studies should farther investigate the relationship between the likely animal dispersers of the Monteverde area and *M. velutina*. Doing a study with the bananas when they are fully mature, ripe, and opened on their own would be interesting to see if the bananas are falling off the plants, or if birds or mammals are taking them. It is important to continue to research the dispersal system and the factors that affect the successful germination of *M. velutina*. Limiting standing water and fruit production appears the best way to discourage their expansion.

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Caste Functional Specialization and the Non-Traditional Roles of Minima in *Atta cephalotes* Leaf-cutting Ants

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ABSTRACT

Division of labor allows societies to specialize, become more efficient, and complete more complex behaviors. Leaf-cutting ants (genera *Atta* and Acromyrmex) have become the dominant neotropical herbivores due to the high degree of polyethism in their colonies. It has traditionally been thought that morphology determined the role an individual ant would play in the colony (caste polyethism). The smallest size class, minima, was historically assigned the roles of hitchhiking, fungal garden care, and cleaning. Foraging was attributed to medium sized ants, media, and defense to the largest workers, called soldiers). However, there has been an increasing amount of research of *Atta* species which shows that minima may be involved in a much more diverse range of roles including defense and trail maintenance. This study consists of two experiments both designed to investigate the size of ants fulfilling a variety of roles in *Atta cephalotes*. In the first experiment, the response of minima (≤3mm) and larger ants (media) to a crushed nestmate was compared. The proportion of minima on the trail increased by 8% when presented with a crushed nestmate, showing they were recruited to aid with defense as opposed to media or soldiers. Also, the mean number of minima swarming the stimuli was much higher than that of the larger size class (14.314 ± 1.660 compared to 0.971 ± 0.156). In the second experiment, ants performing each of 4 roles were measured; (1) foraging, (2) hitchhiking (3) non-foraging behavior (4) ants responding to a crushed nestmate. There was no significant difference between the mean head size of any of the roles, but ants that swarmed around crushed nestmates tended to be smaller. The size distributions of each role also showed a large degree of overlap. This shows that there is no task partitioning based on size between roles performed by the smallest class of ants. All of these results suggest that morphology is not the only factor which determines role in leaf cutting ants.

RESUMEN

La división de labores permite a las sociedades especializarse, volverse más eficiente, y completar comportamientos más complejos. Las zompopas (generos *Atta* y *Acromyrmex*) se han convertido en los herbivoros dominantes del neotropico gracias al alto grado de polietismo en sus colonias. Se ha pensado que tradicionalmente la morfología determina el papel que una hormiga individual va a jugar en la colonia (polietismo de castas). La casta más pequeña, minima, se les ha asignado el papel de pedir aventones, cuido del jardín del hongo, y limpieza. El forrajeo se ha atribuido a las hormigas de tamaño mediano, media, y la defensa a las trabajadoras más grandes, llamados soldados. Sin embargo, ha habido un aumento en la cantidad de investigación en las especies de *Atta* que muestran que las minima pueden estar involucradas en un rango mucho más diverso de papeles incluyendo defensa y mantenimiento de los senderos. Este estudio consiste de dos experimentos ambos diseñados para investigar el tamaño de las hormigas cumpliendo una variedad de papeles en *Atta cephalotes*. En el primer experimento, las respuestas de las minima (≤3mm) y hormigas más grandes a un compañero de nido aplastado fueron comparados. La proporción de minimas en el sender aumentó en 8% cuando se les presentó con un compañero de nido aplastado, mostrando que fueron recrutadas para ayudar con defensa lo opuesto a las media y los soldados. También, el número promedio de minima agregandose al estímulo fue mucho mayor que aquellas de las clases más grandes (14.314 ± 1.660 comparado a 0.971 ± 0.156). En el segundo experimento, las hormigas realizando cada uno de los 4 papeles fueron medidas: (1) forrajeo, (2) aventones, (3) comportamiento de no-forrajeo (4) hormigas respondiendo a compañeros de nido aplastados. No hay diferencia significativa entre el promedio del tamaño de la cabeza de ninguno de los papeles, pero las hormigas que se agregan alrededor de las compañeras de nido aplastadas tienden a ser más pequeñas. La distribución de tamaños de cada papel también muestra un alto grado de traslape. Esto muestra que no hay partición en las labores basado en el tamaño entre los papeles realizados por las clases más pequeñas de hormigas. Todos estos resultados sugieren que la morfología no es el único factor que determina el papel en las zompopas.

INTRODUCTION

Division of labor allows societies to specialize and perform tasks efficiently and effectively. Partitioning roles and increasing social intricacy enables species to perform more complex behaviors (Wilson 1990). Eusocial insects are a hallmark example of division of labor (Griffiths & Hughes 2010). In eusocial insects division of labor is described in terms of polyethism, the functional specialization of individuals in the colony. Polytheism can occur between individuals of a different age (temporal polytheism) or between individuals of different morphologies (caste polyethism) (Hölldobler& Wilson 1990). Leaf cutter ants have one of the most extreme cases of polyethism among social insects. They have over 26 roles involved in colony life which are divided between specialized groups (Hölldobler& Wilson 1990). This division of labor has enabled them to act as the dominant herbivores in Neotropical ecosystems, foraging up to 8-15% of available leaf area in Neotropical forests (Leal et al. 2014). Understanding how tasks are partitioned within a colony is necessary to understanding how leaf cutters have become such a successful taxon.

It has traditionally been assumed that morphology directly determines the role an ant will perform in the colony (Hölldobler& Wilson 1990). Colonies exhibit a huge degree of morphological variation, with an 8x difference in head width and 200x difference of dry body weight exhibited in *Atta sextens* (Griffiths & Hughes 2010). As leaf cutters exhibit a continuous variation in ant size any attempt to lump them into discrete groups is somewhat arbitrary (Hölldobler& Wilson 1990). Wilson (1980) was the first to define a set of physical castes based on 'role clusters' which were classified as mostly separated polytheism curves. To make the curves, he first identified four worker castes (1) gardener-nurses (2) within nest generalists (3) foragers-excavators and (4) defenders. (Wilson 1980). Next, he measured the frequency with which ants of a given head width performed each task and found that although there was some overlap there was a notable peak in head-width frequency for each group, increasing from castes 1-4 (Wilson 1980). Over time this division has been more or less upheld and increasingly linked to ant size in literature. The smallest workers (minima) perform the gardener-nurses and within-nest tasks, while medium sized ants (media) cut and forage leaves and the largest group of workers (soldiers) act as defenders (Linksvayerr et al. 2002).

There have been studies which cast doubt on this hard and fast division. There is growing evidence that size and role might not be as tightly coupled and deterministic as previously thought. This is particularly true for minima, the smallest group of workers. Minima were traditionally thought to clean leaf fragments, help prepare the substrate for the fungus, care for the garden, and "hitchhike" on the leaf fragments carried back to the colony by foragers (Vieira-Neto et al. 2006, Griffiths & Hughes 2010). Many studies have investigated the purpose of their presence on leaf fragments with the majority suggesting they provide protection form parasitoids and clean potentially harmful spores from the leaf (Vieira-Neto et al. 2006). There have multiple attempts to quantitatively describe the size of ants which perform these roles. Hughes and

Goulson (2001) defined minima as ants with a head with of <1.4mm in *Atta capiguara* Another study defined *Acromyrmex echintaor* “minors” as individuals with a head width <1.2mm (Larson et al. 2014).

Although there is debate about the qualitative definition of a minima, it was generally assumed that this smallest class was limited to the roles of clearing, fungal garden care, hitchhiking, and defense against parasitoids (Vieira-Neto et al. 2006, Griffiths & Hughes 2010). However, there is emerging evidence that small ants perform other functions previously designated to ants of other size classes (Hughes & Goulson 2001, Whitehouse & Jaffe 1995, Dupuis & Harrison 2017). *Atta laevigata* minims (defined as less than 5mm in length) respond most aggressively to conspecific enemies whereas the soldier caste respond only to stimulated vertebrates that try to attack the nest entrance (Whitehouse & Jaffe 1995). In *A. capiguara* ants smaller than 5mm are also more likely to respond to conspecific or interspecific threats (Hughes & Goulson 2001). It has also been shown that *Atta cephalotes* minima will assist with trail maintenance (Dupuis & Harrison 2017). *A. cephalotes* castes were also shown to respond flexibly to the removal of other castes and assume responsibility for tasks usually allocated to the removed class (Dupuis & Harrison 2017). These studies a few leaf cutter *Atta* species support the idea that size is not deterministic of role and that caste definitions may be more fluid than previously assumed. More studies with different species are necessary to test the generality of these studies.

In this study, I tested whether size and role are as tightly linked as traditionally thought in *A. cephalotes*. Specifically, I investigated the non-traditional roles which the smallest class ants perform in this species. I focused on defense and foraging, two roles traditionally assigned to larger castes in this species (Wilson 1983, Hölldobler& Wilson 1990, Linksvayerr et al. 2002) In the case of defense, I compared the response to an alarm stimuli between castes to evaluate whether smaller ants are involved in defense as in *A. capiguara* (Hughes & Goulson 2001). In the case of foraging, I tested the assumption that minima are too small to assist in foraging (Wilson 1983, Linksvayerr et al. 2002, Vieira-Neto et al. 2006, Griffiths & Hughes 2010). A Previous study of *A. cephalotes* (Wilson 1983) determined that in order to cut vegetation of average thickness a worker must have a minimum mean head width of 1.6mm and to carry fragments of minimum head width of 1.4 mm was needed. Forgers in Costa Rica appeared to be just as small as ants observed hitchhiking or patrolling the trail (personal observation). Hence, I specifically evaluated whether there is overlap in the size of ants acting as foragers and those hitchhiking or simply present on the trail as“non-foragers”. Through comparing the size distribution of roles carried out by smaller ants, this study also assessed if there was labor partitioning based on size within the smallest class of *A. cephalotes* visible outside of the nest.

MATERIALS AND METHODS

Study Site

Colonies were located in 2 adjacent sites in Los Planos, on the pacific coast of the Tilarán mountain range in Monteverde, Costa Rica. The first site, La Finca San Fransico de Asis, was a private patch of young secondary forest. The second location was a neighboring horse ranch, La Caballeriza El Rodeo, composed of a mixture of pasture and secondary forest patches. All trials were conducted in November and October of 2017 between 8am-12pm.

Experimental Procedure

Defense test

To test defensive response to a potential threat, colonies were presented a crushed nestmate following Hughes and Goulson (2001) to measure traffic and aggregation on the crushed nestmate by different castes focusing on the proportion of ants that were minima. Leaf-cutters have been shown to respond to the pheromones of a crushed nestmate, both in *Acromyrmex echinatior* (Norman et al. 2014) and *Atta sexdens* (Veloso Francelino et al. 2008). Traffic was measured before and during each trial in situations where a crushed nestmaste was present (defense test) and where it was absent (control). For a 2 minute period the number of minima and media ants passing a point was recorded. Minima were identified visually as ants ≤ 3mm. After traffic was recorded, the test stimulus was placed on the side of the foraging trail. Trials were conducted on foraging trails 1-2m from the nest entrance where responses are more likely to occur (Hughes and Goulson 2001). For the defense test, an average sized media forager was selected from the trail (approximately 5mm body length as determined visually in the field) and crushed using a metal rod. The entire body was then placed to the side of the trail. As a control, a small metal screw 5mm in length was placed on the trail. For both the control and the defense trials, traffic was measured for 2 minutes after the stimulus was placed on the trail. At the end of the 2 minutes the number of ants of minima and media swarming the object was counted. Control and defense trials were performed sequentially but the order was alternated to avoid any additive affects. Each trail was left undisturbed for at least 30 minutes between trials to allow it to return to baseline traffic. Seven colonies were tested with five replicates of both defense and control trials per colony. For two colonies, all ants which swarmed the crushed nestmate were collected in order to measure their size.

Size Comparison

To determine the size of small ants performing various roles 10 ants were collected from each of three roles: (1) hitchhiking (2) non-foragers and (3) foragers. Hitchhikers were identified as ants riding on leaf fragments towards the nest. Occasionally, ants were observed quickly climb on and off a leaf fragment. To avoid collecting these ants, hitchhikers were defined as any ant riding on a leaf fragment for more than 10 seconds (Linksvayer et al. 2002). Non-foragers were defined as ants travelling in the direction of the nest entrance without a leaf fragment. It was assumed that these ants did not participate in foraging behavior as otherwise they should have been returning with a leaf fragment. Foragers were identified as any ant carrying plant material in the direction of the nest entrance. As the goal of this experiment was to determine if there is overlap in the size range of small ants in different roles, only the smallest foragers were collected. Trails were observed for an hour and the smallest 10 foragers, as determined visually, were collected. During this same period, the first 10 hitchhikers and non-foragers were collected to get a random distribution of the size on ants in those roles. Ants were collected from 5 colonies, for a total of 50 ants of each role. Additionally, the ants collected from the defense test, were measured and compared. Twenty ants in the swarm around the crushed nestmate were collected from each of two colonies.

Photos were taken of the head of every collected individual through a dissecting microscope. Head width was measured from the photos using the software ImageJ at the broadest part of the head, directly behind the eyes.

RESULTS

Defense Test

Traffic was expressed as the proportion on minima to large foragers on trails before and after the treatment (crushed ants vs control). The proportion of minima varied between treatment and pre/post measurements (Linear Mixed Model , LMM, controlling for dependency within colony and trail (random effects) F=12.636, df=1,110, p=0.0006, Figure 1). Specifically, traffic was composed of about 45% minima before the object (crushed ant or control) was placed on the trail and even after when the control objected was placed on the trail. There was a significant increase in the proportion of minima on the trail when presented with the crushed nestmate was placed on the trail (45% to 53%). In other words, the only caste that responded to the stimulus was the minima. No soldiers were every observed responding to either test. Additionally, no foragers actively carrying a leaf or hitchhikers ever responded to the trials.

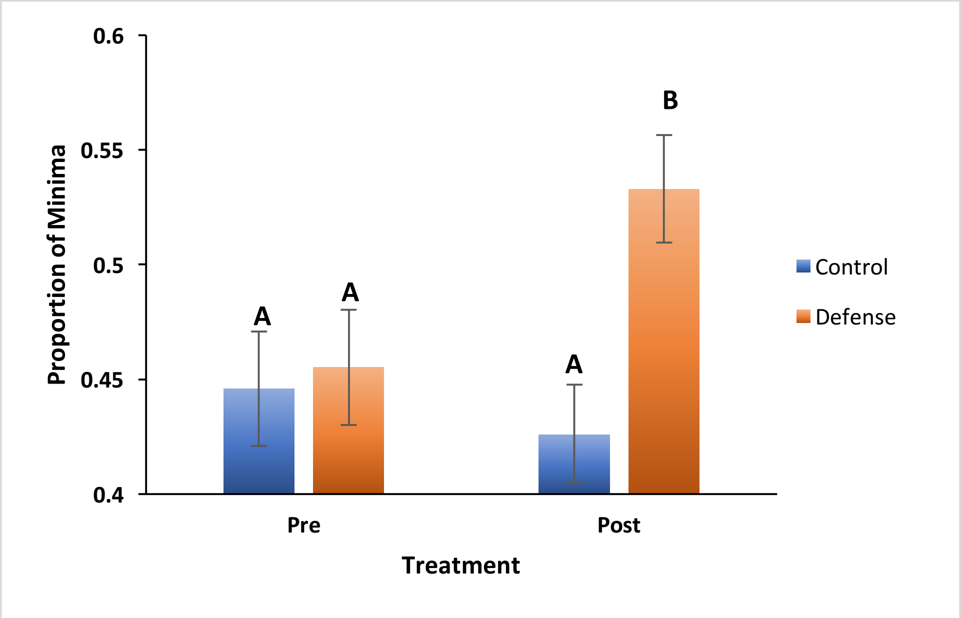

Figure 1. Proportion of *Atta cephalotes* minima counted in a 2 minute period on a foraging trail before and during treatment. Minima were identified visually as any ant ≤ 3mm. During the control treatment a small metal screw was placed on the trail. During the defense test a crushed nestmate was placed near the trail. The baseline proportion was equivalent for both treatments and was the same for the proportion of minima measured during the control trial. There was an increase of 8% in the proportion of minima on the trail after the defense stimuli was placed on the trail. Error bars show ± one standard error, n=35 for each treatment. Letters above error bars show significant differences according to post hoc Tukey tests. Include the sample sizes in the figure caption. Same for all other figures with means.

In addition to traffic, the number of ants of each given size class that swarmed the stimuli were counted. The ants which swarmed are described as “responders”. The number of ants differed between treatment and caste (Generalized Linear Mixed Model with Poisson distribution, colony and trail as random effects: Chisq=5.967, df=1 P=0.015, Figure 2). When presented with the control, more often than not, no ants swarmed the metal screw. Out of 35 trials, minima only swarmed the screw 6 times with no more than 2 responding per trial. Only 3 control trials had any media swarm, with only 1 ant on each of the three occasions. There was no statistical difference between the response of either size group to the control stimuli

When presented with the defense stimuli both size classes were observed swarming the stimuli, but many more minima swarmed than media (figure 2). Out of the 35 trials there were 13 instances where zero media swarmed, and no more than three were ever observed in the swarm. There were no occasions in which zero minima swarmed the crushed nestmate.

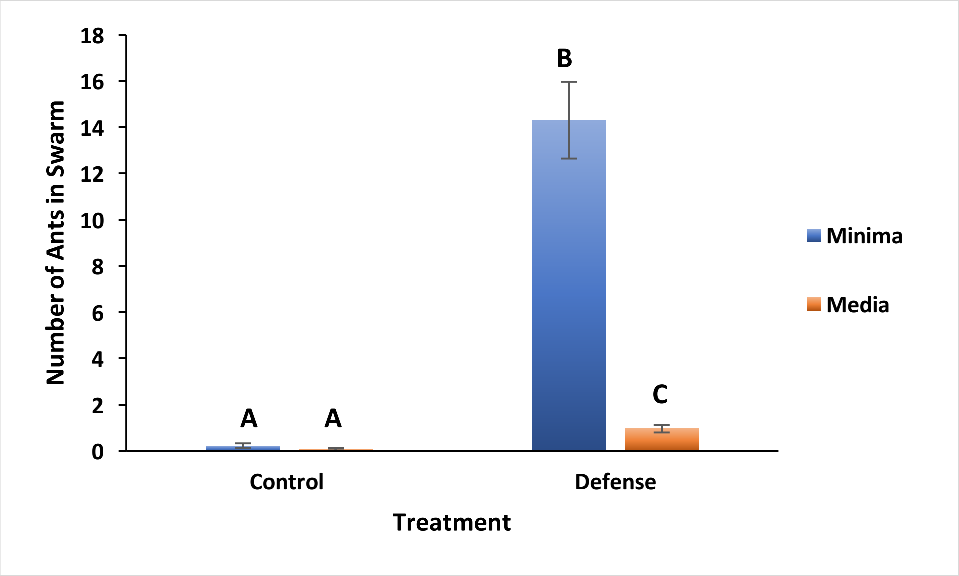

Figure 2. The number of *Atta cephalotes* media and minima swarming either a control (metal screw) or defense (crushed nestmate about 5mm body length) stimuli. Minima were identified as ants less than 3mm in body length and all ants larger were classified at media. There were practically no ants of either caste which responded to the control stimuli. The number of both ants was higher in response to the defense stimuli. However, the number of minima which responded was much higher (mean of 14.314 ± 1.660, n=35 compared to 0.971 ± 0.156, n=35). Error bars show ± one standard error. Letters above error bars show significant differences according to post hoc Tukey tests.

Size Comparison

The hitchhikers, foragers, and non-foragers collected were clearly not different in head size (figure 3) (LMM, F=0.233, df=2, 156, p=0.793). However, when compared with the size of ants that swarmed around crushed ants, the ants which swarmed, the “responders” trend to be smaller than the ants collected in the other roles (F=2.188, df= 3, 195, p=0.091).

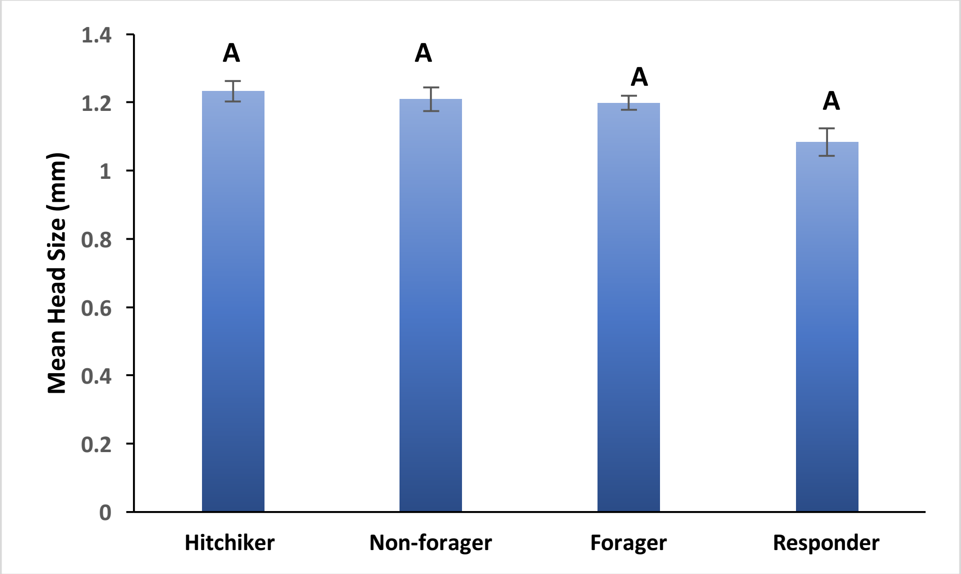

Figure 3. The mean head size of *Atta Cephalotes* ants collected performing 4 roles. Hitchhikers (n=51) were riding leaf fragments, foragers (n=51) were carrying leaf fragments, non-foragers (n=61) were walking towards the nest entrance without leaf material, and responders (n=40) were the ants which swarmed a crushed nestmate. Head size was defined as the width at the widest point of the head and was measured using ImageJ. There was no significant difference ($p>0.05$) between the size of ants in any role, however responders trended towards being slightly smaller. Means are presented with ± one standard error. Letters above error bars show significant differences according to post hoc Tukey tests.

It is also interesting to note that the size distributions of the different roles largely overlap (figure 4). The large majority of the foragers fell between 1-1.5mm head width, about the middle of the spectrum. The smallest observed foragers were selected, so this result demonstrates that the smallest foragers are equal in size to an average sized hitchhiker or non-forager. The distribution of the responders also overlaps, but it more heavily laden on the lower end of the size range.

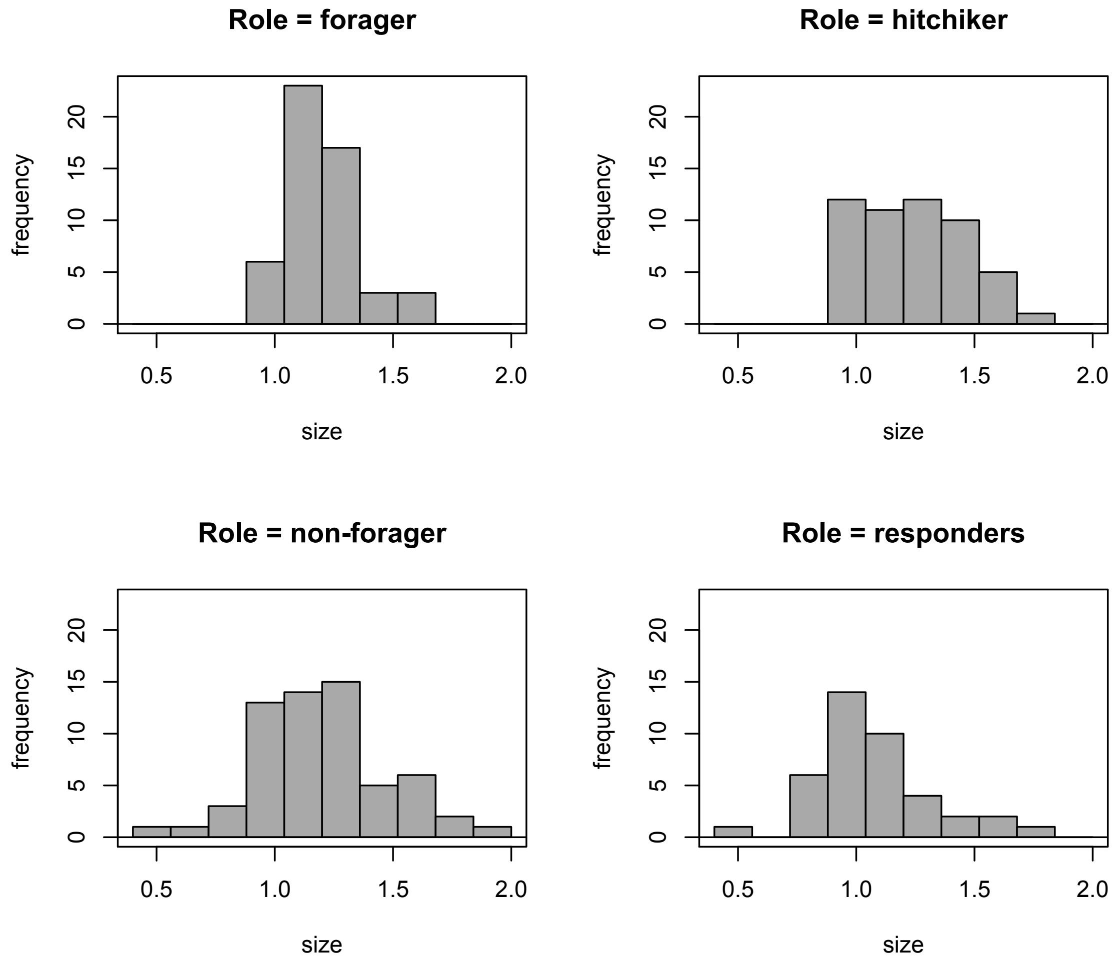

Figure 4. The distribution of head width (mm) between *Atta cephalotes* fulfilling different roles. Foragers, hitchhikers, and non-foragers were collected from 5 colonies. Foragers were identified as they were carrying leaf fragments. The smallest 10 foragers observed in an hour were collected. Hitchhikers were observed riding leaf fragments and the first 10 observed were collected. Non-foragers were defined as ants walking towards the nest entrance and the first 10 0besrved were collected. Responders were the ants which swarmed a crushed nestmate placed on the foraging trail in experiment 1. There is overlap between distributions of all size roles.

DISCUSSION

Defense Test

The minima were recruited to the defense stimuli much more than media. These results are consistent with the findings that *A. capiguara* minima are more aggressive in response to a crushed nest mate than larger workers (Hughes and Goulson 2001) and that *A. laevigata* minima are more responsive to a conspecific threat (Whitehouse and Jaffe 1996). This contradicts the traditional idea that the soldier class would be tasked with defending the colony. Soldiers do respond to the threat of a vertebrate enemy attacking the nest entrance, but in other situations defense is left to smaller ants (Whitehouse & Jaffe 1996).

This may be because smaller ants are better at detecting intraspecific and conspecific enemies (Houghes & Goulson 2010). They are more aware of vibrations resulting from stribulations, a signal of alarmed ants (Houghes & Goulson 2010). They are also a better alarm system as they a more efficient way of transmitting information. Producing minima requires a much smaller energy investment than larger ants, therefore it is better to have many minima patrolling and communicating than just a few soldiers (Hughes & Guolson 2010). These patrolling minima would be the first to respond and be recruited to a detected threat (Hughes & Goulson 2010).

It is also possible that relying on minima may not be just energy efficient, but also a strategically superior defense system (Hughes & Goulson 2010). As most conspecific and intraspecific conflicts occur in the open, it is assumed that battles follow the square law which assumes individuals are killed at a proportional to that of the individuals were in the opposing side. In situations where the square law is in play it is better to have many poor fighters (minima) than a few good fighters (media or soldiers). (Whitehouse & Jaffe 1996).

Minima may also be critical to leaving a pheromone marking on the battle site (Whithouse & Jaffe 1996). Workers are better oriented in areas marked with the chemical mark of their territory and are thus more effective fighters (Salzemann & Jaffe 1990). Conversely, ants fighting in an area marked with the pheromones of a foreign colony are more likely to adopt a submissive posture (Salzemann & Jaffe 1990). Having a large number of minima ensures that many individuals will be in the area who can leave a chemical mark on the battle field (Whitehouse & Jaffe 1996). Whatever the mechanism, the result indicates that small ants are very involved in the response to a potential threat in *A. cephalotes*.

Minima as Foragers

Traditional understanding of labor division in *A. cephalotes,* and leaf cutters in general, assumed that morphology determines the role performed in the colony (Wilson 1983). Historically, minima were believed to be involved in cleaning, care of the fungal gardening, and hitchhiking while all foraging was carried out by larger individuals. It was assumed that minima were too small to forage (Vieira-Neto et al. 2006, Griffiths & Hughes 2010, Linksvayer et al. 2002). There have been attempts to quantify this, with Wilson (1983) defining foragers as ants no smaller than 1.4mm. The results of this study contradict these definitions.

No statistical difference was observed between the mean head width of ants in any of the 4 roles measured, defense included. This result shows that minima are involved in foraging. This phenomena has never been described in leaf cutting ants, and contradicts current understanding of the role of minima. Only the smallest observed foragers were collected and the majority were around the average size of hitchhikers or non-foragers, and generally slightly larger than the ants which swarmed the crushed nestmate. The majority of foragers observed were larger than those measured in this study. Foraging and cutting have physiological size constraints in terms of the weight an ant can carry, the thickness of material that can be cut, and the speed at which the ant can travel (Rudolph & Loudon 1986). It has been shown that colony must have foragers of at least 1.6mm in order to cut material of average thickness (Wilson 1983). It is also worth noting that an ant does not need to cut in order to carry (Schofield et al. 2011). It is possible minima are just carrying and not cutting. However, that would still be a novel result as it has been assumed no ant less than 1.4mm can carry (Wilson 1983).

Size also places limitations on how quickly an ant can carry material. Body size is correlated to speed, with larger ants traveling more quickly (Rudolph & Loudon 1986). It may seem counterintuitive to have smaller workers involved in foraging if they are less efficient. However, if they are capable of carrying plant material and do not have another pressing role it makes sense that minima would assist with foraging. Especially in less active trails where a slower ant is less likely to slow down the flow of traffic (Freeman & Chaves-Campos 2016). This study was conducted around 1300m elevation, where leaf-cutter trails are generally less active than in the lowlands (Houghes & Goulson 2010). It is possible that more smaller ants were observed than would have been seen at a different study site.

Minima in Non-traditional Roles

It appears that ant size and role are not as tightly linked in *A. cephalotes* leaf-cutter ants as previously thought. Instead, the smallest class of *A. cephalotes* perform every role of which they are capable of, including foraging, hitchhiking, defense, and non-forging behavior (i.e presence outside of the colony most likely for defense or trail maintenance (Dupuis & Harrison 2017).

Of course, there are still physical constraints on which roles an ant can fulfill. Cutting requires a minimum head width, and larger foragers are more efficient (Rudolph & Loudon 1986). Conversely, there is likely a maximum size of possible hitchhiker as otherwise the forager would be too weighed down by the load of her hitchhiker to forage efficiently (Rudolph & Loudon 1986). Although there may be exclusion at the ends of the spectrum, it appears as though small ants perform all the castes to the degree to which they are able. Size is just a physiological constraint and not the determining factor.

It is also interesting to compare the distributions of ant sizes. All of the roles had largely overlapping distributions. It is not clear whether all minima act in all the roles or are faithful to a given role. Wilson (1990) noted that there could be polyethism at a finer scale, within the smallest size class. However, the overlapping size distributions shown here suggest that if there is task partitioning at a small scale it is not based on size.

CONCLUSION

The results of this study show that the smallest class of *A.cephalotes* perform many roles not traditionally associated with them. They take an active role in defense and foraging. These results add to the growing body of evidence drawn from studies of other *Atta species (A. capiguara, A. laevigata* and *A. cephalotes*) which challenge the traditional roles of minima (Whitehouse & Jaffe 1995, Hughes & Goulson 2001, Dupuis & Harrison 2017). Together these studies suggest that this pattern can be generalized to all leaf-cutter ants. This contradicts the assumption that morphology determines the role an ant will take in a colony. Leaf-cutter ants have one of the most complex systems of caste polyethism. The intricacy of their social structure has allowed them to become the dominant herbivores of the Neotropics (Leal et al. 2014). The results of this study show that the division of labor operates at a level beyond just ant morphology and is even more complex than previously thought.

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Neighbor-stranger discrimination in leaf cutter ants (*Atta cephalotes)*

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ABSTRACT

Territorial animals use aggressive behaviors as a defense mechanism. Some species respond with more aggression towards strangers than towards their neighbors, known as the dear enemy phenomenon (DEP). Territoriality is common among many insects; such as leaf cutter ants, however only one study has been done to test the DEP on one species of leaf cutter ants, *Acromyrmex lobicornis.* In this study, the DEP was tested on the leaf cutter ants, *Atta cephalotes.* To test this phenomenon ten focal colonies were chosen with three treatments, a control (ants from the same colony), neighbors (ants from the closest neighboring colony) and stranger (ants from a far away colony) were introduced into the focal colony. Higher aggression levels were observed towards strangers than to neighbors than to control (mean score for control=0.92+/-0.15, for neighbor=2.22+/-, for strangers=3.35+/-0.2). A score of 0 indicates the ant was ignored, a score of 2 indicates touching, a score of 3 indicates biting of the legs and/or antennas, a score of 4 indicates biting of the abdomen/body and a score of 5 indicates the ant was dragged or pinned down and held. Another test was performed to determine if levels of aggression increased with increasing distances from the colony. Three focal colonies were tested with five other colonies from progressively farther distances. All strangers received around the same levels of aggression after around 78 meters (mean score for control=0.8+/-0.24, for neighbors=2.07+/-0.28, for stranger 2=3.67+/-0.37, for stranger 3=3.53+/-0.32, for stranger 4=4+/-0.31, for stranger 5=3.27+/-0.42). This is likely because the average foraging trails of *A. cephalotes* is around 78 meters, therefore after a certain distance the ants never come into contact with each other. These results confirm the DEP in *A. cephalotes*, after a certain distance all intruders are treated with equal aggression likely due to habituation.

RESUMEN

Animales territoriales usan comportamientos agresivos como mecanismo de defensa. Algunas especies responden con mayor agresión hacia extraños que vecinos, lo cual es conocido como el Efecto Querido Enemigo (EQE). Territorialidad es común entre insectos tales como las hormigas cortadoras de hojas, sin embargo solo un estudio se ha realizado para probar EQE en una de las especies de hormigas cortadoras de hojas *Atta cephalotes.* Para probar este fenómeno se escogieron 10 colonias focales con tres tratamientos, control(hormigas de la misma colonia), vecinos(hormigas de la colonia mas cercana) y extraños(hormigas de una colonia lejana) las cuales fueron introducidas en una colonia focal. Altos niveles de agresión fueron observados hacia extraños que vecinos o control (puntaje promedio control=0.92+/-0.15, vecino=2.22+/-, extraño=3.35+/-0.2). Un puntaje de 0 indica que la hormiga fue ignorada, 2 indica que fue tocada, puntaje 3 indica mordedura en piernas y/o antennas, puntaje 4 indica mordedura en el abdomen/cuerpo y puntaje 5 indica que la hormiga fue arrastrada y retenida. Otra prueba fue realizada para determinar si los niveles de agresión incrementaban con respecto a la distancia de la colonia. Tres colonias focales fueron analizadas con respecto a otras 5 colonias con distancias cada vez más lejana progresivamente. Todos los extraños recibieron aproximadamente los mismos niveles de agresión después de los 78 metros (puntaje promedio para control=0.8+/-0.24, para vecinos=2.07+/-0.28, para extraño 2=3.67+/-0.37, extraño 3=3.53+/-0.32, extraño 4=4+/-0.31, extraño 5=3.27+/-0.42). Esto es porque los senderos de forrajeo promedio de *A. cephalotes* son alrededor de 78 metros, por tanto depués de cierta distancia las hormigas nunca entraron en contacto con otras. Estos resultados confirman EQE en *A.cephalotes* después de cierta distancia todos los intrusos son tratados con agresión debido a la habituación.

INTRODUCTION

Territorial behavior is the process by an individual or group of species actively defends an established area against conspecific intruders who threaten to use the limited resources that exist in this area (Creasey et al. 2014). Not all conspecific intruders pose the same level of threat (Brown 1964). Animals have developed methods in order to save energy. One such method is the 'neighbor-stranger' discrimination theory which describes how territorial animals can distinguish between different intruders (Falls 1982). Neighbors are treated with less aggression than strangers because they pose less of a threat (Brown 1964).

Numerous territorial species show more aggressive behaviors towards strangers than towards their neighbors (Heinze et al. 1996). This behavior is often referred to as "the dear enemy phenomenon" (DEP) which asserts that strangers from more distant territories pose a bigger threat than neighbors from nearby territories (Temeles 1994). This is likely because the strangers are potentially searching for new territories and become a threat to resources (Temeles 1994). It makes sense not to fight with neighbors because you are more likely to come into contact with them more often and since they are already established nearby they pose less of a threat to resources than strangers who may be searching for new territory and resources. The less aggressive behaviors towards neighbors of territorial species is considered adaptive in that fights are less frequent thus avoiding the costs of many fights (Jaeger 1981). Evidence for neighbor-stranger discrimination has been found in five major taxa, including birds, mammals, fish, amphibians and insects (Falls et al. 1988). The "dear enemy" behavior is common among social insects (Heinze et al 1996).

Territorial behavior is common in species with fixed nests or colonies, such as ants, that have to defend their territory from other species that are a possible threat for limited resources (Hölldobler and Lumsden 1980). Many ant species show varying levels of aggressiveness towards their neighbors and strangers from far away colonies, indicating some form of inter-colony recognition, they can distinguish ants from their own colonies from other intruder ants (Bonavita-Cougourdan et al. 1987).

The mechanisms behind neighbor-stranger recognition in ants are not well known (Dimmarco et al. 2010). One possible mechanism for the variation in aggressive behavior of ants towards their neighbors and strangers is habituation to a persistent odor stiumulus until it is overlooked (Orivel et al. 1997; Langen et al. 2000). It may be possible that the ants in one colony that may forage near ants in another colony have habituated to recognize the different pheromone odors produced by familiar ants. The alternative mechanism, genetic divergence, is where ants become less genetically similar as they come from colonies of greater distances, and therefore are more aggressive towards ants from greater distances then they are to ants in which they are more genetically related (Dimmarco et al. 2010).

Leaf-cutter ants (genera *Atta* and *Acromyrmex*) are good systems to study the DEP because they show intraspecific aggression in order to protect their territories (Höldobler and Wilson 1990). Recognition is achieved through chemical cues (Hernández et al. 2006). Only one study has tested for the DEP in *Acromyrmex lobicornis* and found support for the DEP (Dimmarco et al. 2010). Dimmarco et al. (2010) found differential aggressiveness by neighbor status to be related simply to being neighbors or not and unrelated to the distance between the colonies which suggests this is due to habituation and not genetic divergence.

Leaf cutter ants, *Atta cephalotes* use pheromones, as a discrimination cue (Hernández et al. 2006). It is known that *A. cephalotes* mostly forage within 78 meters of their colonies (Cherrett 1968). If the habituation hypothesis is correct, colonies will react the same towards all intruders after around 78 meters because they are unlikely to come into contact with colonies beyond that distance most of the time.

More studies are necessary to evaluate whether the results of the study by Dimmarco et al. (2010) apply to other species of leaf cutter ants. In this study, I tested the DEP on, *A. cephalotes,* by conducting intrusions by ants within the same colonies, ants from neighboring colonies and ants from distant colonies (strangers) in order to evaluate their levels of aggressiveness towards the intruders. I also looked at distance and if increasing the distance of the strangers from the focal colony would increase the aggression levels or if after a certain distance they are treated the same.

MATERIALS AND METHODS

Study Site

Data were collected over one month on sunny days between 8:00 am and 12:00 pm in November 2017. The study was conducted on two properties, Caballeriza El Rodeo which is mainly horse pasture with small secondary forest patches, and Finca San Francisco de Asis, a private forest reserve, in Santa Elena, Puntarenas Province, Costa Rica. The Holdrige Life Zone is tropical premontane wet forest. Colonies of *A. cephalotes* were located in and around forests edges and pastures.

Strangers vs Neighbors

Ten colonies of *A. cephalotes* were located in and around forest edge and in pastures. These colonies were used as the focal colonies to test aggression against ants from different colonies. Three treatments were tested on each focal nest, a control, neighbor and stranger treatment. For each focal colony, five foraging ants from that colony were taken and marked with a paint pen and then placed in containers for around 10 minutes before being reintroduced into the focal colonies (control ants). The process of marking ants with acrylic pens does not produce alterations in behavior and is often used to mark ants (Roulston et al. 2003; Ballari et al. 2007). The neighbor treatment was similar to the control except the five foraging ants were collected from the closest neighboring colony, around 50 meters from the focal colony. Finally, five stranger ants were taken from a distant colony at least 200 meters away from the focal colony and were again marked and put into containers before being introduced into the focal colony. All treatments were done in the same way in order to assure all foraging ants being tested had the same conditions.

Fifteen foraging ants, five from each of the three treatments (control, neighbor and stranger) were placed into the foraging trail, alternating between control, neighbor then stranger ants, of the focal colony within 1 meter of the nest entrance. The ants were observed for one minute after being introduced into the focal colony and the highest level of aggression observed was recorded. Aggression levels were assigned based on an aggression scale from 0-5 based on the scale used by Diamarco et al. (2010). The different levels were 0=the ant introduced to the focal colony was ignored, 1=there was antennation between the ants, 2=the ants were touched on other parts of their body, 3=biting of the legs or antennas, 4=biting the abdomen/body, and 5=the ant was dragged, or pinned down and held.

The individual plastic containers were then cleaned with alcohol to minimize the mixing of odors between the ant colonies since the foraging ants will deposit colony specific pheromone chemicals (Hölldobler & Wilson, 1996). The process was then repeated for all ten focal colonies.

Distance from colony

Three focal colonies were located for this experiment. For each colony, six treatments were tested. The treatments were of increasing distances from the focal nest. Starting with the control, then the closest neighbor, and four more colonies at progressively farther distances from the focal nest. They were labeled as stranger 2, stranger 3, stranger 4 and stranger 5 (Figure 1). Five ants from each treatment were marked, put into containers and then reintroduced into the foraging trail of the focal colony. The aggression scores were recorded.

Each treatment was done on a different day on the three focal colonies to prevent the possibility that ants get progressively upset upset during the day if multiple treatments were conducted on the same day. Each day a different treatment was tested on all three of the focal colonies, using the same methods as used in the stranger vs. neighbor experiment. The order in which the treatments were tested was random to control for potential increased response over the days. On day one the fourth strangers (average distance of 125 m away) were tested. On the second day the second strangers (average distance of 88 m) were tested. On the third day the neighbors, or first strangers (average distance of 55 m) were tested. On the fourth day the control (ants from the focal colony) were tested. On the fifth day the fifth strangers (average distance of 242 m) were tested. Finally, on the sixth day the third strangers (average distance of 178 m away) were tested (Figure 1).

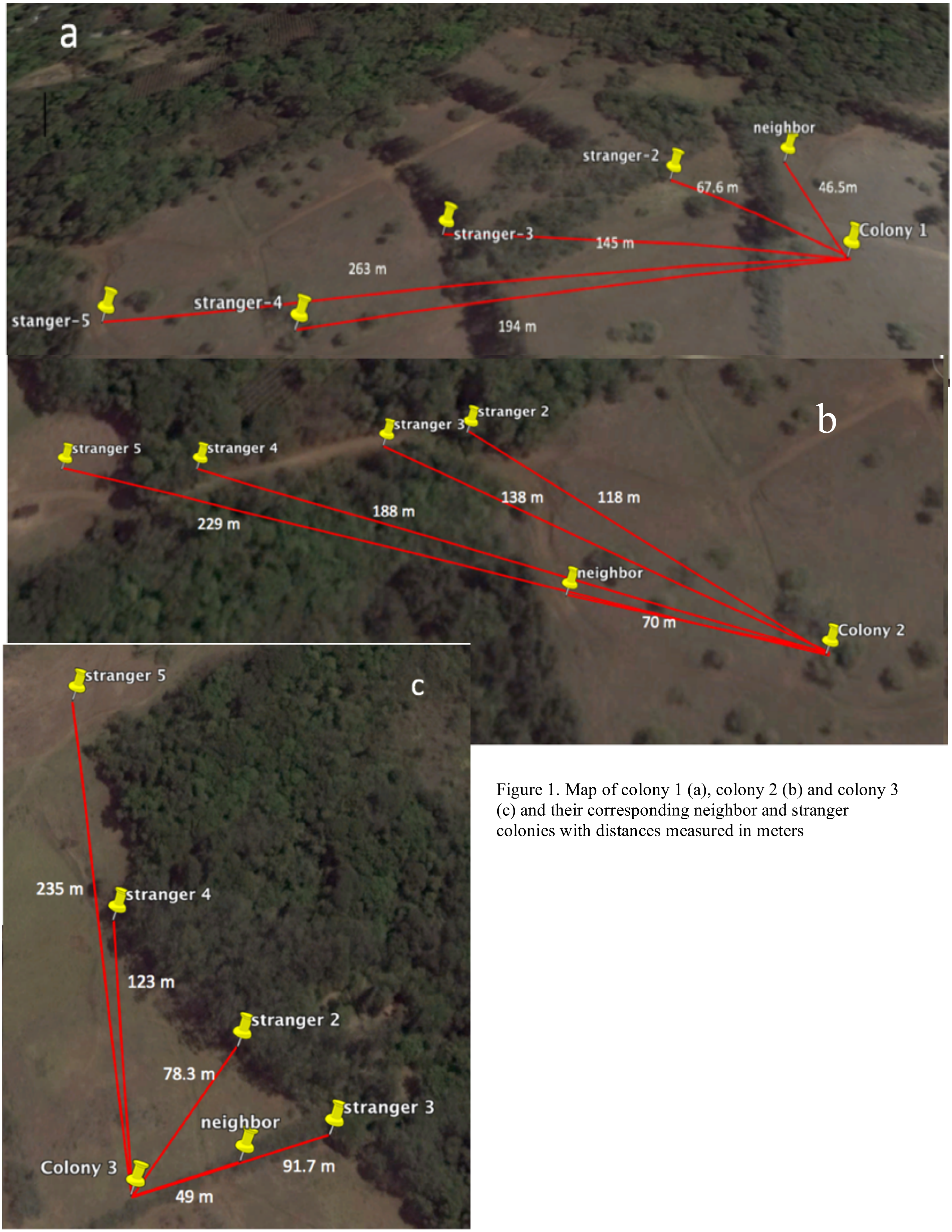

Figure 1. Map of colony 1 (a), colony 2 (b) and colony 3 (c) and their corresponding neighbor and stranger colonies with distances measured in meters

Results

Strangers vs. Neighbors

Focal colonies responded with different levels of aggression to ants from three different treatments (Generalized Linear Mixed Model with poisson distribution and colony included as random effect: chi-square=74.01, p=0.0001). Ants were more aggressive towards strangers than towards their neighbors than to control ants. Ants from the same colony only received antennation (score 1), ants from neighboring colonies were only touched in other body parts (score 2). While the strangers received biting (score 3) (Figure 2).

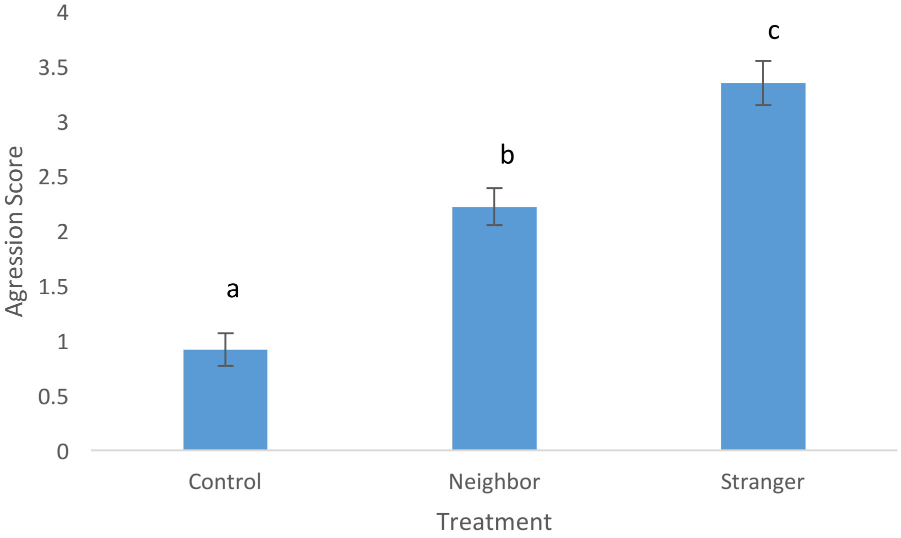

Figure 2: Mean aggression scores of *Atta cephalotes* ants when other foraging ants from the same colony (control), closest neighbor (neighbor) and a far away colony (stranger) were introduced into the foraging trail of the focal (control) colony. Means with different letters are significantly different according to Turkey post-hoc tests. Means are presented with +/- 1 standard error. A higher aggression score indicates more aggressive behaviors while a score of 0 indicates no aggression observed.

Distance from colony

In general ants respond more aggressively to strangers than to neighbors than to control ants (Generalized Linear Mixed Model with poisson distribution and colony included as random effect: X^2=47.08, p=0.0001), mirroring the results from the previous experiments but the average response to the four stranger treatments (stranger-2, stranger-3, stranger-4 and stranger-5) was similar (Figure 3). The mean aggression score was the lowest for the control (ants from the focal colony) with a mean score of 1 indicating antennation between the ants. Followed by the neighbors (ants from the closest colony) with a mean aggression score of 2 indicating the ants were touched by others and finally the most aggression was observed in the stranger colonies, which all showed a similar mean aggression score between 3 and 4 indicating the ants were bitten on the legs or antennas or they were bitten on their abdomen or bodies (Figure 3).

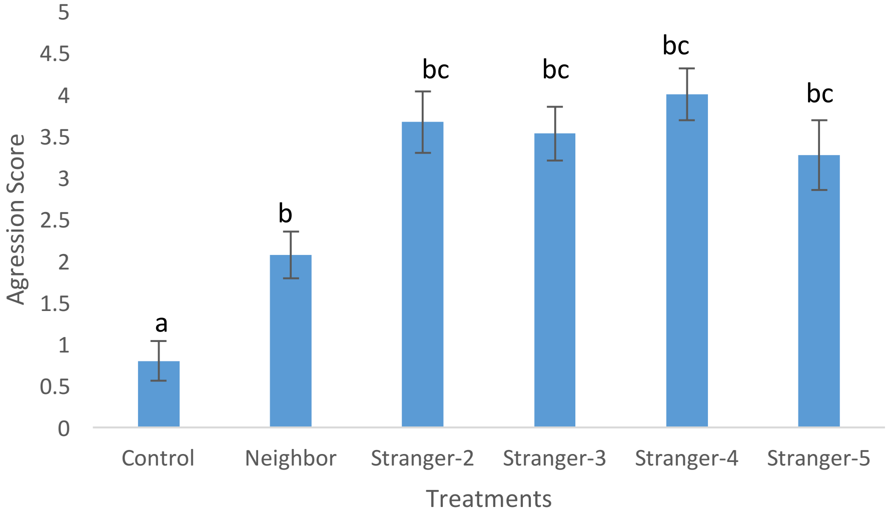

Figure 3: Mean aggression scores of Atta cephalotes ants when foraging ants from colonies at different distances were introduced into the focal (control) colonies. Ants from the control came from the focal colony, neighbor ants came from the closest colony to the focal colony and stranger-2 through stranger-5 ants came from colonies at progressively farther distances from the focal colony. Means with different letters are significantly different from one another (Tukey post-hoc test). Post-hoc comparisons between the neighbor and stranger categories are marginally not significant (p around 0.1) whereas comparisons between stranger categories are clearly not significant (p>0.90). Bars represent +/- standard error. A higher aggression score indicates more aggressive behaviors while a score of 0 indicates no aggression observed.

DISCUSSION

Similar to the only other study done on leaf cutter ants (Dimarco et al. 2010), I found that *A. cephalotes*, were more aggressive towards strangers from far away colonies than ants from their closest neighboring colonies, which supports the dear enemy hypothesis. This indicates that *A. cephalotes* can discriminate ants from stable adjacent colonies from other colonies, and vary their response accordingly (Temeles 1994). One possible explanation for the variation in aggressive behavior is habituation (Orivel et al. 1997; Langen et al. 2000). They use odors to distinguish ants from their colony and from other colonies (Hernández et al.2006). The ants could learn to recognize the different odors of foraging ants in different nearby colonies, while the far away colonies remain foreign and pose a greater threat. One possible threat could be that one colonies fungal gardens has become infected, as this is their only source of food they are forced to leave in search of new territory and may end up in the foraging trails of another colony (Wirth et al. 2013). A second and more likely threat could be that a new queen is establishing a colony too close to an already existing colony and the new foragers are getting into the foraging trails of an existing colony (Hölldber and Wilson 1990). In both cases the resources are being threatened.

Similar to the results in the other study done on leaf cutter ants, my results support the habituation hypothesis (Dimarco et al. 2010). It is known that *A, cephalotes* forage within 78 meters of their colonies (Cherrett 1968), so after 78 meter all ants are treated the same since they never come into contact with each other and therefore do not have the opportunity to learn their smells. All of the neighboring colonies were within 78 meters, so it is likely that they come into contact with the focal colony and have learned that its not worth fighting with as much aggression as this colony is already established and sharing resources.

Ants were found to be more aggressive towards strangers from far away colonies than towards their close neighbors, however the distance from the colony did not impact their aggression levels. They were equally aggressive towards all stranger ants and consistently less aggressive towards the ants from their closest neighbor colony. The colonies often experienced overlap of their home ranges and foraging trails, which is common among many ant species (Heinze 1996). This could indicate that they come into contact more often with ants from close colonies, but after a certain distance all they no longer come into contact and treat all the ants from distant colonies the same. The closest stranger colony was around 68 m away while most of the stranger colonies tested were at distances greater than 78 m away from the focal colonies (Figure 1). *A. cephalotes* mostly forage between 30 and 78 m, only 2 cases in which they forage farther than 78 m from their nest (Cherrett 1968). Therefore, it is likely that all stranger colonies test never come into contact with the focal colony. Another study done found the opposite to be true, they observed an increase in aggression between ants from different species with increasing distance from the colony (Heinze et al. 1996).

Another possible explanation for their differing aggression levels towards neighbors and strangers is the genetics of the ants. Ants from closer neighboring colonies may be more genetically related to the ants in the focal colonies, and they might be less inclined to attack their relatives than non-related stranger ants. The previous study on leaf cutter ants looked at the genetic divergence between colonies, but failed to find significant evidence to support genetics as a mechanism for the dear enemy hypothesis (Dimarco et al. 2010). This is not a likely explanation for my results since I did not see an increase in aggression and distance increased, instead I saw aggression level off after around 78 meters from the colony. More studies on genetics should be done in order to eliminate the genetic factor in the case of *A. cephalotes*.

In conclusion, my results show that the dear enemy phenomenon is present in *A. cephalotes* ants. They more aggressive towards ants from farther away stranger colonies than those from their closest neighboring colonies. However, the distance from the colonies did not increase consistently with the aggression levels. All strangers were treated the same, after a certain distance from the focal colony. This is only the second experiment of this kind with leaf cutter ants and the first experiment done of *Atta* ants, and suggest that the dear enemy phenomenon is a common behavior in leaf cutter ants.

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Horn Morphology and Mating Success in Neotropical Dung Beetle *Ontherus pseudodidymus*

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Abstract – Weaponry are exhibited by many different species and employed as tools to gain reproductive advantage over members of the same species. Dimorphisms in morphology of weaponry often correspond to differences in competitive success among males, even creating major/minor archetypes based on trait exaggeration. Well-studied tunneling horned beetles (THB) tend to exhibit weapon exaggeration in this way, although it has been shown in some systems that weapon allometry alone is not enough to describe reproductive success. Few studies describe how relatively small horns or unusual morphologies are used to win contests. *Ontherus pseudodidymus* is a neotropical THB that exhibits unusual dimorphism in male horns. Some males have a simple single horn (SHM) while others end in a broadly notched or forked morphology (FHM). Males were measured for mass, prothorax width and horn length and paired based on similar size but differing horn morphology in sexual competition trials to identify potential trends in reproductive success based on horn morphology. SHM had on average 127% longer horns than FHM, contradicting previous observations in size difference. Males were heavier on average than females by 111%, and showed no telltale breakpoint or other feature pointing towards major/minor male classifications. SHM had a positive horn allometry while FHM did not show a clear allometric trend. The probability of winning is highest for SHM when they have larger horns and body sizes. FHM had the best chance of victory with little difference in horn length but larger relative body size to their competitor. Selection seems to favor both traditional exaggeration as well as potentially less costly alternate morphology in some conditions.

Resumen- Armas son exhibidas por muchas especies y usadas como herramientas para ganar ventaja reproductiva sobre miembros de la misma especie. El dimorfismo en la morfología de las armas responde usualmente a diferencias en el éxito competitivo entre machos, incluso creando mayor/menor arqueotipo basado en la exageración del rasgo. Estudios de escarabajos cuernudos de tuneles tienden a exhibir una exageración en sus armas de este modo, aunque ha sido demostrado que en algunos sistemas solo esta alometría no es suficiente para describir su éxito reproductivo. Algunos estudios describen como relativamente cuernos pequeños o morfologías inusuales son usadas para ganar competencias. *Ontherus pseudodidymus* es un escarabajo cuernudo neotropical que exhibe dimorfismo de forma inusual en machos. Algunos machos tienen un cuerno simple (CS) mientras otros terminan en una amplia ramificación (CR). Se midió la masa de los machos, ancho del protórax, largo del cuerno y fueron emparejados con tamaño similar pero diferente morfología de cuerno en pruebas de competencia sexual para identificar potenciales tendencias en el éxito reproductivo basado en morfología de cuernos. CS tuvieron en promedio 127% cuernos más largos que CR, contradiciendo previas observaciones en diferencia del tamaño. CS tuvo una alometría positiva mientras que CR no mostró una tendencia clara en su alometría. La probabilidad de ganar es mayor para CS cuando estos poseen cuernos largos y tamaño corporal. CR tuvo el mayor chance de victoria con pequeña diferencia en el tamaño del cuerno pero mayor tamaño corporal que su competidor. La selección parece favorecer ambas, exageración tradicional así como un menor costo potencial de la morfología alterna en algunas condiciones.

INTRODUCTION

Many different species of animals employ weaponry or ornamentation in the process of courtship. Darwin hypothesized that the driving force behind the development of ornamental structures was sexual selection; females choosing males with more prominent features as an indicator of their fitness and suitability as a mate (Darwin 1871). Weapons, however, are used in more direct intraspecific competition over mates by fighting off competitors or defending territory and resources mates find desirable (West-Eberhard 1983). The maintenance of exaggerated weaponry or structures is assumed to be costly to the fitness of an organism, increasing mortality and stress through increased resource needs or higher parasitism or predation (Andersson 1982, Zuk & Kolluru 1998), the tradeoff being that the structures provide reproductive advantage over less developed competitors. Historically, the majority of theoretical models of selection have focused on the role of female preference in ornaments, while there is no defined framework for weapon development and selection through male-male interactions (Emlen 2008). On a basic level, existing theories tie size or exaggeration of weapons to their likelihood of winning combat and securing their mate or territory (Emlen 2008). In example, weapon size in bovids is predictable based on the intensity of selection they experience (Bro-Jørgensen 2007). Selection thus favors exaggerated weaponry as males with better weaponry should have more reproductive success, resulting in the incredible diversity of weaponry observed in many different groups of animals (Emlen 2008).

Several species of beetles employ ornaments, coloration and/or weapons that they use in the courtship process (Emlen & Nijhout 2000), many with horns used in combat with competitors over females. Horned beetles have been used in many studies of sexual selection as model organisms due to their diverse weaponry and behavioral traits (Emlen & Nijhout 2000, Emlen 2008), as well as their relatively short generation times enabling extensive testing (Emlen 1997). Tunneling dung beetles show an especially diverse set of weaponry, as their mating occurs in brood chambers constructed by females beneath piles of dung. Males who arrive first attempt to block rivals from passing and reaching the female, and during combat they employ their horns to shove, flip and otherwise force the opposing male out of the way (Emlen 2008). Horn exaggeration has been linked to lineages confined to tunneling behavior in dung beetles, while loss of horns entirely has been linked to groups deviating from tunnels in favor of surface level dung rolling (Emlen & Keith Philips 2006).

The relationship between weapon size and body size characteristics, or allometry, offers insight into the strategies used by tunneling dung beetles to gain mates. Weapons can have positive allometry, or faster relative growth to body size, slower relative growth (negative allometry) or grow around the same rate (isometric allometry). Although the size of a beetle's horn tends to scale with body size, in some species there is a dimorphism among horned males that separate "major" males with proportionately much larger horns from "minor" males (Emlen 1996, Gadgil 1972). Major males have proportionally longer horns (i.e. steep positive allometry) and tend to be larger in size, while minors are often smaller than females and have very short horns with different allometry relationships compared to majors (Emlen 1996, Cummings 2015). Morphological groups correspond to behavioral groups in these beetles. Major males use their large horns to win contests over access to females while minor males construct auxiliary tunnels to avoid confrontation (Moczek & Emlen 2000).

Although most studied horned beetles demonstrate an allometric basis of dimorphism, this is potentially due to sampling bias in organism selection based on fascination with the most extreme examples of exaggeration or difference (Bonduriansky 2007), or more complex selective forces acting on a given trait such as using weaponry for courtship or other non-competitive interactions (Eberhard & Gutiérrez 1991). Very few studies have described how tunneling horned beetles with relatively small horns or unusual morphologies win contests (Pomfret & Knell 2006). Characterizing the allometric relationship of tunneling horned beetles with different unusual morphologies and the effect of morphology on winning access to females can help elucidate whether common selective pressures operate to shape horn morphologies in general (Pomfret & Knell 2006, McCullough & Simmons 2016)

Ontherus pseudodidymus is a species of neotropical burrowing dung beetle that exhibits unusual horn morphology dimorphism in males (Génier 1996). Larger males have a "broadly notched" or forked morphology of their horns while smaller males have a simple single horn (Génier 1996). Although variation in horn size in this species is on the low side of the spectrum compared to most studied horned beetles, other males in the genus show smaller simpler horns. This may suggest that horn size and morphology is the target of sexual selection in *O. pseudodidymus.* The mating behavior in the entire genus has been poorly studied but the few studies conducted indicate that the genus digs tunnels under dung (Sánchez & Genise 2008).For this reason, it is expected that males used their horns in contest within tunnels to win access to the females that construct brood chambers within the tunnels (Emlen 2008)

In this study I provide the first allometric characterization of horn morphology in *O. Pseudodidymus* to evaluate whether this structure show signatures of sexual selection. Specifically, I tested whether horn size shows a positive allometry. I also conduced standard competition trails in observation chambers (Emlen 1994) to evaluate whether horn size and morphology are the main factors explaining access to females through behavioral strategies.

MATERIALS AND METHODS

STUDY ORGANISM – *O. pseudodidymus* is a fairly abundant species of burrowing dung beetle found in the neotropics from Nicaragua to Northern Panama (Génier 1996). Individuals are black or dark brown, with some iridescence on their elytra (Génier 1996). Males have a back-wards curved horn of either a single or forked point at the end extending up from their heads (Génier 1996, Fig. 1), while females have no horn but small bumps at around the same position (Génier 1996). All individuals collected were separated out by gender and placed in a 100mL plastic sample cup with a thin layer of dirt at the bottom. While beetles were not being handled, the cups were secured with fine mesh and a rubber band to prevent escape. Separation of the beetles prevents fighting and copulation. Beetles were massed after separation and each individual's prothorax was measured. At this time, males were also assigned horn morphologies based on qualitative observation.

Figure 1. Horn morphologies of male *Ontherus pseudodidymus* beetles. The left image is an example of single horn morphology while the right is an example of the forked morphology. The individuals above were captured in lower montane rainforest on the pacific slope of Costa Rica.

STUDY SITE – All individuals were collected between 1550 and 1700 meters above sea level in the forest reserve of the Estación Biológica Monteverde, in Monteverde, Costa Rica. Sites were in the lower montane rainforest Holdridge life zone (Haber 2000). Collection areas were at least 10 meters from trail to avoid edge effects caused by clearing. Collection occurred from October 17 to November 14 after 10:00 am. In concordance with similar studies in the same area, there was higher abundance on days following rains than dry days (Cummings 2015).

BEETLE COLLECTION—Sixteen pitfall traps were set 10m away from trails and each other along transects. Holes were dug in the ground to place 10cm deep plastic cups with their rims even with ground level (Fig. 2a). Cups were half filled with dirt and baited with pig dung, in small clumps, around the size of a marble or ping pong ball, on top of the dirt. Plastic plates were erected on wooden skewers to deflect rainwater (Fig. 2b), although the site of the trap appeared influence whether a trap would flood more than attempts to create drainage could compensate. Pig dung was anywhere from 1-4 days old, but fresher and more damp dung was more effective at attracting individuals (personal observation). The time of day traps were set did not affect collection over the course of the study. Pitfall traps were left overnight after being set, and collected after 10am the following day. Cups were removed from the ground, and the contents were dumped into a plate, sorted through for individuals of the study species and then discarded. Traps were then reset and rebaited. This style of trapping was selected due to high success in previous studies (Cummings 2015, Gregory *et al.* 2015).

Figure 2. Trap design showing a) in-ground setup with dirt and bait fill and b) use of skewers and a plate to deflect rainwater from the pitfall trap. Traps were used to collect *Ontherus pseudodidymus* individuals in the neotropical lower montane rainforest near the Estación Biológica Monteverde between 1550 and 1700 m a.s.l. Traps were left overnight and collected after 10 a.m. the following day.

MORPHOLOGICAL MEASUREMENTS - Body measurements were taken for all individuals immediately following collection, which consisted of mass in grams and prothorax width in millimeters as an indicator of body size. Following competitions, all males were chilled for approximately 5-10 minutes to slow body movements and escape behavior. Individuals were then placed on their side and photographed through a dissection microscope next to a line of 0.5cm to isolate their horn profile. These images were used to measure horn length in the image analysis program ImageJ (Abramoff *et al.* 2004). A total of 38 males and 23 females were measured in these ways. Allometric relationships were tested in accordance to standards set by previous studies (Kodric-Brown *et al.* 2006), transforming measurements to log scale. Log-adjusted models were tested for linearity because they appeared linear and fit well.

COMPETITION TRIALS – Brood chamber tests (Figs. 3a-c) were conducted to test intraspecific competition between male *O. pseudodidymus* beetles. Chambers with removable glass panels were placed horizontally and filled with dirt, leaving about 10cm of room at the top. The glass was replaced, and the chamber stood upright so that a ping-pong ball sized clump of dung could be placed on top. Individuals that had been isolated for 24 hours were placed into competition groups consisting of 1 female and 2 males. Males were paired with each other based on similarity in mass and prothorax width but with different horn morphologies, while females were chosen haphazardly. The females were placed into the chambers first, directly onto the dung. Females immediately began tunneling through the dung and into the dirt, and left alone for 10 minutes to begin construction of brood chambers. Tunnels appeared to be between 6 and 12 cm deep when tanks were disassembled (personal observation). The males were then placed into the tanks near the entrance to the female's tunnel, which after lying motionless for a short amount of time they quickly walked towards and entered. Chambers were sealed with fine netting and duct tape and left for 24 hours. After this period, the chambers were unsealed and gently laid flat, then after removing the top glass panel and dung the dirt was slowly disturbed until all beetles had been located. Whichever male was found closest to the female at this time was considered the winner. Males who were considered winners were always within 2 cm of the female at the time of review, while losers were found anywhere from on the far side of the winning male to as far away as the tank would allow. Males were identified by their horn morphology and placed back into their containers, while females were promptly released. Chambers were cleaned out to make them ready for the next round of trials. Seventeen sets of beetles were successfully tested during the study.

STATISTICAL ANALYSIS – Logistic regression was used to analyze the interaction of factors and competition outcome in accordance with prior studies (McCullough & Simmons 2016). In analysis, winning was set to a value of 1 and losing to 0, so models could be fit to the results more completely (McCullough & Simmons 2016, Cummings 2015). For allometry, log transformed measurements were tested for their linearity to test the fit of various allometric relationships. Linear modeling was chosen at this stage following the precedent set in other studies (McCullough & Emlen 2013, Kodric-Brown *et al.* 2006) and as the graphed data appeared linear in nature. Prothorax width was selected as an indicator of body size for this analysis as it has been found to represent the overall organism's size well (Pomfret & Knell 2006, Emlen 1996, Eberhard & Gutiérrez 1991).

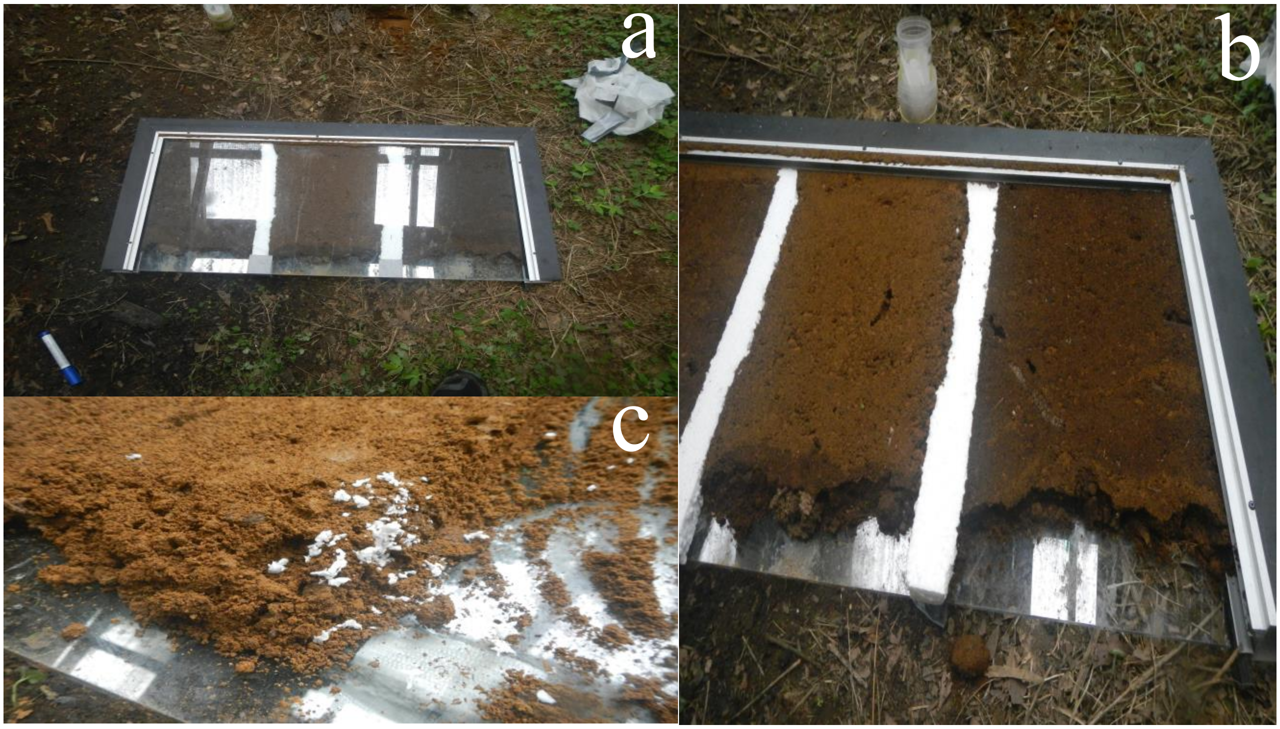

Figure 3. Brood chamber tanks showing a) the closed and complete tank, b) the tank open with dividers between trial groupings and visible tunnels/dung lining and c) a tunnel cross section uncovered in the competition assessment and trial disassembly. Male *O. pseudodidymus* of different horn morphologies were paired with each other based on similar mass and body size for trials. Trials were left to run for 24 hours then disassembled to assign winners and losers.

RESULTS

MORPHOLOGICAL MEASUREMENTS - In this study 38 male *O. pseudodidymus* and 23 females were measured. 17 of those females were used to conduct 17 brood chamber tests with 34 males. In both males and females, prothorax width showed a significant correlation to mass, with each gram of mass increase prothorax width increases by 10.4mm (put in more biologically relevant perspective, 1.04 mm for every 0.1 of a gram) (Fig. 4; Linear Regression; F = 150.3, df = 1,59, $R^2 = 0.718$, $p < 0.0005$).

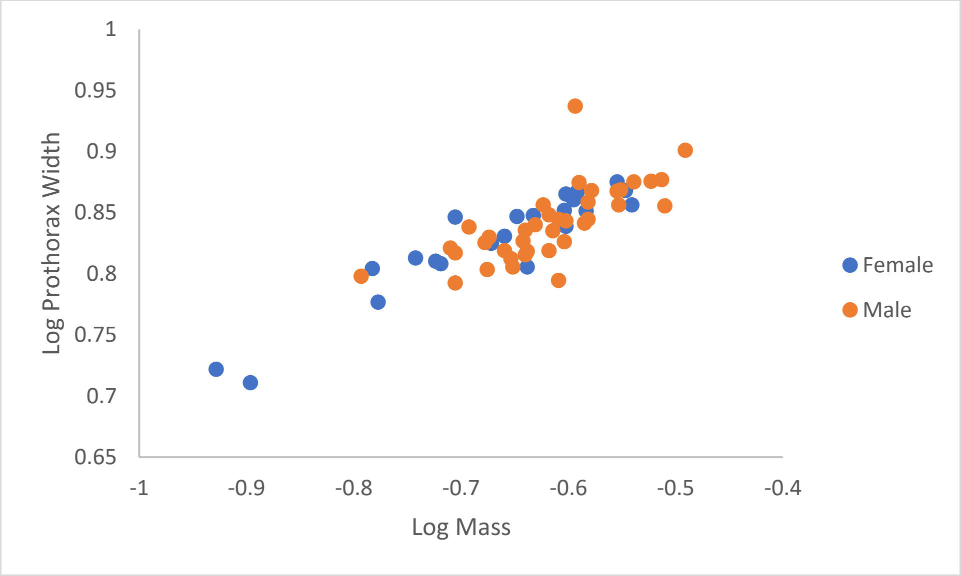

Figure 4. In both males and females of *O. pseudodidymus* (n=61), prothorax width increased with mass ($p<0.0005$, $R^2=0.718$). As mass increased, each 0.1g conferred a 1.04 mm increase in prothorax width. Individuals were weighed and measured immediately after capture in 16 pitfall traps in Costa Rica.

On average the mass of males (0.244 ± 0.0057 (SE) g) was 111% higher than the average mass of females (Two Sample T Test, p = 0.0297, Fig. 5; 0.221 ± 0.0098 (SE) g). Female's prothorax width was normally distributed, ranging from 5.14mm to 7.94mm, as was mass, ranging from 0.118 g to 0.298 g (Fig. 6a-b). Among males, mass and horn length were evenly distributed, with mass ranging from 0.161 g to 0.346 g, and horn length ranging from 0.04 cm to 0.16 cm (Fig. 7 a-b). Prothorax width was not normally distributed, with most individuals having between 6.22 mm and 7.3 mm, while few had 7.85 mm to 8.95 mm widths (Fig. 7c). Single horn morph males had on average (0.101 ± 0.006 (SE) cm) 129% longer horns than forked morph males (Two Sample T Test, p = 0.0145, Fig. 8; 0.079 ± 0.006 (SE) cm).

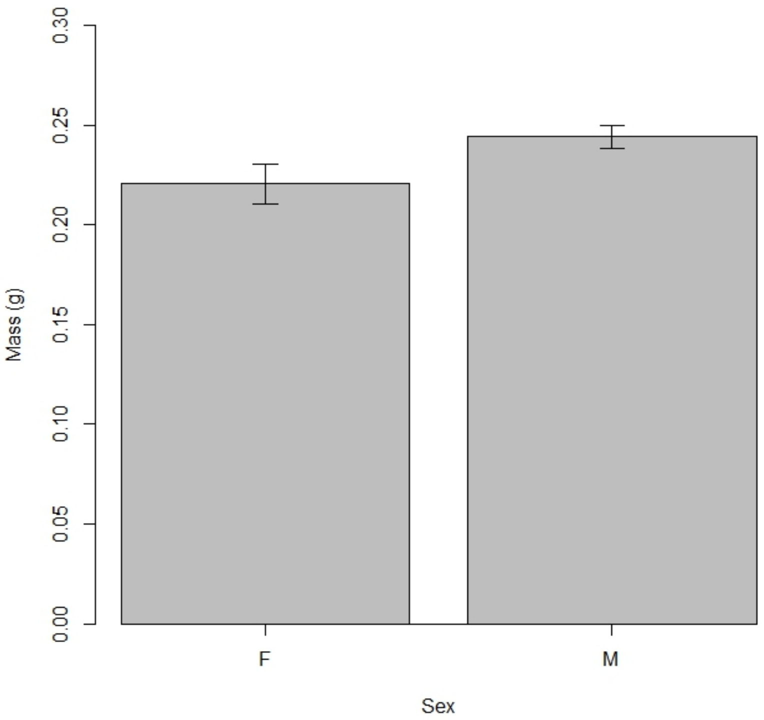

Figure 5. *O. pseudodidymus* males (n=61) collected in lower montane tropical rainforest are on average (± standard error) heavier than their female counterparts (p=0.029). Individuals were massed immediately after capture and separation to control for variation over time.

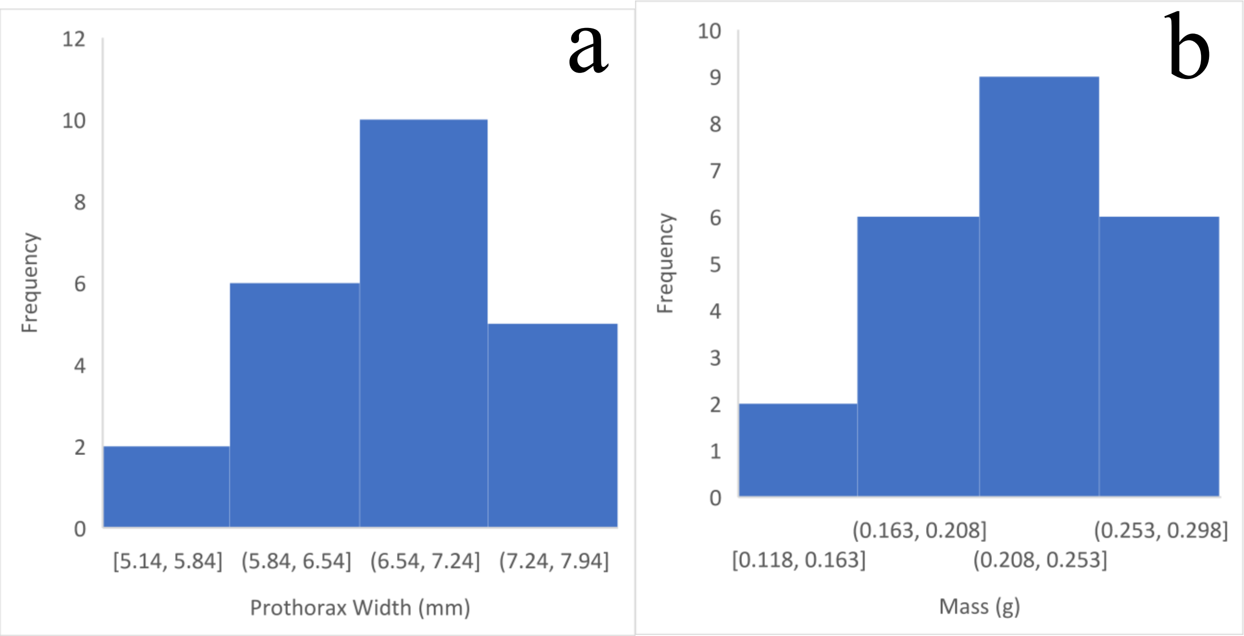

Figure 6. Size distributions of 23 female *O. pseudodidymus* beetles. a) shows the distribution of prothorax widths recorded in this sample, ranging from 5.14 to 7.94mm, and b) shows the distribution of masses recorded, ranging from 0.118 to 0.298 g. Both measurements are normally distributed.

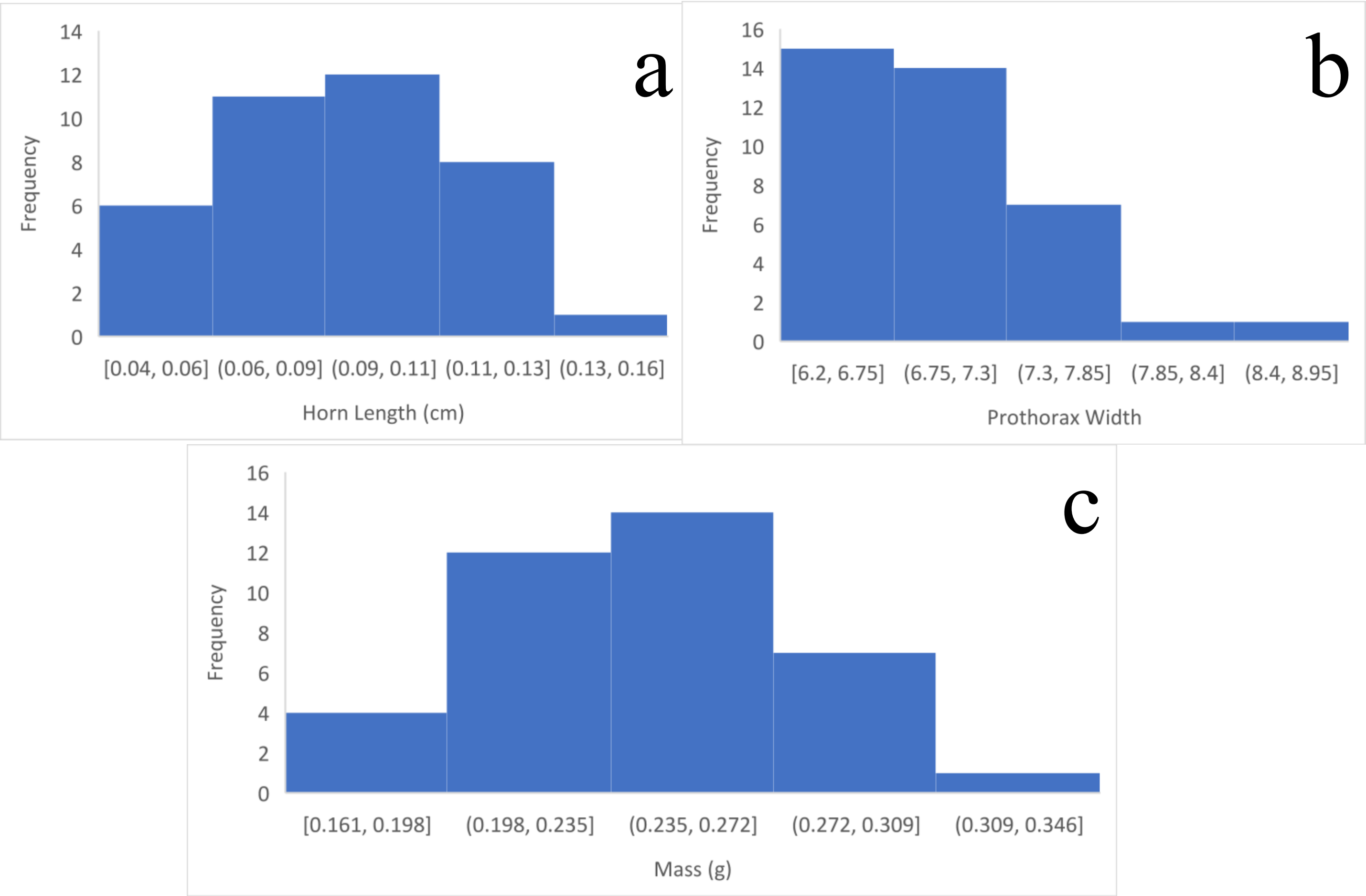

Figure 7. Size distribution of 38 male *O. pseudodidymus* for measured morphological traits a) horn length, which is normally distributed, showing most males centered around intermediate lengths, b) showing a non-normal distribution of prothorax width, many males have between 6.2 and 7.3 mm prothoraxes while very few have extremely large measurements, and c) mass which is normally distributed, with few males at either extreme.

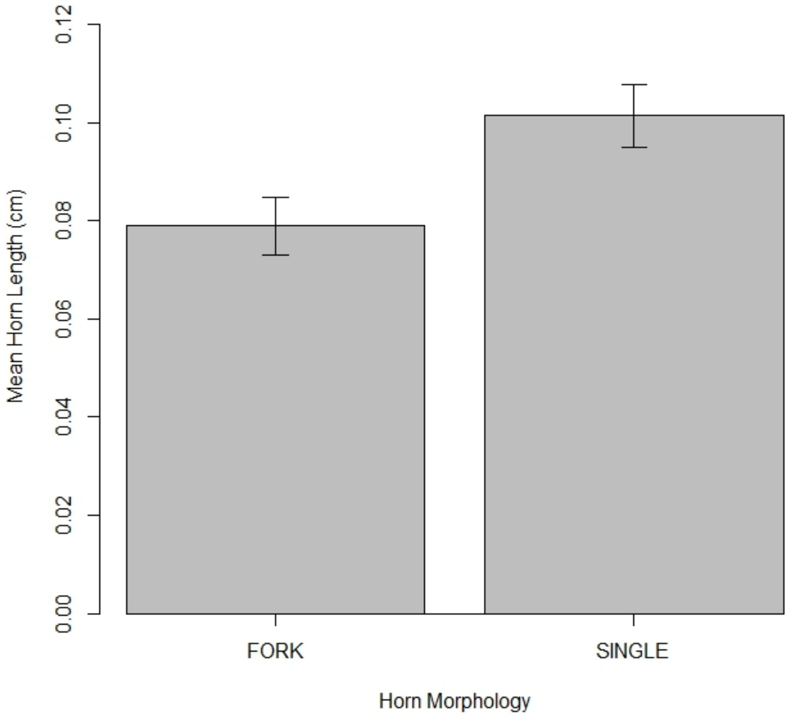

Figure 8. Male *O. pseudodidymus* average horn length varies with horn morphology (p=0.015), with single horn morph males displaying horns on average 129% longer than forked males in this sample. Horn measures were taken using image analysis of the side profile of the beetle's head in proximity to a known scale of 0.5 cm.

HORN ALLOMETRY – The unadjusted data for both single horned males and forked males did not appear linear (Fig. 9), so it was log adjusted to test for allometric relationships. A linear model was found to be the best fit in describing horn to body allometry in single horned males (Linear Regression; F= 6.819, df = 1, 15, R^2=0.313, p < 0.001; Fig. 10). This relationship matches a positive allometry as the slope is greater than 1 (Fig 10). Forked horn males did not significantly fit a linear model, showing a nonsignificant negative allometry (Linear Regression; F = 0.1834, df = 1,19, p = 0.673; Fig. 10).

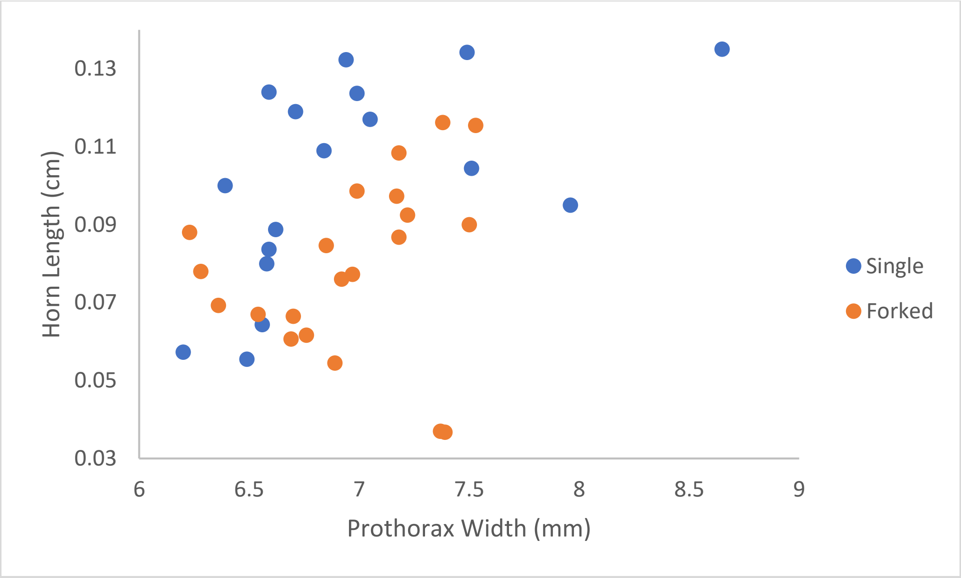

Figure 9. Unadjusted morphological relationship for both single and forked horn male *O. pseudodidymus*. Individuals were measured for body size after collection and for horn length following brood chamber testing. As neither group appeared to have a clear linear relationship, measurements were assessed again after log transformation to investigate allometric models.

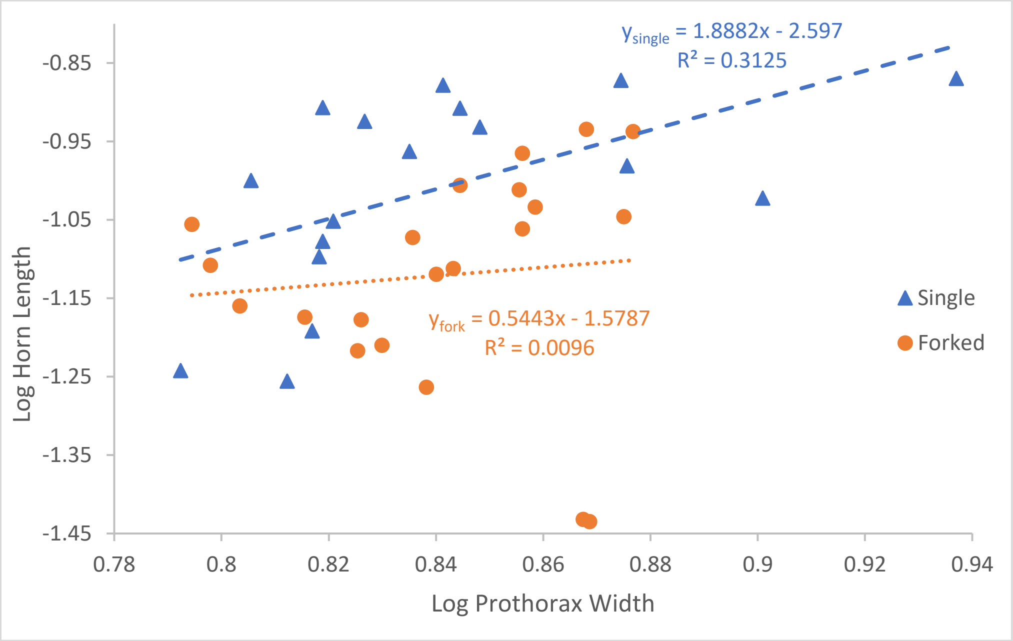

Figure 10. Allometric relationships between prothorax width and horn length in both single and forked morph males. Single horn males showed a significant positive allometry ($p < 0.001$) in horn length with a fit for a linear relationship while adjusted. Forked horn males showed a nonsignificant negative allometry ($p = 0.673$) that did not significantly fit the linear model.

BROOD CHAMBER TESTS – In all possible combinations of independent variables and interactions, only the three-way interaction between horn difference, prothorax width difference and horn morphology was significant in its relationship to trial outcome ($p = 0.002$, Chi square = 9.19; Table 1). Single horned males had the highest chance of winning when paired against a male of much smaller horn and prothorax size, with their overall likelihood of winning corresponding to the magnitude of weapon size difference in their favor (Fig.11). Forked horn males had the highest chance of winning with essentially no difference in horn length, but greater difference in prothorax width. Their chance of winning increased most sharply as prothorax width difference increased in their favor but horn length difference stayed near zero (Fig. 11).

Table 1. Several different models were tested for significance in relation to brood chamber competition outcome. The isolated morphological characteristics were nonsignificant in determining outcome. Each two-way interaction appears to have no significant influence over outcome, but the final three-way interaction of all characteristics was highly significant.

	Chi Square	**p value**
A. Log Diff. Horn	0.591	0.442
B. Log Diff. Prothorax	2.456	0.117
C. Morphology	1.052	0.305
	Interactions	
A x B	0.000	1.000
A x C	0.000	1.000
B x C	0.000	1.000
A x B x C	9.190	0.0024

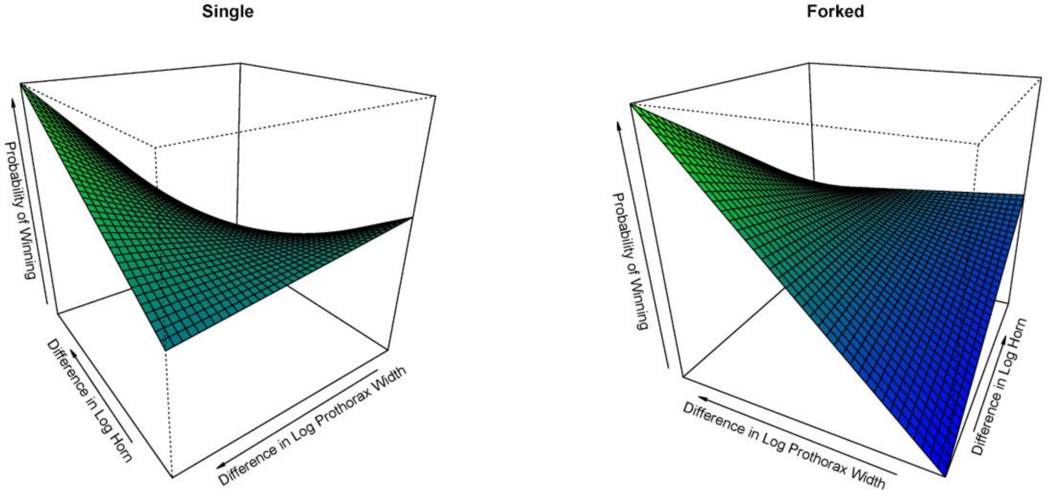

Figure 11. Outcome modeling of competition trials for each horn morphology. Note that the horizontal axes of each graph are not the same between graphs. Single horned males increased the likelihood of victory as their horn size outmatched that of their opponent with greater and greater magnitude. Forked horned males were the most likely victor when pitted against males of similar horn size but lesser prothorax width, although they may also perform well with a larger horn than their opponent. Males were pitted against others of similar mass and size but differing horn morphology.

DISCUSSION

Single horn males tended to have much larger horns than their forked counterparts, as well as showing a positive allometry for accelerated growth relative to other body features. This contradicts the observations previously made by Génier that forked males are larger (Génier

1996), but supports the findings of the rest of this study. Larger horns are sexually selectively favored in single horned males based on these findings but the same cannot be said for forked males. The role of sexual selection in influencing their horn length is somewhat unclear from an allometric perspective, although the brood chamber testing reveals that being generally larger in body size should be favored at least to a point. Forked males do best in combat when their horns are approximately the same as their opponents, at least when paired against single horn males. Similarly, they have a higher chance of winning if they have a larger horn but smaller or equivalent body size, so horn length must play some role in their strategy, even if exaggeration is not favored. Allometry supports this conclusion but does not impart a precise relationship, as no significant trend was found for forked males. On the theoretical front, it has been found in a species of collard lizard that weapon performance confers a reproductive advantage rather than weapon size (Lappin & Husak 2005). Forked males may generally have a higher performance weapon that enables them to exploit or overcome the generic advantage exaggerated single horned males generally have. It could also be less costly for forked males to develop their weapons as they do not show the need to grow them as extensively than single males to be successful. In this way, the forked morphology capitalizes on a less expensive strategy that conveys a potentially invaluable increase in an individual's competitiveness. The forked morphology, if less costly, may even extend longevity of individuals relative to the normal morphology (Robinson *et al.* 2006), conferring increased overall reproductive advantage within an individual's lifetime.

As the initial focus of this study was to follow the observations of Génier (1996) to establish major/minor male classes focused on horn morphology, opponents were selected based on similar body size and differing horn morphology, which in turn caused little variation in horn length between competing pairs, as well as other characteristics. Through assessing different models, it was shown that no applicable model "break point" of scaled horn length to any other trait exists within this species. Among males measured for this study, the average male is significantly heavier than any given female, which also points to a lack of minor class males, although there may be much smaller male within the larger population. To that end, though, there are no detectable major or minor males of any kind observed within the context of this study. This suggests that horn length is the target of sexual selection overall, even though the intensity differs between morphology. An ideal expansion upon the roots this study has provided would include a much more extensive system of competitive pairings among all possible combinations of trait size and horn morphology that should be analyzed in a multivariate fashion (Pomfret and Knell 2006), used here to show underlying selective differences in male archetypes. A more diverse set of competitive pairs will shed light onto how the trends in size and morphology extend into natural competitive scenarios. Pomfret and Knell (2006) also found non-normal prothorax distribution when comparing their species' morphological groups. This would expand on the dynamics that lead individuals to reproductive success in systems that appear to deviate from generalized frameworks in part or whole.

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Episodic memory and return intervals of hummingbirds to feeders with fixed nectar-renewal rates

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ABSTRACT

Episodic memory is the mechanism by which many foragers remember where and when they encountered food and what food they found. The use of episodic memory contributes to forager fitness as it improves foraging efficiency. Hummingbirds are expected to exhibit episodic memory because they feed from flowers with great spatial and temporal variation in nectar quality. The cognitive ability of hummingbirds to remembering the timing and location of high quality nectar production has only been tested in the temperate zone. This study tested the capacity of tropical montane hummingbirds to learn when and where nectar is produced. Pairs of feeders that differed in nectar-renewal rate (10 vs 20 minutes after being drained) were provided to wild hummingbirds in the cloud forest of Monteverde, Costa Rica (elevation 1500 m) across several sites. Return intervals by hummingbirds of various species were recorded over the course of several days. Across all species, an adjustment of return interval times towards 10 minutes was observed over the course of the study period. The mean return intervals of the Violet Sabrewing only differed significantly from 10 minutes on the first day. The Green Crowned Brilliant and the Purple Throated Mountain Gem exhibited strong adjustments in mean return intervals towards 10 minutes to both feeders by the last day. These results suggest that hummingbirds in the tropical cloud forest use episodic memory as hummingbirds in the temperate zone do, indicating that episodic memory is a general trait in this taxon.

RESUMEN

La memoria episódica es el mecanismo por el cual varios forrajeadores recuerdan cuando y donde encuentran comida y que comida encuentran. El uso de memoria episódica contribuye al éxito de forrajeo al mejorar la eficiencia de forrajeo. Se espera que los colibríes exiban memoria episodica debido a que se alimentan de flores con una gran variación espacial y temporal en la calidad del néctar. La habilidad cognitiva de los colibríes de recorder el tiempo y ubicación del néctar de alta calidad ha sido probabo únicamente en la zona templada. Este estudio prueba la capacidad de colibríes tropicales de montaña para aprender cuando y donde se produce el néctar. Pares de comederos que difieren en la tasa de renovación de néctar (10 vs 20 minutos después de ser vaciados) se presentaron a colibríes en el bosque nuboso de Monteverde, Costa Rica (elevación 1500 m) a lo largo de varios sitios. Los intervalos de retorno de los colibríes de varias especies se tomaron a lo largo de varios días. A través de todas las especies, un ajuste en los intervalos de retorno hacia los 10 minutos se observó a lo largo del período de estudio. El interval de retorno promedio del Ala de sable Violáceo solo difiere significativamente a los 10 minutos durante el primer día. El Brillante Frentiverde y el Colibrí Montañés Gorgimorado exhiben un ajuste fuerte en los intervalos de retorno promedio hacia los 10 minutos en ambos comederos para el último día. Estos resultados sugieren que los colibríes en el bosque nuboso tropical usan la memoria episódica a como lo hacen los colibríes en la zona templada, indicando que la memoria episódica es una característica general de este taxon.

Key words: forager behavior, pollinator fitness, cloud forest

INTRODUCTION

A forager's fitness depends on their ability to maximize energy intake and minimize energy expended in their search for food. To do this, many foragers remember where they previously found food, what food was available there, and when they encountered food (Allen & Fortin 2013). For example, scrub jays remember what kinds of food they cache and where and when they cache it (Clayton & Dickinson 1998). This memory of what, where, and when is called episodic memory and was once thought unique to humans, though recent studies have observed its relevance to a variety of animal species (Dere et al. 2006).

Nectar foragers are expected to show episodic memory because they are constantly confronted with a variety of flowers that vary in nectar concentration, spatial location and renewal rate (Feinsinger 1978, Feinsinger et al. 1986, Temeles et al. 2002, Chalcoff et al. 2006). The ability of some individuals to remember nectar quality of widely distributed individual flowers over time after visiting them should increase their fitness in comparison to individuals with poor memory (Henderson et al. 2004, González-Gómez et al. 2014). Understanding decision-making in pollinating animals is not only necessary to understand the selective pressures that shaped their natural histories, but also to identify the limitations of their persistence, along with those of the plants that depend on them for pollination. For instance, as climate change affects the phenology (Miller-Rushing & Inouye 2009) and distribution of flowering plants (Kelly & Goulden 2008, Loarie et al. 2009), it is important that pollinators harbor the capacity for mechanisms such as episodic memory so that they may continue to pollinate flowering plants and conserve biodiversity.

Hummingbirds pollinate thousands of plant species in the tropics (Bawa & Hadley 1990) and therefore would benefit from episodic memory. Territorial hummingbirds in the temperate zone are known to exhibit episodic memory. Several studies conducted in South American temperate zones demonstrated the ability for Green-backed Firecrown hummingbirds to adjust their foraging behaviors to correspond with flower nectar-renewal rates (González-Gómez et al. 2014, González-Gómez et al. 2011a, González-Gómez et al. 2011b). Individuals changed their visitation times to match the nectar-renewal rate of the most profitable nectar source within several hours. Experiments conducted in North American temperate zones found similar changes to forager behavior in response to different nectar-renewal rates in Rufous hummingbirds and evidence that color cues help hummingbirds learn flower refill schedules (Healy & Hurly 2003, Henderson et al. 2006, Samuels et al. 2014). These findings suggest that hummingbird fitness is maintained by their use of episodic memory for foraging success.

Very few studies have been conducted in the tropics to evaluate the capacity of hummingbirds to learn nectar-renewal rates. Traplining Long-tailed Hermit hummingbirds, non-territorial hummingbirds who travel between many flower patches to forage for nectar, in the tropical lowland wet forests of Costa Rica were found to adjust adjusted visits to fixed-interval schedules of food presentation in feeders (Gill 1988). Hummingbirds in tropical montane forest deal with species with extremely variable secretion rates (Feinsinger 1978) so it is expected that they utilize episodic like memory for foraging as well. No study has evaluated this hypothesis in any tropical montane forest. The current study aimed to evaluate the capacity of wild hummingbirds in the tropical pre-montane forest to adjust their return intervals to feeders of two different fixed nectar replenishment rates, using methods modeled after the temporal training in the study by González-Gómez et al. 2011a.

MATERIALS AND METHODS

STUDY SITE. The study was conducted at the Estación Biológica in the cloud forest of Monteverde, Costa Rica, with an altitude of 1,500 m and a mean annual precipitation of 2501.675 mm (Figure 1). The mean annual temperature of Monteverde is 18°C. The study was conducted during the wet season in October and November, 2017.

Figure 1. Map of Costa Rica showing the location of the Monteverde Cloud Forest where the study was conducted in October and November, 2017 (Monteverde Cloud Forest Reserve). The study aimed to evaluate the capacity of wild, hummingbirds to adjust their return intervals to feeders of fixed nectar replenishment rates.

INITIAL TRAINING. The experiment contained a training period in which individuals encountered feeders and incorporated them into their foraging route. Training feeders were typical commercial hummingbird feeders with four artificial flowers for nectar extraction. Feeders were hung in the forest edge about 1.5 m above the ground and at least 20 m apart and filled with a 25% nectar solution. Feeders were kept full for at least two days prior to experimentation to maintain hummingbird return.

EXPERIMENTAL PROTOCOL. Feeders from the initial training period were replaced by two experimental feeders hung at least 30 cm apart about 1.5 m above the ground (Figure 2). Each experimental feeder contained one artificial flower constructed from a sealed syringe tip (Figure 3A). Flowers contained 40 µL of a 25% nectar solution. One feeder was designated the 10-minute feeder and was refilled 10 mins after being drained. The other feeder was designated the 20-minute feeder and was refilled 20 mins after being drained. Flowers were refilled by hand (Figure 3B).

Figure 2. Spatial arrangement of experimental feeders refilled with nectar at different rates. Feeders were used to evaluate the capacity of wild, hummingbirds to adjust their return intervals to feeders of different fixed nectar replenishment rates in October and November, 2017.

Figure 3. A. Experimental feeder with one artificial flower containing 40 µL of a 25% nectar solution. B. Refill of the experimental feeder by hand. The two experimental feeders were refilled at different fixed rates and the return intervals of wild, hummingbirds were analyzed for signs of episodic memory in the cloud forest of Monteverde, Costa Rica in October and November, 2017.

When a hummingbird encountered the feeders, the specific feeder that was visited, the species of the visitor, and the time of the visit was recorded. A visit was defined as a hummingbird inserting its beak into the artificial flower. The amounts of time that elapsed between visits by the same species (return interval) were calculated. Experiments took place between 8:00 AM and 12:00 PM each day. The first hour was considered a training period and only the last three hours were included in the experimental analysis. Only days in which species returned 15 or more times within the last three hours of observation were analyzed, with a goal of obtaining data from at least 7 visits per feeder in the last three hours, an adequate sample size for sufficient statistical power.

RESULTS

The majority of the time, individuals visited both feeders upon return. Some hummingbirds were observed chasing away other hummingbirds, but none stayed full-time to defend any feeder. Sometimes more than one bird of the same species was observed around one feeder (up to 3 at one time). This indicates that more than one bird of each species was visiting the feeders.

Of the six species that visited the feeders, three species had days in which individuals returned 15 or more times within the last three hours of observation: The Violet Sabrewing (VS), the Green Crowned Brilliant (GCB), and the Purple-throated Mountain Gem (PTMG). A Wilcoxan rank sum test showed that across all species and all days of the experiment, return intervals to the 10 and 20-minute feeders did not differ significantly between one another (Table 1C, Table 2C, Table 3C).

VIOLET SABREWING. One sample t-tests were performed to determine whether the mean return intervals differed significantly from 10 and 20 mins, the nectar-refill rates for the two feeders, on each day. For the VS, the mean return intervals to both the 10 and 20-minute feeders were close to 4 mins the first day (Figure 4A) and were significantly different than 10 and 20 mins (Table 1A and B). The following days showed mean return intervals to both feeders that were not significantly different from 10 mins (Table 1A) and that were significantly different or showed trends of differentiation from 20 mins (Table 1B).

Table 1. Table showing the results of one sample t-tests used to determine if mean return intervals of Violet Sabrewing hummingbirds to feeders with different nectar replenishment rates were significantly different from (A) 10 mins and (B) 20 mins, the nectar-renewal rates of the two feeders. (C) A Wilcoxan rank sum test demonstrated that the mean return intervals did not differ significantly between feeder types each day. The study aimed to evaluate the capacity of wild hummingbirds to adjust their return intervals to feeders of fixed nectar replenishment rates in the cloud forest of Monteverde, Costa Rica, in October and November, 2017.

A.

	10-minute Feeder			20-minute Feeder		
Date	*t*	*df*	*p*-value	*t*	*df*	*p*-value
10/30	-8.85	31	$p < 0.0001$	-8.03	36	$p < 0.0001$
11/4	0.34	10	0.74	-0.46	13	0.65
11/8	-0.39	12	0.71	0.93	10	0.37
11/9	-4.26	16	6.04e-4	-4.85	15	2.13e-4

B.

	10-minute Feeder			20-minute Feeder		
Date	*t*	*df*	*p*-value	*t*	*df*	*p*-value
10/30	-23.15	31	$p < 0.0001$	-21.28	36	$p < 0.0001$
11/4	-2.33	10	0.04	-3.71	13	2.60e-3
11/8	-3.99	12	1.81e-3	-1.77	10	0.11
11/9	-4.26	16	6.04e-4	-4.85	15	2.13e-4

C.

Wilcoxan rank sum test	
W	*p*-value
612	0.81
89	0.52
53.5	0.30
130	0.84

GREEN CROWNED BRILLIANT. The mean return intervals to both feeders of the GCB were not significantly different from 10 mins except for the mean return interval to the 20-minute feeder on 11/4 (Table 2A). The return interval to the 20-minute feeder on this day also differed significantly from 20 mins, as it was close to 4 mins on 11/4 (Table 2B). The mean return intervals to both feeders on 11/8 did not differ significantly from 20 mins (Table 2B). However, the high mean return intervals may be attributed to the large variation in return intervals observed on 11/8 (Figure 4B). Mean return intervals showed a trend away from 20 mins and became closer to 10 mins in the last three days (Figure 4B, Table 2A and B).

Table 2. Table showing the results of one sample t-tests used to determine if mean return intervals of Green Crowned Brilliant hummingbirds to feeders with different nectar replenishment rates were significantly different from (A) 10 mins and (B) 20 mins, the nectar-renewal rates of the two feeders. (C) A Wilcoxan rank sum test demonstrated that the mean return intervals did not differ significantly between feeder types each day. The study aimed to evaluate the elasticity of foraging behaviors of wild hummingbirds regarding their return intervals to feeders of varying nectar replenishment rates in the cloud forest of Monteverde, Costa Rica, in October and November, 2017.

A.

	10-minute Feeder			20-minute Feeder		
Date	*t*	*df*	*p*-value	*t*	*df*	*p*-value
10/30	1.15	7	0.29	1.58	11	0.14
11/4	-1.32	18	0.20	-2.89	17	0.01
11/8	0.93	7	0.38	0.95	6	0.38
11/9	1.18	8	0.27	-1.18	8	0.27
11/10	0.85	12	0.41	1.05	12	0.32
11/13	2.00	10	0.07	1.89	9	0.09
11/14	0.76	10	0.47	-0.05	11	0.96

B.

	10-minute Feeder			20-minute Feeder		
Date	*t*	*df*	*p*-value	*t*	*df*	*p*-value
10/30	-1.15	7	0.29	-1.53	11	0.15
11/4	-6.88	18	$p < 0.0001$	-9.39	17	$p < 0.0001$
11/8	0.15	7	0.89	0.26	6	0.80
11/9	-0.34	8	0.74	-0.34	8	0.74
11/10	-2.60	12	0.02	-2.53	12	0.03
11/13	-2.15	10	0.06	-2.50	9	0.03
11/14	-2.03	10	0.07	-6.15	11	$p < 0.0001$

C.

Wilcoxan rank sum test	
W	*p*-value
45.5	0.877
200.5	0.3739
28.5	1
40.5	1
81.5	0.8976
56	0.9718
70	0.828

PURPLE-THROATED MOUNTAIN GEM. The mean return intervals of the PTMG to both feeders were not significantly different from 10 mins across all days (Table 3A) and were significantly different from 20 mins across all days (Table 3B). The mean return intervals to both feeders became closer to 10 mins each day (Figure 4C).

Table 3. Table showing the results of a one sample t-test used to determine if mean return intervals of Purple-throated Mountain Gem hummingbirds to feeders with different nectar replenishment rates were significantly different from (A) 10 mins and (B) 20 mins, the nectar-renewal rates of the two feeders. (C) A Wilcoxan rank sum test demonstrated that the mean return intervals did not differ significantly between feeder types each day. The study aimed to evaluate the elasticity of foraging behaviors of wild hummingbirds regarding their return intervals to feeders of varying nectar replenishment rates in the cloud forest of Monteverde, Costa Rica, in October and November, 2017.

A.

	10-minute Feeder			20-minute Feeder		
Date	*t*	*df*	*p*-value	*t*	*df*	*p*-value
11/9	1.52	10	0.16	0.72	6	0.50
11/10	1.15	8	0.28	1.08	8	0.31
11/13	0.69	13	0.50	0.33	11	0.75

B.

	10-minute Feeder			20-minute Feeder		
Date	*t*	*df*	*p*-value	*t*	*df*	*p*-value
11/9	1.52	10	0.16	0.72	6	0.50
11/10	1.15	8	0.28	1.08	8	0.31
11/13	0.69	13	0.50	0.33	11	0.75

C.

Wilcoxan rank sum test	
W	*p*-value
44	0.649
42.5	0.894
93	0.6522

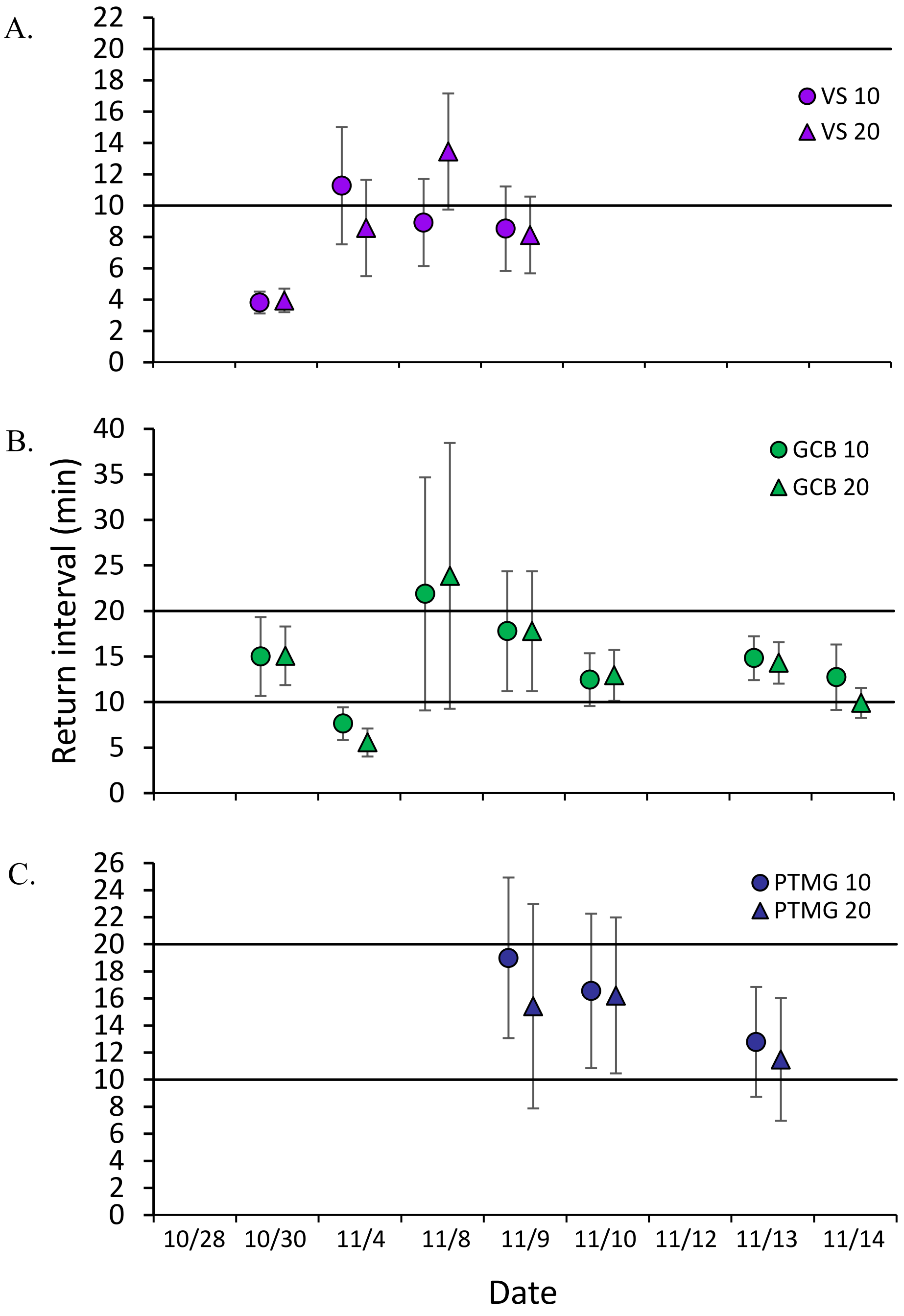

Figure 4. Graphs depicting the mean return intervals of hummingbirds to feeders of differing nectar-renewal rates over the course of several days. Data from days in which individuals did not return 15 or more times within the last three hours of observation were excluded from analysis. Circles depict mean return intervals to 10-minute feeders and triangles depict mean return intervals to 20-minute feeders. Error bars represent standard error. A. Mean return intervals of the VS. B. Mean return intervals of the GCB. C. Mean return interval of the PTMG. The study aimed to evaluate the elasticity of foraging behaviors of wild, hummingbirds regarding their return intervals to feeders of varying nectar replenishment rates in the cloud forest of Monteverde, Costa Rica, in October and November, 2017.

DISCUSSION

Three species of hummingbirds adjusted their foraging behavior to correspond with the optimal nectar-renewal rate, 10 mins, over the course of nine days, suggesting that they used episodic memory to remember the location of nectar sources and when they were replenished.

The VS exhibited clear change in mean return interval time across the days and relatively small variance in return interval time each day, indicating that VS were adjusting their return times to match the 10-minute feeder's refill rate. Though the mean return intervals were significantly lower than 10 mins the first day, they adjusted quickly towards 10 min return intervals in the subsequent days, suggesting that they remembered the refill rate of 10-minute feeders after the first day. The GCB also exhibited a change in mean return intervals across days. Though return intervals were irregular for the first several days, mean return intervals close to 10 mins with small variance were observed in the later days of the study, suggesting that they remembered the optimal refill rate by the fifth day. The PTMG showed an adjustment of mean return intervals towards 10 mins from higher averages between 15 and 19 mins on the first day. Variance in return interval times was relatively large for the PTMG which may explain the relatively higher mean return intervals. Variance got smaller with each day of the study, however, suggesting that the PTMG was refining its return intervals towards the optimum nectar-renewal rate.

For all species, there was no significant difference between the mean return intervals to the 10 and 20-minute feeders. This could simply be explained by the practically non-existent energy cost associated with checking the 20-minute feeder while visiting the 10-minute feeder. Every time that a hummingbird returned to feeders within range of the optimal return interval, it obtained a nectar reward from the 10-minute feeder and some of the time it encountered nectar in the 20-minute feeder. Since the feeders were not far from one another, the energy cost of visiting both feeders upon return was not great enough to discourage checking both the 10 and 20-minute feeders each time.

In previous studies, hummingbirds learned the optimal return intervals for two feeders with different nectar-replenishment rates, but the feeders also differed in nectar concentration, with the feeder with lower nectar concentration refilling more frequently than the feeder with higher nectar concentration (González-Gómez et al. 2011a, González-Gómez et al. 2011b, González-Gómez et al. 2014). Because the feeders differed in nectar concentration as well as nectar-replenishment rate, they offered different rewards; a lower quality nectar more frequently and a high-quality nectar less frequently. The current study provided the same nectar reward at two different rates and, therefore, provided less pressure than previous studies for the hummingbirds to learn the nectar-replenishment rates of both feeders. This explains the differences in behavior between the subjects in the current study and previous ones.

In the current study, subjects adjusted return intervals over the course of 9 days to match the nectar-renewal rate of the optimal, 10-minute feeder. In previous studies, the changes occurred over the course of a few hours (González-Gómez et al. 2011a, González-Gómez et al. 2011b, González-Gómez et al. 2014). This difference may be explained by the aforementioned differences in nectar rewards that increased the pressure to learn in previous studies compared to the current one. It may also be explained by the differences in foraging techniques between individuals of the past and current studies. In the studies by González-Gómez et al., test subjects were territorial Green-backed Firecrown hummingbirds who stayed to defend the experimental feeders. This may explain how subjects were able to learn nectar-replenishment rates so quickly; individuals were the only ones drinking from the feeders as opposed to the current study where many different individuals visited the feeders. Another explanation for the slower learning time in the current study is that training feeders were available during experimental trials, providing a more reliable source of nectar than the experimental feeders. The availability of the training feeders reduced the hummingbirds' dependency on the experimental feeders, perhaps reducing pressure on them to learn the nectar-replenishment rates of the experimental feeders.

Competitive ability may have influenced the refinement of each species' adjustment of return intervals to correspond with the optimal nectar-renewal rate. The VS, for example, was the largest and most aggressive hummingbird of the study and is considered highest in the dominance hierarchy out of the hummingbirds analyzed (O'Brien 2015). The VS was also observed chasing other species of hummingbirds away from feeders, preventing smaller hummingbirds from recognizing the feeder as a reliable food source and perhaps limiting their ability to learn the nectar refill rates or to return when they wanted to return even if they learned. This may explain why the mean return intervals of the PTMG, a smaller and less aggressive species, were higher and had more variance than the more dominant VS.

According to the results, hummingbirds in the tropical pre-montane cloud forest partially adjust their return intervals to flower patches of varying nectar-renewal rates to maximize nectar reward and minimize energy costs. It is noteworthy that even though test subjects were not territorial, many different hummingbirds drank from the feeders, and training feeders provided indefinite supplies of nectar during the experimental trials, the data showed evidence of episodic memory through overall changes to the mean return intervals to both feeders over the course of the study. A future study conducted in the tropics might more closely emulate previous studies by González-Gómez et al. by observing territorial hummingbird responses to differences in nectar concentration and nectar-replenishment rates, providing a better source of comparison between the foraging behavior of hummingbirds in the tropics vs temperate zones. The overall capacity for hummingbirds to adjust their foraging behavior in response to different nectar availabilities creates optimism their persistence in a changing environment. The similarities between the changes in behavior by hummingbirds in the tropic and temperate zones in response to different nectar sources provides evidence that the use of episodic memory during foraging is a common trait of the taxon.

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Nectar secondary compounds, sucrose concentration and palatability to a butterfly pollinator (*Heliconius melpomene*)

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ABSTRACT

Plants have evolved secondary compounds to defend against herbivory. Secondary compounds also occur in the nectar of many flowering plants, possibly deterring pollinators and negatively impacting pollination. The Nutrient-Toxin Titration Theory predicts that more nutritious nectar could compensate for the presence of toxins and maintain pollinator visits. I tested multiple solutions containing different concentrations of sucrose with different concentrations of quinine or nicotine, both naturally occurring alkaloids found in floral nectar. *Heliconius melpomene* butterflies drank less sucrose solution when nicotine was present (on average 15.12 +/- 2.12 µl SE vs 31.15 +/- 4.36 µl SE) and drank more quinine-sucrose solutions at lower quinine concentrations (on average 47.20 +/- 2.94 µl SE vs 65.27 +/- 4.07 µl SE). *H. melpomene* tend to prefer solutions with 20% sucrose by weight, regardless of alkaloid concentration. An increase in sucrose concentration somewhat compensates for the presence of alkaloids tested up to a 20% sucrose concentration. However plants may never fully be able to compensate for toxic nectar, as shown by higher amounts of nectar taken when no alkaloids were present.

RESUMEN

Las plantas han evolucionado compuestos secundarios para defenderse contra la herbivoría. Los compuestos secundarios también ocurren en el néctar de varias plantas que producen flores, posiblemente disuadiendo los polinizadores e impactando negativamente la polinización. La teoría de Tritación de Nutrientes-Toxinas predice que el néctar más nutritivo puede compensar por la presencia de tóxinas y mantener las visitas de los polinizadores. Probé diferentes disoluciones con diferentes concentraciones de sacarosa que contienen diferentes concentraciones de quinina o nicotina, ambos alcaloides que ocurren naturalmente en el néctar floral. Las mariposas *Heliconius melpomene* toman menos soluciones de sacarosa cuando hay nicotina presente (promedio 15.12 +/- 2.12 µl SE vs 31.15 +/- 4.36 µl SE) y toman más soluciones de sacarosa-quinina con las menores concentraciones de quinina (en promedio 47.20 +/- 2.94 µl SE vs 65.27 +/- 4.07 µl SE). *H. melpomene* tiende a preferir soluciones de 20% de sacarosa por peso, sin importar la concentración de los alcaloides. Un aumento en la concentración de sacarosa de alguna manera compensa por la presencia de alcaloides probados hasta un 20% de la concentración de sacarosa. Sin embargo las plantas pueden no ser totalmente capaces de compensar por el néctar tóxico, como es demostrado por las grandes cantidades de néctar consumidos cuando los alcaloides no están presentes.

INTRODUCTION

The coevolution of plants and herbivores plays an important role in plant biochemistry (Coley et al. 1985; Coley & Barone 1996). Many plant species develop secondary compounds in their tissues that help them defend against herbivores (Freeland & Janzen 1974). Surprisingly, these compounds are also found in the nectar of many plant species. Secondary compounds with known toxicity to herbivores have been found in at least 21 plant families (Gegear et al. 2007). This phenomenon is called toxic nectar. Toxic nectar could have a negative effect on the fitness of plants by deterring pollinators and reducing pollination (Alder & Irwin 2005). The Nutrient-Toxin Titration Theory (NTT; Cipollini & Levey 1997b) states that plants compensate for bitter tasting compounds in their nectar by increasing nectar quality in other ways.

The exact reason for the existence of toxic nectar has yet to be determined. Toxic nectar may be a byproduct of the presence of defense compounds in other tissues (Alder 2000).

Selection in favor of defense compounds may result in the unavoidable inclusion of secondary compounds in nectar. For example, increases in herbivory are positively linked to nectar alkaloid concentrations (Alder 2006). Alternatively, toxic nectar may deter nectar robbers while still attracting pollinators (Alder & Irwin 2005), however there is little research to support this idea. Toxic nectar in *Catalpa speciosa* plants deterred nectar robbers by reducing the volume of nectar that they stole and inducing erratic behavior after consumption, however effects on pollinators were unknown (Stephenson 1981). Similar to the nectar robber hypothesis, toxic nectar may be a way for plants to control which pollinators visit their flowers. The attraction of a pollinator to a plant is influenced by a variety of characteristics such as nectar viscosity, sugar type, and flower morphology. It is possible that nectar secondary compounds act as chemical filters for pollinators. Bitter nectar has been shown to deter certain species of hummingbirds but not others (Johnson et al. 2006). Such filters may be beneficial because specialized pollinators may be more effective pollinators by carrying more pollen and collecting a more genetically diverse array of pollen from distant locations (Betts et al. 2015).

Although the reason for toxic nectar is unclear, it has been demonstrated to interfere with pollination. For example, nectar alkaloids decrease the rate of flower visitation and length of stay in both nectar robbers and pollinators (Cipollini and Levey 1997a; Masters 1991). Plants may counter negative impacts of nectar secondary compounds by increasing nectar quality in other ways. The NTT predicts that a plant will compensate for undesirable compounds in their nectar or fruit by increasing the nutritional quality of the reward (Cipollini & Levey 1997b). Such compensation may come in the form of higher concentrations of nutrients or higher caloric value. For example, unpleasant tastes of secondary compounds in nectar may be masked by higher sucrose concentrations (Glendinning 2002). Increasing sucrose concentration increases the caloric value of the rewards and improves the taste of nectar. This theory relies on the ability of nutrients to fully compensate for the presence of secondary compounds so that pollination and dispersal rates are unaffected (Cipollini & Levey 1997a).

Butterflies are important pollinators of many plant species. In some cases, their pollination behavior can be impacted by nectar alkaloids, reducing the amount of nectar they imbibe and rate of visitation (Master 1991), but not in all cases (Landlot & Lenczewski 1993). Some butterflies sequester alkaloids to use as anti-predator defense and are not deterred by nectar alkaloids (Edgar 1982; Nishida 1994). *Heliconius melpomene* (Nymphalidae: Heliconiinae) is a common neotropical nectar-feeding butterfly. It does not sequester alkaloids, so it does not have a predisposed preference for alkaloids. Here, I determine *H. melpomene's* sucrose concentration preference in the absence of alkaloids and the volume imbibed. I also assess the impact of nicotine and quinine, types of nectar alkaloids, on quantity of nectar imbibed. I test the interaction of sucrose concentration with nicotine and quinine to see if changes in sucrose concentration compensate for the presence of bitter alkaloids in nectar.

METHODS

Study Site

This study was conducted at the Monteverde Butterfly Garden, Monteverde, Costa Rica. The butterflies were enclosed in a series of large flight enclosures with glass and mesh walls. The enclosure maintains a relatively steady temperature but it is still partially exposed to surrounding temperature fluctuations. All individuals were collected from the same room every day.

Study Organism

Heliconius melpomene butterflies are solely nectar feeders. They are long-lived butterflies that are also known to consume amino acids from pollen (Gilbert 1972). They insert their proboscis into flower to extract nectar through suction feeding. They are most active in the morning and afternoon on sunny days.

Sucrose Solutions

Sucrose solutions of 0%, 5%, 10%, 20%, and 40% sucrose by weight were made with white table sugar and spring water (Table 1). 10 ml of each solution were made and stored in closed glass vials. These vials were stored at room temperature between trials.

Table 1. Composition of non-toxic sucrose solutions of varying sucrose concentrations. Solutions were tested on *Heliconius melpomene* butterflies in the Monteverde Butterfly Garden, Monteverde, Costa Rica. Common white table sugar was used to make the solution. 10 ml of each solution was made. 100 µl of solution was offered to each butterfly.

Solution	Sucrose Concentration (% by weight)
Non-Toxic	0%
	5%
	10%
	20%
	40%

Nicotine Solutions

Nicotine solutions were made using tobacco leaves from cigarettes, white table sugar, and spring water (Table 2). 0.9mg of nicotine was extracted from one cigarette. The tobacco leaves were placed in a glass container with enough methanol to cover the leaves (approximately 5 ml). This was then placed in a vibrating water bath for 30 minutes. The nicotine leaves were removed from the methanol. The methanol solution was then filtered. The glass container and the methanol solution were placed in a food dryer at 75 ˚C until the methanol evaporated. The remaining nicotine in the glass container was then mixed with 30 ml of water and divided evenly between three glass vials.

Table 2. Composition of nicotine and control solutions with varying concentrations of sucrose. Solutions were tested on *Heliconius melpomene* butterflies in the Monteverde Butterfly Garden, Monteverde, Costa Rica. Nicotine was extracted from cigarette tobacco leaves using methanol. Common white table sugar was used as the sucrose. 10 ml of each solution was made. 100 µl of solution was offered to each butterfly.

Nicotine Solution	Sucrose Concentration (% by weight)	Nicotine Concentration
Nicotine	10%	30 mg/L
Nicotine	20%	30 mg/L
Nicotine	40%	30 mg/L
Control	10%	-
Control	20%	-
Control	40%	-

One gram of sucrose was added to the first vial to create a solution of 30 mg nicotine/L + 10% sucrose (Table 2). Two grams of sucrose was added to the second vial in order to create a solution of 30 mg nicotine/L + 20% sucrose. Four grams of sucrose was added to the third vial in order to create a solution of 30 mg nicotine/L + 40% sucrose. An additional three solutions of 10%, 20%, and 40% sucrose by weight were made without nicotine to act as the control solutions. The nicotine concentration is within standard concentrations found in nature, which can range between 4.9 mg/L and 505.8 mg/L (Ji et al. 2000).

Quinine Solutions

Quinine solutions were made using sucrose, spring water, and quinine water (Canada Dry Tonic Water; Table 3). Quinine water contains 80 mg quinine/L and 10% sucrose (Ohira et al. 2013). The sucrose content was confirmed using a refractometer. The quinine water was poured into a large metal bowl to remove all carbonation before making the solutions.

Ten ml of quinine water was added to three glass vials. The first vial remained unaltered to serve as the solution of 80 mg quinine/L + 10% sucrose. One gram of sucrose was added to the second vial to create a solution of 80 mg quinine/L + 20% sucrose. Three grams of sucrose were added to the third vial to create a solution of 80 mg quinine/L + 40% sucrose. One ml of quinine water and 9.0 ml of water were added to three new glass vials. 0.9 g of sucrose was added to the first vial in order to create a solution of 8 mg quinine/L + 10% sucrose. 1.9 g of sucrose was added to the second vial in order to create a solution of 8 mg quinine/L + 20% sucrose. 3.9 g of sucrose was added to the third vial to create a solution of 8 mg quinine/L + 40% sucrose (Table 3). These concentrations are within standard concentrations found in nature, which can range between 4.9 mg/L and 505.8 mg/L (Ji et al. 2000).

Table 3. Composition of high quinine and low quinine concentration solutions with varying levels of sucrose concentration. Solutions were tested on *Heliconius melpomene* butterflies in the Monteverde Butterfly Garden, Monteverde, Costa Rica. Common white table sugar was used as the sucrose. The quinine came from quinine water. 10 ml of each solution was made. 100 µl of solution was offered to each butterfly.

Quinine Solution	Sucrose Concentration (% by weight)	Quinine Concentration
Low	10%	8 mg/L
Low	20%	8 mg/L
Low	40%	8 mg/L
High	10%	80 mg/L
High	20%	80 mg/L
High	40%	80 mg/L

Preference of Sucrose Concentration, Quinine, and Nicotine

In the morning before feeding, butterflies were held in a mesh cage for two hours. The cage was kept indoors to maintain a steady temperature. Each butterfly was tested one at a time, once per day. Each butterfly was given a unique marking on the underside of its wings to distinguish individuals and ensure that no butterfly was tested more than once per day. The butterflies were released into the garden at the end of the day. To control for the effects of weather on butterfly hunger and behavior, multiple types of solutions were tested on each day and all feeding trials were under an incandescent bulb of 60 watts placed 30 cm from the feeding butterfly.

The five non-toxic sucrose solutions, six quinine solutions, and six nicotine solutions were tested by placing a single drop of 100 µl of the desired solution on a piece of wax paper using a 100 µl microcapillary tube. A butterfly was taken from the holding chamber by holding it gently at the base of its wings. Its proboscis was uncurled into the solution using the tip of a paperclip. The proboscis was held in the solution for three seconds before the paperclip was removed. If the butterfly did not start feeding on the first attempt, two additional attempts were made. When the butterfly started to feed, its wings were released and it was allowed to feed freely. The butterfly was returned to the holding chamber if did not feed, or at the end of its feeding trial. Each solution was tested on 17 butterflies. The volume imbibed was measured by subtracting the volume that remained from the initial volume.

RESULTS

Sucrose Concentration Preference

Butterflies tended to favor 20% sucrose over the other concentrations (ANOVA, F = 12.51, df = 4, $p < 0.0001$; Figure 1). Butterflies drank more of the 20% sucrose (59.61 +/- 6.99 µl SE) compared to 0% sucrose (10.11 +/- 1.22 µl SE), 5% sucrose (33.40 +/- 5.20 µl SE), and 10% sucrose (41.94 +/- 5.73 µl SE; Fisher LSD, $p < 0.05$; Figure 1). Butterflies consumed 22.38% more of the 20% sucrose than the 40% sucrose (46.27 +/- 4.98 µl SE), however this difference was not significant (Fisher LSD, p = 0.073; Figure 1).

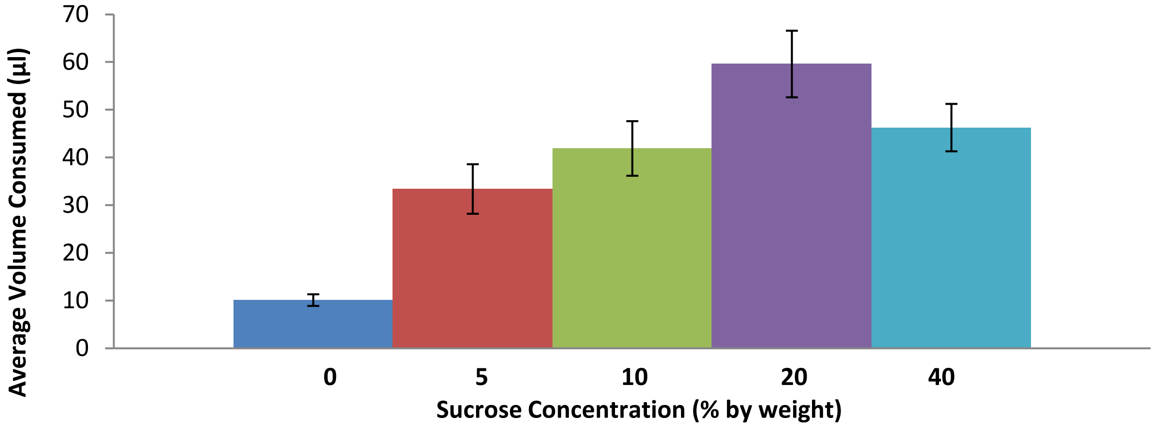

Figure 1. Average solution consumption of 0%, 5%, 10%, 20%, and 40% sucrose solutions by weight by *Heliconius melpomene* butterflies at the Monteverde Butterfly Garden, Monteverde, Costa Rica (n=17). Solutions were made using common white sugar. Butterflies favored 20% sucrose when compared with 0%, 5%, and 10% (Fisher LSD, $p < 0.05$). Error bars represent standard error of the mean.

Quinine concentration and sucrose concentration

Both sucrose concentration (ANOVA, F = 5.07, df = 2, p = 0.008) and quinine concentration (ANOVA, F = 13.77, df = 1, p = 0.0003) impacted the amount of solution imbibed by *H. melpomene*. In 20% sucrose solutions, low quinine (75.49 +/- 7.19) solution was favored over high quinine solution (58.21 +/- 5.10 µl SE; Fisher LSD, p = 0.043). The same results were found in 40% sucrose solutions; butterflies drank more low quinine solution (63.88 +/- 5.02 µl SE) than high quinine solution (42.72 +/- 4.14 µl SE; Fisher LSD, p = 0.014; Figure 2). In 10% sucrose solutions, butterflies drank 15.78% more low quinine (56.45 +/- 8.17 µl SE) than high quinine (40.67 +/- 5.16 µl SE), but this difference was not quite statistically significant (Fisher LSD, p = 0.064; Figure 2).

Butterflies tended to prefer 20% when controlling for quinine concentration. In high quinine solutions, there was a statistically significant preference for 20% sucrose when compared

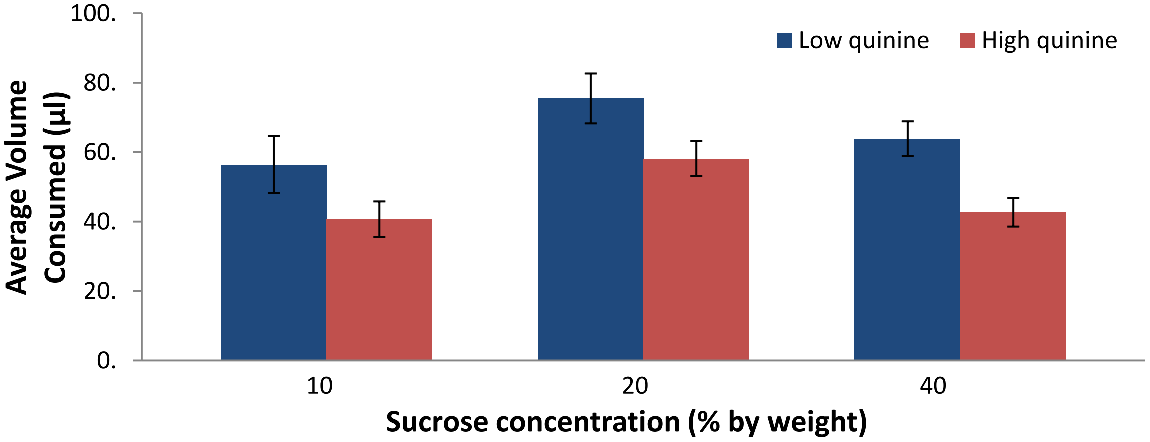

Figure 2. Average volume of quinine solution imbibed by *Heliconius melpomene* butterflies at the Monteverde Butterfly Garden, Monteverde, Costa Rica (n=17). Error bars represent standard error of the mean. Low Quinine solutions contained 8 mg quinine/L. High Quinine solutions contained 80 mg quinine/L. In both 10%, 20%, and 40% sucrose, the butterflies drank a significant amount more low quinine nectar than high quinine nectar (Fisher LSD, $p < 0.05$). Amongst the low quinine solutions and high quinine solutions, the difference between sucrose concentrations shows a trend in favor of 20% sucrose.

with 10% sucrose (Fisher LSD, p = 0.04). There was a trend in favor of the consumption 20% sucrose when compared with 40%, however it was marginally statistically significant (Fisher LSD, p = 0.06). A similar pattern was found in low quinine solutions. Here, the only groups that were statistically different from each other were 10% sucrose and 20% sucrose, in which butterflies drank 33.73% more 20% sucrose than 10% sucrose (Fisher LSD, df = 2, p = 0.026; Figure 2).

Nicotine and sucrose concentration

Statistical analysis showed a preference for control nectar over nicotine nectar. A 2-Way ANOVA showed significant differences between sucrose concentrations (F = 4.84, df = 2, p = 0.009) and between nicotine concentrations (F = 38.19, df = 1, p < 0.00001; Figure 3). In 10% sucrose solutions, control (38.48 +/- 6.61 µl SE) was preferred over nicotine (15.71 +/- 3.16 µl SE; Fisher LSD, p = 0.006). Butterflies always preferred control when controlling for sucrose concentration (Fisher LSD, p < 0.05).

Amongst the nicotine solutions, there were few significant differences in consumption. However, there was a slight trend in favor of the consumption of 20% sucrose (28.15 +/- 4.93 µl SE). Of the control solutions, only significant difference existed between 10% sucrose (38.47 +/- 6.6 µl SE) and 20% sucrose (38.48 +/- 7.76 µl SE), in which butterflies consumed 57.95% more of the 20% sucrose (Fisher LSD, p = 0.007).

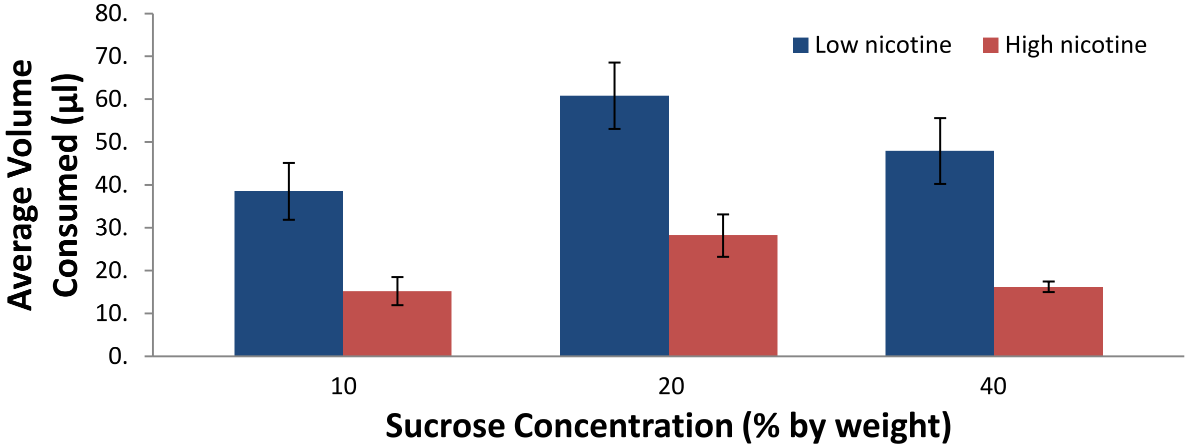

Figure 3. Average volume of nicotine solution imbibed by *Heliconius melpomene* butterflies at the Monteverde Butterfly Garden, Monteverde, Costa Rica (n=17). Error bars represent standard error of the mean. Control solutions contained no nicotine. Nicotine solutions contained 30 mg nicotine/L. There were significant differences between the high nicotine solutions and control solutions of the same sucrose concentration in which control was preferred by butterflies (Fisher LSD, p < 0.05). There is a slight trend in favor of the 20% sucrose solutions regardless of the presence or absence of nicotine, however this trend is not statistically significant in all cases.

DISCUSSION

Floral nectar alkaloids have been shown to deter pollinator butterflies due to their bitter taste. The NTT suggests that plants should be able to compensate for the unpalatability of bitter alkaloids in nectar by increasing sucrose concentration. I examined the effect of sucrose concentration on the palatability of nectar containing quinine and nicotine by measuring volume imbibed. I conducted this study on *H. melpomene* butterflies in the Monteverde Butterfly

Garden, Costa Rica. My data shows that plants are not able to fully compensate for the presence of alkaloids in their nectar.

H. melpomene favored 20% sucrose solutions. Butterflies did not change their preference from 20% sucrose to 40% sucrose in the presence of alkaloids. This finding concurred with past studies that found that most butterfly families preferred nectar with a 20%-25% concentration of sucrose (Cirino 1998; Olsson 2008). The viscosity of the nectar plays an important role in determining solution preference. At higher concentrations of sucrose, the viscosity becomes a limiting factor driving butterfly choice over palatability (Kim 2011). While it is possible that butterflies may prefer the taste of nectars with higher sucrose concentrations, it is too difficult for them to drink it. The small diameter of the proboscis limits the viscosity of the nectar that they can drink (Kingsolver & Daniel 1979). Higher concentrations of sucrose would provide more nutrients per unit of volume, but it would also increase the viscosity of the nectar and decrease the rate at which it is imbibed (May 1985). The nectar viscosity, therefore, limits plants' ability to compensate for the presence of bitter alkaloids, even at relatively low concentrations. Presuming that butterflies are already primarily drinking nectar of the optimal viscosity, plants cannot increase sugar concentration without moving out of the optimal viscosity range.

No matter how much sugar was added, the butterflies always drank less alkaloid nectar than non-alkaloid nectar. Therefore, it may be impossible for a plant to fully compensate for the presence of some secondary compounds in their nectar. The viscosity of nectar limits a plant's ability to compensate. However, sucrose is not the only compound in nectar that provides nutritional benefits. For example, long-lived butterfly species like *H. melpomene* require amino acids in their diets (Gilbert 1972). Studies have shown that butterflies prefer nectars with higher amino acid concentrations (Erhardt & Baker 1990; Mevi-Schutz & Erhardt 2005). It is possible that the NTT still applies to butterflies and nectar with secondary compounds, but with nutrients other than sucrose. Further experiments testing the effects of amino acids and toxic compounds on butterfly preference could lend insight into the importance of various nectar secondary compounds on butterfly preference.

The presence of quinine and nicotine may act as pollinator deterrents, here shown by the significant decrease in the volume of nectar imbibed by *H. melpomene*. However, the butterflies had a stronger negative reaction to nicotine than to quinine. Quinine may not be a strong deterrent of butterflies (Landlot & Lenczewski 1993). They consistently drank a larger volume of quinine solutions than nicotine solutions, indicating that not all defense compounds induce the same reaction amongst pollinators. This response could be an incidental physiological aspect of the organism or an adaptation developed from coevolution. If *H. melpomene* coevolved with plants that had low levels of quinine in their nectar, this could explain its relative tolerance of quinine compared to nicotine.

The negative response of the butterflies to toxic nectar suggests that the presence of secondary compounds in nectar may be a byproduct of herbivore defense. *H. melpomene* does not appear to have adapted to alkaloids in nectar but rather maintained an aversion to their taste. Toxic nectar does not appear to confer any adaptive advantage in attracting pollinators, and instead reduces the palatability of the pollinator reward. And yet plants with toxic nectar are still able to persist in nature. Toxic nectar may actually be an adaptive response in which plants use bitter compounds to select for their preferred pollinator. While *H. melpomene* dislikes nicotine more than quinine, *Bombus terrestris* prefers nicotine to quinine (Tiedeken et al. 2014). Therefore, the type of secondary compound in the nectar has the ability to deter specific pollinators. The exact function of toxic nectars has yet to be definitively determined but its adaptive function and significance plays an important role in understanding the convergence and interaction of herbivore defense and pollinator attraction.

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Evaluation of hummingbird pollination syndrome in flowers of *Besleria princeps*

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ABSTRACT

Pollination syndromes are collections of traits to evolved by plants to attract specific functional groups of pollinators. Hummingbird pollination syndrome is characterized by large, bright, typically red/orange flowers with wide, tubular corollas, large quantities of nectar, and exserted anthers and stigma that are high off the ground. *Besleria princeps*' external morphology aligns with hummingbird pollination syndrome, however there is some disagreement on whether the pollinators of *B. princeps* are hummingbirds or insects though there is no real evidence supporting either claim and ultimately the pollinator is unknown. The aim of this study was to determine how *B. princeps* fits with the hummingbird pollination syndrome and if it is primarily visited by hummingbirds. Flower and nectar characteristics, such as corolla width and length and nectar volume, sugar concentration, replenishment rate, were measured in the field and compared with those of known hummingbird-pollinated species. Hummingbird visitations to *B. princeps* were observed using camera traps. A feeder experiment was conducted to determine if proximity to the ground deterred hummingbird visitation using camera traps and hummingbird feeders hung at heights representing *B. princeps* and plants high enough to not put visitors at risk for terrestrial predators. *B. princeps'* corolla morphology was similar to that of known hummingbird-pollinated species, but nectar volume ($\text{mean}_{B.\ princeps} = 0.40 \pm 0.04$, $\text{mean}_{\text{known}} = 13.60 \pm 5.83$) and sugar concentration ($\text{mean}_{B.\ princeps} = 5.26 \pm 0.74$, $\text{mean}_{\text{known}} = 21.60 \pm 1.66$) were significantly lower. In 1,440 hours of continuous recording camera traps recorded *B. princeps* being visited by hummingbirds only twice. Feeder height had no effect on hummingbird visitation. *B princeps* exhibits some of the traits associated with hummingbird pollination syndrome in flower color and corolla length and width, but it deviates from the syndrome by having included anthers, low nectar volume, low nectar sugar concentration, and a close proximity to the ground. *B. princeps* is not primarily visited by hummingbirds. Its superficial appearance as hummingbird-pollinated plant is deceiving, as its nectar characteristics make it unattractive to hummingbirds. *B. princeps* does not meet the qualifications of hummingbird pollination syndrome. The success of pollination syndromes as predictors is greatly dependent on evaluating all of the relevant flower characteristics, rather than basing predictions on external morphology.

RESUMEN

Los síndromes de polinización son una colección de rasgos que evolucionaron por las plantas para atraer grupos funcionales de polinizadores. El síndrome de polinización de colibríes es caracterizado por flores largas, brillantes, típicamente de colores rojo/anaranjado, con corolas tubulares amplias, largas cantidades de néctar, y con anteras y estigmas expuestas que están elevadas del suelo. La morfología externa de *Besleria princeps* se alinea con el síndrome de polinización por colibríes, sin embargo hay desacuerdo si los polinizadores de *B. princeps* son colibríes o insectos aunque no hay evidencia real que soporte esto y ultimadamente el polinizador es desconocido. El propósito de este estudio fue determinar como *B. princeps* encaja en el síndrome de polinización por colibríes y si es visitado primariamente por los mismos. Las características de las flores y el néctar, tales como ancho y largo de la corola y volumen de néctar, concentración de azúcar, tasa de reposición, se midieron en el campo para comparar con aquellos de especies conocidad polinizadas por colibríes. Las visitas de colibríes a *B. princeps* se observaron usando cámaras trampa. Un experimento con comederos se condujo para determinar si la proximidad al suelo disuade a los colibríes de visitar las flores usando cámaras trampa y comederos de colibrí colgados a elevaciones que representan a *B. princeps* y plantas lo suficientemente altas que no pongan a los visitantes en riesgo potencial por depredadores. La morfología de la corola de *B. princeps* fue similar a especies que se conoce son polinizadas por colibríes, pero el volumen de néctar volume ($\text{promedio}_{B.\ princeps} = 0.40 \pm 0.04$, $\text{promedio}_{\text{known}} = 13.60 \pm 5.83$) y la concentración de azúcar ($\text{promedio}_{B.\ princeps} = 5.26 \pm 0.74$, $\text{promedio}_{\text{known}} = 21.60 \pm 1.66$) fueron significativamente más bajos. En 1

440 horas de grabación continua de las cámaras trampa se grabó unicamente dos visitas de colibrí visitando *B. princeps*. La altura a la que se colocó el comedero no tiene ningún efecto en la visita de colibríes. *B, princeps* exhibe algunas características asociadas con el síndrome de polinización por colibríes en el color de la flor y el tamaño y ancho de la corola, pero se desvia del síndrome al tener anteras interiors, bajo volumen de néctar, baja concentración de azúcar y una proximidad al suelo. *B princeps* no es visitada primariamente por colibríes. Su apariencia superficial a una planta polinizada por colibríes es engañosa, ya que las características del néctar la hace poco atractiva para los colibríes. *B princeps* no reune las cualidades del síndrome de polinización por colibríes. El éxito de los sindromes de polinización como predictores depende ampliamente de la evaluación de todas las características relevantes de las flores, más que de basarse en predicciones de la morfología externa.

INTRODUCTION

Pollination is an important, if not vital, part of a plant's reproductive life cycle and for this reason much of the plant's energy is dedicated to attracting pollinators (Waser & Ollerton 2006). Plants employ many different strategies to encourage pollination: some plants are more generalized and attract all kinds of pollinators, whereas others are more specialized (Waser & Ollerton 2006). How we classify plants as "generalists" or "specialists" depends greatly on our categorization of the plant's pollinators. According to the Pollination Syndrome Hypothesis plants may develop phenotypic traits that reflect specialization for a group of pollinators (Fenster *et al.* 2004). The hypothesis claims that plants evolve in response to the selective pressures of their primary pollinators and that regardless of phylogenetic relationships all plants pollinated by a particular functional group of pollinators should have a set of similar traits that characterize the syndrome (Fenster *et al.* 2004) A pollination syndrome is a suite of floral traits, including rewards, associated with the attraction and utilization of a specific group of animals as pollinators (Fenster *et al.* 2004). For instance bat pollination syndrome is characterized by large bell-shaped flowers with large quantities of dilute nectar that shed pollen at night (Martén-Rodríguez et al. 2009).

Plants primarily pollinated by hummingbirds typically have a specific set of floral traits that constitute the hummingbird pollination syndrome (Fenster *et al.* 2004). Plants specialized for hummingbird pollination tend to have large, bright, typically red/orange flowers with wide, tubular corollas, large quantities of nectar, and exserted anthers and stigma that are high off the ground (Long 1997, Fenster *et al.* 2004, Waser & Ollerton 2006). There are many plants that rely on hummingbirds for pollination and many plants that exhibit all of these traits (Fenster *et al.* 2004). However, there are some hummingbird-pollinated plants that do not possess all of the key traits presumably evolved to attract hummingbirds (Fenster et al. 2004). There are also plants that posses all morphological characteristics of hummingbird-pollinated flowers but are rarely visited by hummingbirds due to little nectar production (Martén-Rodríguez and Fenster 2008) . In general the use of floral traits should not be used as predictors of pollinator when no field data are available (Martén-Rodríguez et al. 2009). More field studies, particularly in the tropics, are necessary to determine whether plants that miss some of the floral traits characteristic of the hummingbird pollination syndrome are indeed primarily pollinated by hummingbirds (Martén-Rodríguez and Fenster 2008).

The family Gesneriaceae displays great floral diversity and many pollination syndromes in the Neotropics but few studies have documented pollinators in the field (Martén-Rodríguez et al. 2009). In this family, congeneric species that look morphologically similar may differ in pollinators. For instance, *Gesneria reticulata* and *Gesneria cuneifolia* are sister species that morphologically conform to the hummingbird pollination syndrome but field studies confirmed that the former relies on self pollination and produces little nectar while the latter is indeed primarily pollinated by hummingbirds and produces much more nectar (Martén-Rodríguez and Fenster 2008).

The genus *Besleria* also includes closely related morphologies that suggest hummingbird pollination in most montane species (Haber 2000). Flowers in this genus exhibit many traits that are part of the hummingbird pollination syndrome but the anthers are included rather than exserted, such as in the shrub *Besleria triflora* for example (Feinsinger *et al.* 1986). Nonetheless, *B. triflora* is clearly pollinated by hummingbirds (Feinsinger *et al.* 1986). The related *B. princeps* is an unresolved case. The flowers are externally similar to those of *B. triflora*, and also exhibit included anthers, but it contrasts with *B. triflora* in that it produces low amounts of nectar (Obegi 1999, Murphy 2003). The latter as led some authors to classify it as insect pollinated (Linhart *et al* 1987, Temeles *et al* 2002) while others consider it hummingbirds pollinated (Haber 2000). Flowers are protandrous with asexual phases (Obegi 1999) so it is possible that only one of the two phases produces low amounts of nectar (Aizen and Basilio 1998) but this possibility remains untested. Another particularity of *B. princeps* that departs from the classic hummingbird pollination syndrome is that it only reaches about 50 cm in height (personal observation) which could increase the risk of predation for hummingbirds from terrestrial predators (Lima 1991). The bright orange flower and long tubular corolla of *B. princeps* should attract hummingbirds (Fenster *et al.* 2004), but its proximity to the ground and low nectar production may make it less attractive to hummingbirds (Lima 1991, Long 1997, Fenster *et al.* 2004). Low height and low nectar volumes may not necessary deter hummingbird visitation because the plant could produce small amounts of highly concentrated nectar to encourage hummingbirds to visit the flowers frequently (Henderson et al. 2006, González-Gómez et al. 2011) in spite of the high risk of predation. Nectar concentration and refilling rates in this species have not been measured to the best of my knowledge.

The aim of this study is to evaluate if *B. princeps* quantitatively aligns with the morphological and nectar characteristics of hummingbird pollination syndrome plant species and determine if *B. princeps* is actually visited by hummingbirds frequently enough to function as its main pollinator. My specific goals were: 1) comparing the flower morphology and nectar characteristics of *B. princeps* against the same features in known hummingbird-pollinated local species, 2) quantifying visitation to *B. princeps* flowers, and 3) determining if close proximity to the ground is a hummingbird deterrent using artificial hummingbird feeders.

MATERIALS AND METHODS

All data was collected in the cloud forest behind Estación Biológica in Monteverde, Costa Rica along or near Principal, División, and Mirador trails. The study had three parts: the first part consisted of collecting data regarding nectar characteristics and flower morphology, the second part was observing pollinator visitation, the third part was an experiment testing the willingness of hummingbirds to visit feeders close to the ground.

Flower Morphology and Nectar Characteristics

Data was collected on the length and width of the corolla, the sex of the flower, nectar volume, sugar concentration, and replenishment rate of each flower. Thirty-six flowers on individual plants were sampled, the fist six were used to learn how to extract nectar and only the data on corolla length and width was used. All samples were taken within an elevation range of 1690-

1790 meters and the majority of the samples were between 1750 and 1790 meters. The sampled flowers were within 0-8.1 meters from the trail. All flowers were on Principal, División, or Mirador trails. The length of the corolla was measured from the aperture of the flower to the end of the bract and width was measured horizontally across the flower's aperture, because it was asymmetrical and horizontal width was the limiting factor for a bird's beak. One–and occasionally two–microliter capillary tubes were used to collect the nectar. The capillary tube was held in the flower for one minute. Because nectar volumes were typically less than one microliter, volume was measured by recording the distance the nectar traveled in the capillary tube in 0.5 millimeter (mm) increments. The value was converted into microliters by measuring the length of the capillary tube in 0.5 mm increments and dividing the total capacity of the capillary tube (in microliters) by the number of 0.5mm increments to create a conversion value representing the volume that each increment of 0.5mm measurements. The distance that the nectar traveled in 0.5 mm was multiplied by the conversion value. Sugar concentration was measured using a hand-held refractometer. The nectar volume and sugar concentration of each flower were measured twice with a five-minute interval between the first and second samples in to record replenishment rate. The sex of the flower was determined visually based on figures from Obegi (1999).

The data from each flower characteristic, excluding sex, were compared against data from the literature. Corolla length and width of *B. princeps* was compared against data on the corolla length and width of flowers preferred by Purple-throated Mountain Gems (Temeles *et al* 2002) in Monteverde, Costa Rica, which were found to visit *B. princeps* (see results). Sugar concentration of *B. princeps* nectar was compared with the sugar concentrations of nectar of known hummingbird-pollinated plants in the mountains of Costa Rica (Baker 1975). The nectar volume of *B. princeps* was compared with the nectar volume of four hummingbird-pollinated plants in Monteverde, Costa Rica (Feinsinger 1986).

Pollinator Visitation

Pollinator visitation observations were taken using eight camera traps placed in eight patches chosen for having clusters of at least three *B. princeps* flowers. Seven patches were on División trail and one patch was on Mirador trail. All patches were within an elevation range of 1750-1770 meters and all cameras were within 0-9 meters from the trail, with a minimum distance of 7.80 meters between cameras. The camera traps were active for 15 days. The number of hummingbird sightings and visits to *B. princeps* were recorded, as well as the species of hummingbird. Data for known hummingbird-pollinated plant visitation was taken from Martén-Rodríguez and Fenster (2008).

Feeder Experiment

The feeder experiment was designed to evaluate hummingbirds' willingness to visit feeders close to the ground. Six hummingbird feeders and six camera traps at two different heights were set up in three patches where flowering *B. princeps* were absent. Artificial nectar was synthesized with a sugar concentration of 15% and each feeder was filled with 157 mL of nectar every other day throughout the experiment. A sugar concentration of 15% was chosen because it was the average sugar concentration of *B. triflora* nectar (Feinsinger et al. 1986) and was within the range of nectar concentrations recorded for *B. princeps* (see results). Six feeders were selected and paired together so that the bases were similar. To test the effects of feeder height on hummingbird visitation, three patches were selected 3-8 meters off División trail and in each patch two feeders were hung between trees, one with its base at 130-150 cm off the ground, and the second feeder directly below it with its base at 50-60 cm off the ground. The feeder close to the ground represented *Besleria princeps*, which is about 20-50 meters tall. The high treatment represented a flower that was outside of the range of terrestrial predators. Camera traps were set up opposite to each feeder and an orange tarp was strung above the feeders to protect them from rain. The camera traps were active for five days and the feeders were refilled and the camera memory cards replaced every other day until they were removed. Camera trap footage was reviewed and number of visits was recorded. Visits constituted a hummingbird drinking from the exposed hole or attempting to drink but being prevented from drinking by some obstruction to the hole. The species of each visitor was identified.

RESULTS

Flower Morphology and Nectar Characteristics

Sex

Sexual phase has no influence on nectar volume or nectar sugar concentration. Nectar sugar concentration and volume were similarly low between male and female sex phases (Table 1, Figure 1, Figure 2).

Table 1. Influence of male and female sexual phases on nectar volume and sugar concentrations of the flowers of *Besleria princeps* in the cloud forest of Monteverde, Costa Rica. Results of a Welch Two Sample t-test comparing nectar volume and sugar concentration in male and female sexual phases.

Volume Sample 1	t = 1.45, df = 23.40, p = 0.16, n = 14
Volume Sample 2	t = 1.34, df = 18.75, p = 0.20, n = 14
Concentration Sample 1	t = -0.47, df = 11.64, p = 0.65, n = 14
Concentration Sample 2	t = -0.30, df = 13.60, p = 0.77, n = 14

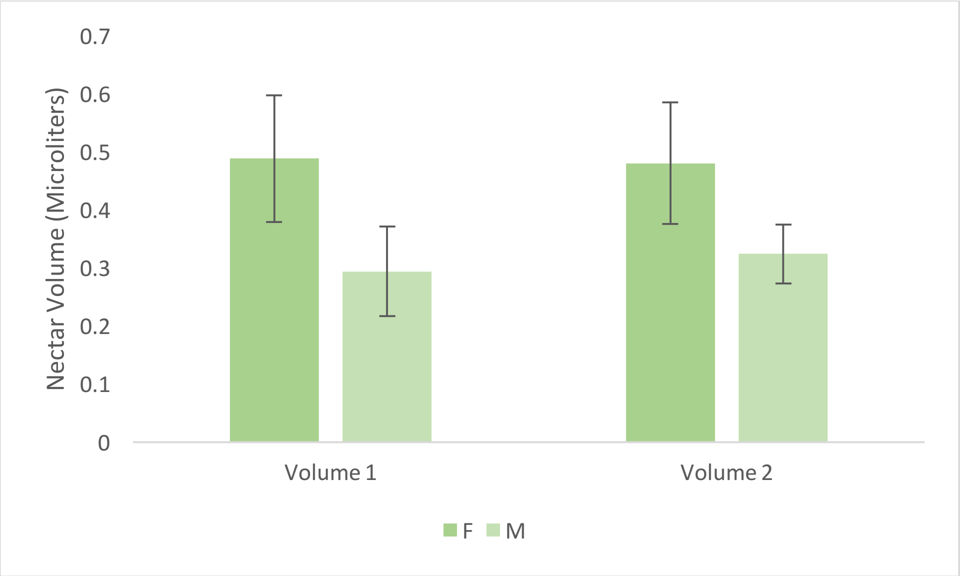

Figure 1. Influence of male (M) and female (F) sexual phases on nectar volume of the flowers of *Besleria princeps* in the cloud forest of Monteverde, Costa Rica. There is no difference in the

nectar volume between the male and female sex phases. The two nectar volumes were sampled within five minutes of each other. Mean volume ± one standard error.

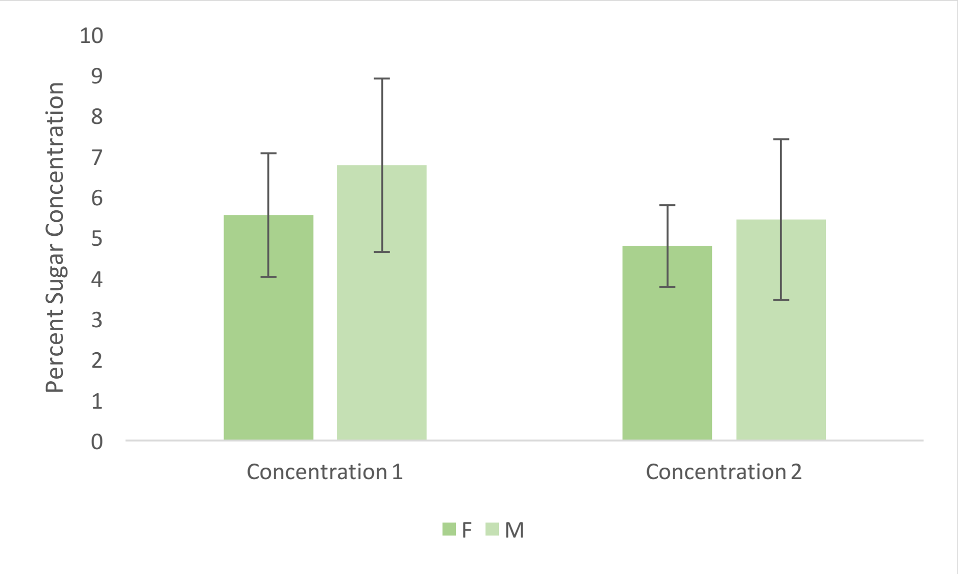

Figure 2. Influence of male (M) and female (F) sexual phases on nectar sugar concentrations of the flowers of *Besleria princeps* in the cloud forest of Monteverde, Costa Rica. There is no difference in the nectar sugar concentration between the male and female sex phases. The two concentrations were sampled within five minutes of each other. Mean sugar concentration ± one standard error.

Short Term Nectar Replacement

Flowers replaced their total nectar volume within five minutes after its extraction. Volumes were similar when the flower was first sampled and when the second sample was extracted (Paired t-test, t = -0.40, df = 29, p = 0.69, n = 30 pairs, Figure 3).

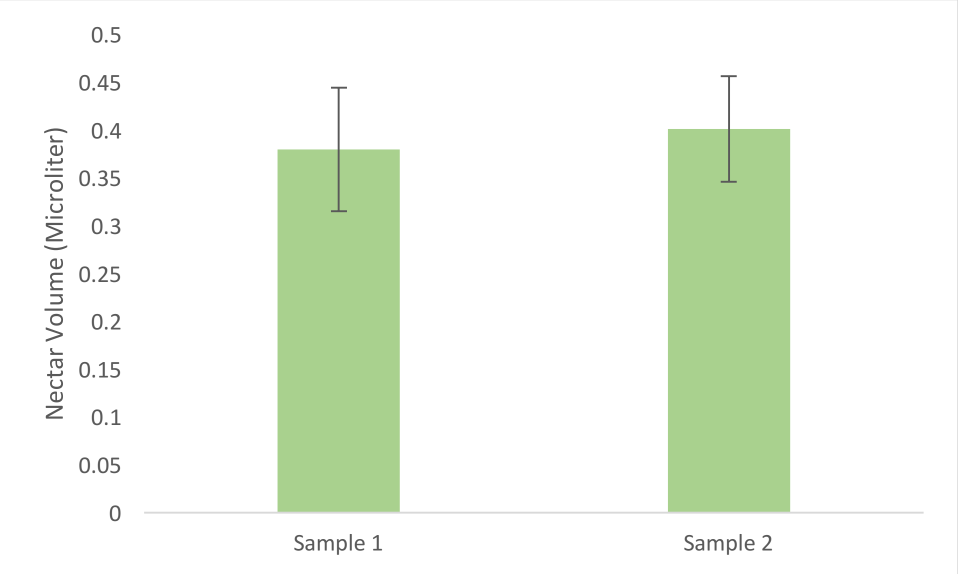

Figure 3. Nectar volume in *Besleria princeps* flowers in the cloud forest of Monteverde, Costa Rica in the first sample and in the second sample taken five minutes later. There is no difference in nectar volume between the first and second samples. Mean nectar volume ± one standard error.

Nectar sugar concentration did not change when the nectar was replenished within five minutes of its initial extraction. Sugar concentrations were similar when the flower was first sampled and when it was sampled again five minutes later (Paired t-test, $t = 1.56$, $df = 12$, $p = 0.14$, $n_1 = 18$, $n_2 = 24$, Figure 4).

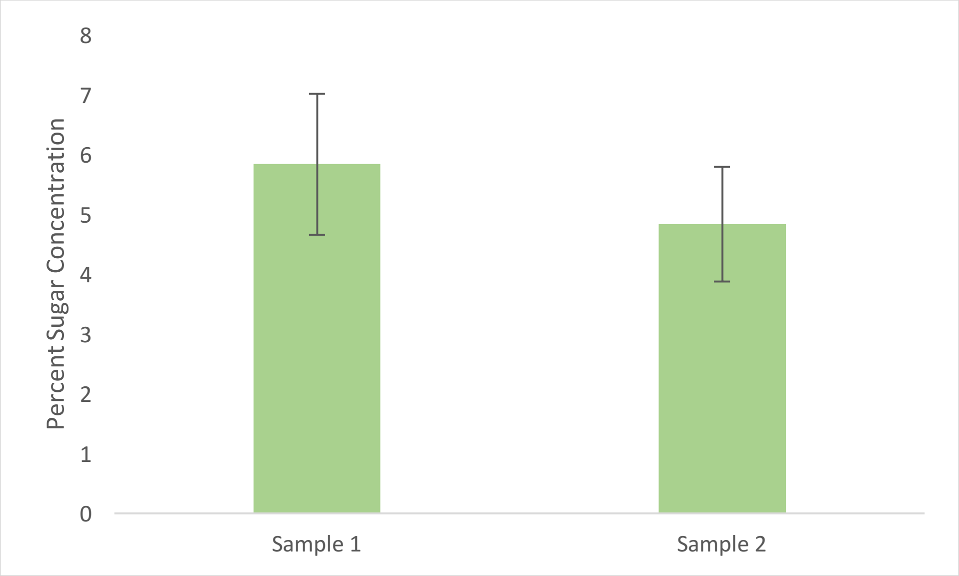

Figure 4. Nectar sugar concentration in *Besleria princeps* flowers in the cloud forest of Monteverde, Costa Rica when first sampled and when sampled a second time five minutes later. There is no difference in nectar sugar concentration between the first and second samples. Mean nectar sugar concentration ± one standard error.

Comparison with Known Values

Corolla Characteristics

B. princeps has a similar corolla length (Welch Two Sample t-test, $t = 0.59$, $df = 7.12$, $p = 0.57$, $n_{B.\ princeps} = 36$, $n_{\text{Known}} = 8$, Figure 5) and width (Welch Two Sample t-test, $t = 0.02$, $df = 7.35$, $p = 0.99$, $n_{B.\ princeps} = 36$, $n_{\text{Known}} = 8$, Figure 6) as known hummingbird-pollinated species in Monteverde, Costa Rica.

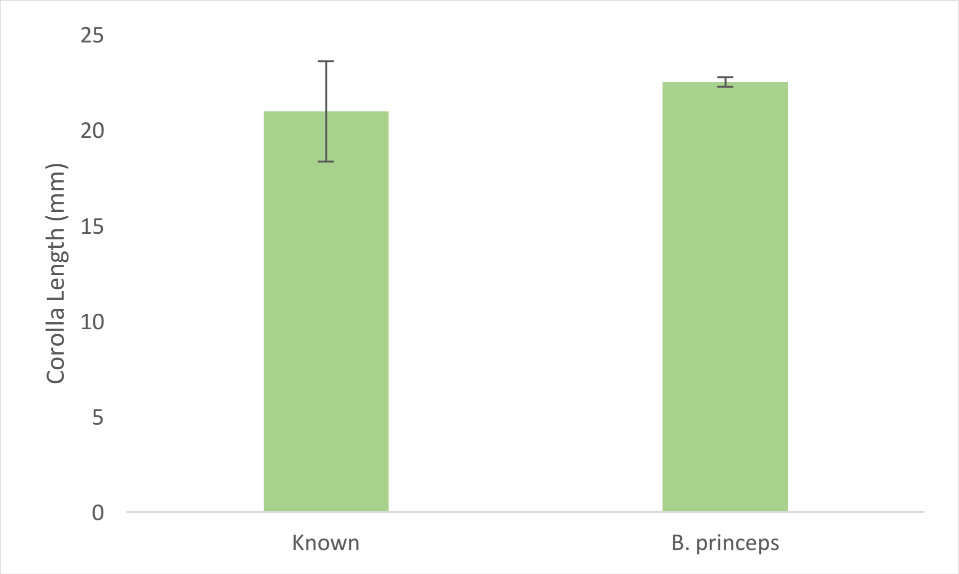

Figure 5. Corolla length in *Besleria princeps* flowers in the cloud forest of Monteverde, Costa Rica compared with the corolla lengths of known hummingbird-pollinated species in Monteverde, Costa Rica. There is no difference between corolla length in *B. princeps* and known hummingbird-pollinated plant species. Mean corolla length ± one standard error.

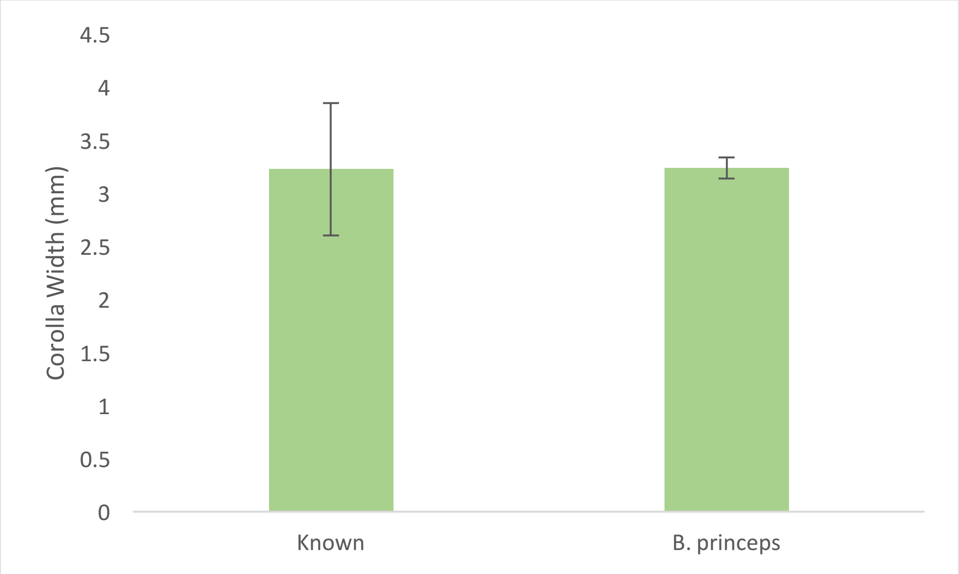

Figure 6. Corolla width in *Besleria princeps* flowers in the cloud forest of Monteverde, Costa Rica compared with the corolla lengths of known hummingbird-pollinated species in Monteverde, Costa Rica. There is no difference between corolla width in *B. princeps* and known hummingbird-pollinated plant species. Mean corolla width ± one standard error.

Nectar Characteristics

The nectar volume of *B. princeps* was much less than the nectar volume of known hummingbird-pollinated species in Monteverde, Costa Rica ($\text{mean}_{B.\ princeps} = 0.40 \pm 0.04$, $\text{mean}_{\text{known}} = 13.60 \pm 5.83$; Independent Wilcoxon Rank Test, w = 0, p = 0.00091, $n_{B.\ princeps} = 60$, $n_{\text{Known}} = 4$, Figure 7).

Nectar volume in *B. princeps* ranged from 0-1.70 microliters and known hummingbird-pollinated species volumes ranged from 3.8-30.5 microliters.

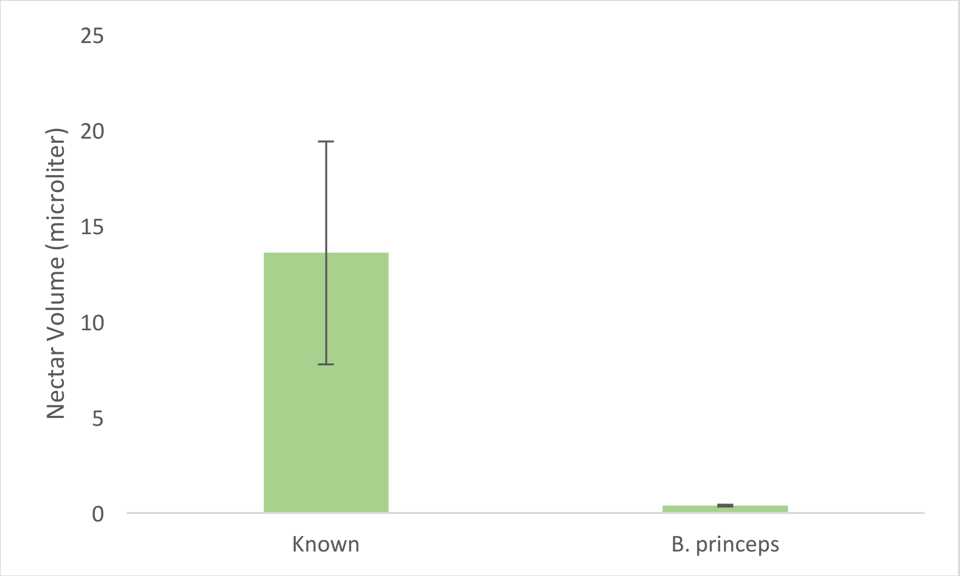

Figure 7. Nectar volume in *Besleria princeps* flowers in the cloud forest of Monteverde, Costa Rica compared with the nectar volumes of known hummingbird-pollinated plant species in the mountains of Costa Rica. There is no difference between nectar volume in *B. princeps* and known hummingbird-pollinated plant species. Mean nectar volume ± one standard error.

The nectar sugar concentration of *B. princeps* was much less than the nectar sugar concentration of known hummingbird-pollinated plant species in the mountains of Costa Rica ($\text{mean}_{B.\ princeps}$ = 5.26 ± 0.74, $\text{mean}_{\text{known}}$= 21.60 ± 1.66; Welch Two Sample t-test, t =-9.00, df = 12.80, p = < 0.0001, $n_{B.\ princeps}$ = 42, n_{Known} = 10, Figure 8). Nectar sugar concentration in *B. princeps* ranged from 0.5-17.5%, and four of the 42 samples fell within a range of 12-17.5%, the range of sugar concentration in known hummingbird-pollinated plants was 16-31%.

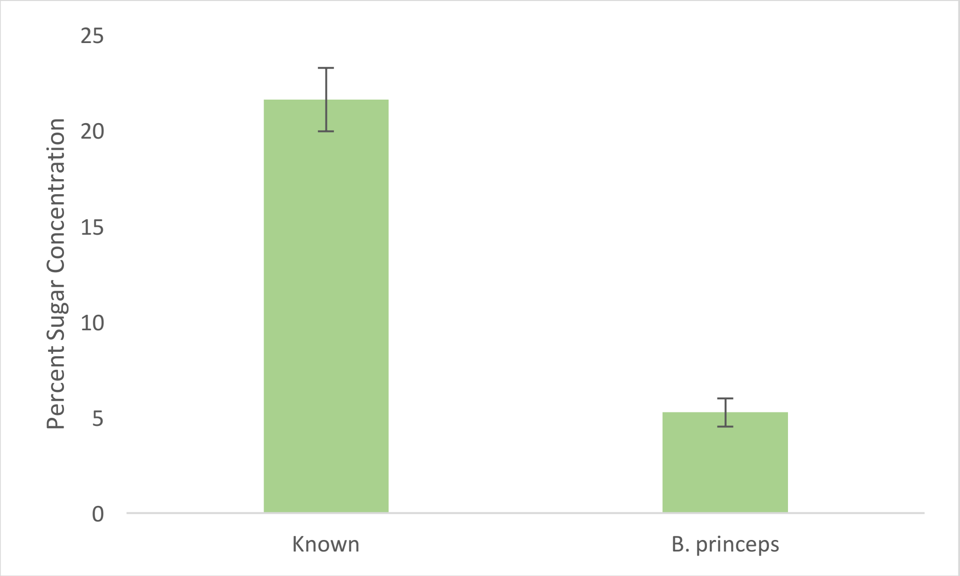

Figure 8. Nectar sugar concentration in *Besleria princeps* flowers in the cloud forest of Monteverde, Costa Rica compared with the nectar sugar concentrations of known hummingbird-pollinated plant species in the mountains of Costa Rica. There is no difference between nectar sugar concentration in *B. princeps* and known hummingbird-pollinated plants. Mean nectar sugar concentration ± one standard error.

Flower Visitation

In the 1,440 combined hours that the eight camera traps were active there were two hummingbird visitations one by a male and one by a female Purple-throated Mountain-gem and one potential visit from an unidentifiable hummingbird. Per hour *B. princeps* was visited 0.001 times. Traditional hummingbird-pollinated plants like *Gesneria citrina* and *G. cuneifolia* were visited 0.194 and 0.146 times per hour respectively. *G. reticulata,* a generalist that was misclassified as hummingbird-pollinated, had a visitation rate of 0.023.

Feeder Experiment

Hummingbirds showed no preference for one feeder height over the other. There was no significant difference in number of hummingbird visits between feeders at high and low positions (Wilcoxon Independent Samples, V = 37, p = 0.76, n = 18, Figure 9).

Figure 9. Influence of feeder height on hummingbird visitation per day in the cloud forest of Monteverde, Costa Rica. Hummingbirds showed no preference based on feeder height. Box shows 25th, 50th, and 75th percentiles and the whisker represents the 95th percentile.

High feeders were visited 86 times and low feeders were visited 61 times. Feeders in both the high and low positions were visited by the Green-crowned Brilliant, the Stripe-tailed Hummingbird, the Coppery-headed Emerald, the Violet Sabrewing, the Green Hermit, and the Purple-throated Mountain-gem, which visited *B. princeps* as well.

DISCUSSION

My results show that although *B. princeps* exhibits some traits associated with hummingbird pollination syndrome (Fenster et al. 2004) it is not frequently visited by hummingbirds. The bright orange color of its flower is a common trait of hummingbird-pollinated species and the length and width of its corolla are similar to those of plant species known to be pollinated by hummingbirds in Monteverde (Fenster et al. 2004). While being high off the ground is a trait included in the pollination syndrome for hummingbird-pollinated species, in this study it was determined that hummingbirds are not deterred from visiting nectar sources by close proximity to the ground, and showed no preference for nectar sources higher off the ground. While the height, or lack there of, of *B. princeps* might not be a deterrent for hummingbirds as proposed at the start of this study, it does not appear that they visit the flowers frequently. Hummingbirds were observed visiting *B. princeps* flowers only twice, both visitors were Purple-throated Mountain-gems, which are also the most frequent visitor of *B. triflora* (Feinsinger *et al* 1986, Temeles et al. 2002). The volume and sugar concentration of the nectar of *B. princeps* is much lower than most hummingbird-pollinated species, and hummingbirds rely on high nectar volume and sugar concentration to fuel their speedy metabolisms (Long 1997). *B. princeps* was able to completely replace its nectar within five minutes of extraction. It may be that the small volume of nectar is compensated for by the rapid replenishment rate, however this would not make up for *B. princeps*' low nectar sugar concentration, as hummingbirds prioritize concentration over replenishment (Castellanos *et al* 2002).

Some closely species in the Gesnerieae family have different pollinators but both appear to fit the hummingbird pollination syndrome (Martén-Rodríguez and Fenster 2009). *B. triflora*, a species known to be pollinated by hummingbirds, has an average nectar sugar concentration of 15% and an average nectar volume of 10.8 microliters (Feinsinger *et al* 1986). The mean sugar concentration of *B. princeps'* nectar is much lower than that of *B. triflora,* but there were a few *B. princeps* flowers that produced nectar with a similar sugar concentration to *B. triflora*. This indicates that while *B. princeps'* nectar does have a low sugar concentration for a hummingbird-pollinated plant and its average concentration is low even compared *B. triflora*, which has a below average sugar concentration compared with other species (Feinsinger *et al* 1986), some flowers do produce nectar with a similar sugar concentration. However, the same does not hold true for *B. princeps'* nectar volume. The highest nectar volume measured for *B. princeps* was only a little over a tenth of the nectar volume of *B. triflora*. *B. princeps* may have an ancestor with higher nectar volume and concentration that was hummingbird-pollinated, though if so *B. princeps* has lost those traits.

B. princeps is visited by hummingbirds despite its low nectar volume and sugar concentration. However, it is visited very rarely. *B. princeps'* visitation rate was two orders of magnitude less than known hummingbird-pollinated plants, and one order of magnitude less than *G. reticulata*, a generalist species that was misclassified as hummingbird-pollinated. If *G. reticulata* is misclassified, it is even more likely that *B. princeps* is also misclassified given that its visitation rate is so much lower. Hummingbirds have learned that despite the appearance of *G. reticulata* as a hummingbird-pollinated plant it contains little nectar and has a low sugar concentration and therefore is not a good resource (Martén-Rodríguez and Fenster 2008). *B. princeps* may be in a similar situation as its external morphology fits the syndrome, but it is rarely visited by hummingbirds and also has a low nectar volume and sugar concentration. Even though *B. princeps* replenished its nectar quickly, this was not enough to regularly attract hummingbirds.

Given the infrequency of hummingbird visitation it is likely that *B. princeps* does not rely on hummingbirds for reproduction. *B. princeps* is not primarily hummingbird pollinated, but it is unclear why it exhibit traits specific to hummingbird pollination syndrome. *Rhytidophyllum leucomallon,* a member of the Gesnerieae family, is a generalist species pollinated by bats, hummingbirds, and insects that has been misclassified as a hummingbird-pollinated by the hummingbird pollination syndrome (Martén-Rodríguez and Fenster 2008, Martén-Rodríguez *et al.* 2009). One possible explanation for generalist species to exhibit traits associated with specialization on a specific type of pollinator is asymmetric specialization (Martén-Rodríguez *et al.* 2009). This occurs when plants specialize on specific pollinator functional groups but the pollinators do not specialize in return, leading to flowers that exhibit certain traits intended to attract, for instance, hummingbirds but because the pollinators are not reciprocating specialization the flower is actually pollinated by insects, bats, *and* hummingbirds making a flower that intends to be a specialist, a generalist (Martén-Rodríguez et al. 2009). *B. princeps* may also be a generalist species that only appears to be exclusively hummingbird-pollinated, but in fact does not rely on hummingbirds for reproduction.

Linhart *et al* (1987) lists *B. princeps* as insect pollinated, though they do not specify what type of insect pollinates it. Nectar concentration of hummingbird and various insect pollinated species is fairly similar (Baker 1975). Because insects are not more likely to be attracted to nectar with a low sugar concentration, there does not appear to be any reason why insects would be attracted to *B. princeps* which exhibits more characteristics of a hummingbird-pollinated plant than an insect pollinated plant. In fact, low sugar concentration in nectar has been used to deter bee visitation (Martén-Rodríguez and Fenster 2008). *B. princeps* may use self-pollination (Linhart *et al* 1987), however, unisexual phases are thought to reduce self-pollination (Obegi 1999). *G. reticulata* is considered to primarily reproduce through autonomous selfing (Martén-Rodríguez and Fenster 2008). This may be seen as an indication that *B. princeps* also has high rates of selfing given the other similarities that it shares with *G. reticulata*. However, *G. pedunculosa* is protandrous, like *B. princeps,* and does not self-pollinate because the stamens curl down below the stigma before it becomes active (Martén-Rodríguez and Fenster 2008). The stamens also appear drop down below the stigma in *B. princeps* (personal observation) supporting the idea that it does not rely on self-pollination. *B. princeps* does not primarily rely on pollination for reproduction (Murphy 2003). *B. princeps* grows in an environment well suited to vegetative reproduction through fragmentation (Obegi 1999), as the area is wet and experiences high levels of disturbance (Murphy 2003). *B. princeps* has been found to reproduce more frequently using fragmentation than seed germination (Murphy 2003), indicating that while it does produce fruits, *B. princeps* primarily reproduces without pollination.

In conclusion, *B. princeps* does not appear to be primarily pollinated by hummingbirds. *B. princeps'* flowers do exhibit some of the morphological traits of associated with hummingbird pollination syndrome (Fenster et al. 2004). However, its lack of height, included anthers, small amount of nectar, and low sugar concentration deviate from the hummingbird pollination syndrome (Fenster et al. 2004). While anther position and height do not affect hummingbird visitation, *B. princeps'* nectar characteristics are unattractive to hummingbirds. Looking exclusively at external morphology one would incorrectly classify *B. princeps* as a hummingbird-pollinated plant using the hummingbird pollination syndrome, however the nectar characteristics of the species do not align with the syndrome and indicate that it is not primarily pollinated by hummingbirds. Pollination syndromes can be useful when all traits are evaluated, however superficial traits are not accurate indicators of how well a plant aligns with the syndrome, and pollination syndromes can not be used in place of field work.

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Fruit pulp and seed caching behavior by agoutis (*Dasyprocta punctata*)

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ABSTRACT

Agouti caching behavior may be key to high plant diversity in Neotropical forests. Caches can be forgotten, making agoutis important seed dispersers. Rodent seed caching is likely to increase with nutritional value of the seed. Fruit pulp may promote caching directly through satiation or by agoutis selectively caching more nutritious food. In this study, agouti (*Dasyprocta punctata*), caching behavior with entire jocote (*Spondias purpurea*; Anacardiaceae) fruits were compared to jocote seeds with the pulp removed. Feeding sites were set up 1-2m off trail in secondary forest. Each site contained five tagged whole fruits and five tagged bare seeds. Seeds were either cached, consumed, or untouched after 3-4 hours of placement. Fruit pulp was eaten most often ($\bar{x}$ = 20 per day of 50 whole fruits placed) and statistically more than the entire fruit ($\bar{x}$ = 3.3846 per day of 50 placed) or manipulated seeds ($\bar{x}$ = 0.3846 per day of 50 placed). Entire fruits ($\bar{x}$ = 2.6923 per day of 50 placed) were cached more than seeds that either had their fruit pulps consumed by the agoutis ($\bar{x}$ = 0.3846 20 per day of 50 whole fruits placed) or were presented without pulp ($\bar{x}$ = 0.6154 per day of 50 placed). Therefore, whole fruits protect their seeds from agouti seed predation and increased their likelihood of being cached, which is essential to their eventual dispersal and germination.

RESUMEN

El comportamiento de acumulación de las guatusas puede ser la clave para la alta diversidad de plantas en los bosques Neotropicales. Las semillas acumuladas pueden ser olvidadas, haciendo a las guatusas importantes dispersores de semillas. El comportamiento de acumulación de los roedores es probable que aumente con el valor nutricional de la semilla. La pulpa de la fruta puede promover el comportamiento de acumulación por saciedad o por las guatusas seleccionando alimentos más nutritivos. En este estudio, el comportamiento de acumulación de la guatusa (Dasyprocta punctata) con jocotes (Spondias purpurea: Anacardiaceae) enteros se compararon con semillas de jocote con la pulpa removida. Estaciones de alimentación se ubicaron a 1-2 m afuera de los senderos en bosque secundario. Cada sitio contenía cinco frutos enteros con marcas y cinco semillas peladas con marca. Las semillas fueron acumuladas, consumidas, o no tocadas después de 3-4 horas de su colocación. La pulpa de la fruta fue consumida más frecuentemente ($\bar{x}$ = 20 por día de los 50 frutos colocados) y estadísticamente más que los frutos enteros ($\bar{x}$ = 3.3846 por día de los 50 colocados) o las semillas manipuladas ($\bar{x}$ = 0.3846 por día de las 50 colocadas). Los frutos enteros ($\bar{x}$ = 2.6923 por día de las 50 colocadas) se acumularon más frecuentemente que las semillas que tanto tuvieron la pulpa consumida por las guatusas ($\bar{x}$ = 0.3846 20 por día de los 50 frutos colocados) o que se presentaron sin la pulpa ($\bar{x}$ = 0.6154 por día de los 50 colocados). Así, los frutos enteros protegen las semillas de la depredación de semillas por las guatusas y aumenta la probabliidad de ser acumuladas, lo cual es esencial para su eventual dispersión y germinación.

INTRODUCTION

Some seed predators partake in cache hoarding, which likely plays a vital role in forest dynamics (Wang and Chen 2009), and may determine why diversity is so high in tropical forests (Jansen and Forget 2001). Hoarding makes seed dispersers of otherwise seed predators. Caching occurs when seed predators store seeds for periods of food scarcity. Caches are moved from site of seed discovery to lessen pilferage (Jansen and Forget 2001). These caches, if forgotten, can aid plant recruitment and may be important for seed dispersal (Donald and Boutin 2011) as seeds dispersed further from the parent plant have greater fitness and promote genetic diversity (Tilman 1997).

Rodents cache seeds and can also determine the nutritional content of the seed (Wang and Chen 2009). This ability is crucial, for when food is scarce they rely almost entirely on cached reserves (Jansen and Forget 2001). Therefore, food that rodents perceive as more valuable is likely to be cached first. Food value is most likely determined by energy, nutrient content and storage life (Jansen and Forget 2001), which suggests that seeds that are surrounded by a fruit pulp may be favored more by seed predators for either eating or caching (Wang and Chen 2009). On the other hand, density or offering a reward to the disperser (Nathan and Muller-Landau 2000) through satiation may determine the likelihood of caching (Guimarães *et al.* 2006). Masting fruits produce large seed crops with long intervals between to assure satiation and subsequent caching (Jansen and Forget 2001). The Quick-Meal Hypothesis predicts that fruit pulp encourages animal satiation, and will reduce the risk of seed predation and promote hoarding/caching, but many rodent-dispersed seeds do not have any pulp at all, leaving the question of the role of fruit pulp (Guimarães *et al.* 2006). A study with agoutis (*Dasyprocta leporine*) in South America tested the Quick Meal Hypothesis to try to determine what the presence of fruit pulp would be to the agoutis. Their results did not show any difference between caching *Hymeneae courbaril* (Fabaceae: Caesalpinaceae) seeds with and without the fruit's dry, sawdust-like pulp (Guimarães Jr *et al*. 2006).

While Guimarães (2006) did not find a preference by the agoutis toward the dry fruit pulp, there are still many large-seeded genera that are dispersed rodents have pulp to promote satiation and dispersal (Guimarães Jr *et al*. 2006). The presence of a fruit pulp that is juicier may be more preferred by agoutis if presented with that type of pulp and a bare seed removed of its juicy fruit pulp. This study aims to determine how the presence of juicy fruit pulp impacts the caching behavior of the Central American Agouti (*Dasyprocta punctata*). Caching behavior was evaluated with artificial feeding sites containing whole commercial jocote fruits and jocote seeds.

METHODS

Study Area

This study focused on fruit pulp and caching behavior of Central American Agoutis in secondary forest surrounding the Estación Biológica in Monteverde, Costa Rica. It is a tropical premontane wet forest at approximately 1500m above sea level. The feeding sites were placed in such a manner that open areas, trails, and steep slopes were avoided, yet contained native species that created a closed canopy (Figure 1). The study was conducted from mid-October to mid-November, which is the end of the wet season (Haber *et al*. 2000).

Figure 1. A typical study site with native secondary successional species creating a closed canopy. Feeding sites were located 1-2m away from trails. Here caching behavior of the Central American Agouti (*Dasyprocta punctata*) was observed with seeds surrounded by fruit pulp and those for which pulp was removed. All sites were located near the Estación Biológica in Monteverde, Costa Rica at approximately 1500m.

Study Species

Agoutis (*D. punctata*) are large contributors to seed predation in low- to middle-elevation rain forests from Southern Mexico to Ecuador. Their typical size is 50cm with a weight around 3kg. Foraging and sleeping are solitary activities for them, but they do live in monogamous pairs, and produce litters once or twice a year with 1-2 young each time. They den in tunnels, hollow trees, or under logs or brush piles. They typically occupy home ranges that are 2-3ha in size and are very common diurnal mammals. Their typical diet consists mainly of seeds but will occasionally eat fruits or fungi, and will scatter-hoard food in caches when the abundance of food is high. (Janzen 1983). Lastly, agoutis are unable to register red coloration, so they were not impacted by the methods used in this study (Farias Rocha 2009).

Camera Traps

Camera traps were set up at two sites per day for the first few days. This was to ensure agouti presence and high levels of activity were the study would be conducted. The camera traps showed that agoutis would consume jocotes and other diurnal mammals did not prefer them. Out of approximately 50 videos gathered from those traps, only a few showed the presence of white-nosed coatis (*Nasura narica*) at the feeding sites. While they were present, the data from the videos show that their presence was not overwhelming and they were not great influence on the data.

Feeding stations

To create the feeding sites, a total of ten tagged commercial jocotes, five whole fruits and five bare seeds, were placed in a single site. To get just the seeds of the jocotes, the fruit pulp had to be removed by hand using a knife did (Figure 2). The fruit pulp was removed in such a manner that the endocarp of the seed was exposed. For the first two days, six sites were placed 1-2m off the trail, then for the rest of data collection, ten sites were set up each day. Sites were placed 5m meters away from each other and 5m meters away from previous site locations, except for the first day. Sites were placed between 8-9am and left for 3-4 hours.

Figure 2. Preparation of jocotes to be used in feeding site to test if fruit pulp influences caching and predation behaviors of agoutis. Fruit pulps were removed by hand to expose the endocarp of the seed. Each site contained ten jocotes, five whole fruits and five bare seeds. Site materials were prepared the day before and preserved in the refrigerator to delay rotting. Materials were taken out in the morning and brought to room temperature before placement between 8-9am.

Seed tagging

Once 50 seeds were cleaned of pulp and 50 whole fruits had been chosen, they were all marked with tags (Figure 3). Using a Dremel, holes were drilled through the seeds, and for the case of the fruits, a hole was drilled through the fruit pulp and the seed. Fishing line was slipped through the hole, tied, and cut so there was a tail of approximately 15 cm in length. A piece of pink flagging tape was tied at the end of the fishing line 6 cm in length. For a whole fruit, a cut was made in the middle of the flagging tape to distinguish if the cached seed came from a seed covered with fruit or one with the pulp removed (see Gálvez *et al*. 2009).

Foraging Observations

After 3-4 hours, the sites were relocated and foraging observations were recorded. The jocotes were eaten, cached, or left untouched, and observations were recorded in eight categories: eaten whole fruits (fruit pulp and seed), fruit pulps, and bare seeds, cached fruits, seeds that were removed of their fruit by the agoutis, and bare seeds, and lastly, untouched fruits and seeds. A 5m radius sweep was also conducted around the site to accurately determine fate of the jocotes, fruits and seeds. From personal observation, the agoutis would eat within 1-2m of the site, if not at the site directly. If the entire fruit or bare seed had been consumed, then only the tag would remain, and if the fruit pulp was eaten, then a seed that was attached or next to a tag with a cut in the flagging tape would remain. If either had been cached, they could typically be relocated within 5m of the feed site as found from personal observations. Rarely would a cached object would not be relocated, but if it could not, then it was assumed to be cached due to their observed feeding habitats in relation to site placement along with the lack of tag remnants within 5m. All that could be retrieve, were retrieved so that any confusion or overlap with materials from other days were minimized.

Figure 3. Jocote feeding site used to test if fruit pulp impacted seed predation and caching by agoutis. Each site contained ten total commercial jocotes (*Spondias purpurea*), half whole jocote and half manipulated seeds. Each were tagged using a Dremel, fishing line, and pink flagging tape. Whole fruits had a cut made in the flagging to determine their origin, especially when fruit pulps were predated. Sites were set up 1-2m off trail, 5m away from each other, and at least 5m away from previous site locations. Sites were set out between 8-9am for 3-4 hours before fate of seeds was recorded.

RESULTS

Eaten

Feeding sites contain whole jocotes and jocote seeds, and agouti consumption of fruits differed significantly between the two treatments: with and without pulp (Friedman chi-squared = 20.939, df = 2, p-value = <0.001). Consumption of just fruit pulp (leaving the intact seed but not caching it) was much more frequent ($\overline{x}$ = 20 per day of 50 whole fruits placed) compared to eating both fruit with seed ($\overline{x}$ = 3.3846 per day of 50 whole fruits placed) and seeds with pulp previously removed ($\overline{x}$ = 0.3846 per day of 50 bare seeds placed). A pairwise comparison test among the three treatments showed they were all significantly different from one another (A-B p = 0.0059, A-C p = 0.0092, B-C p = 0.0007; Figure 4).

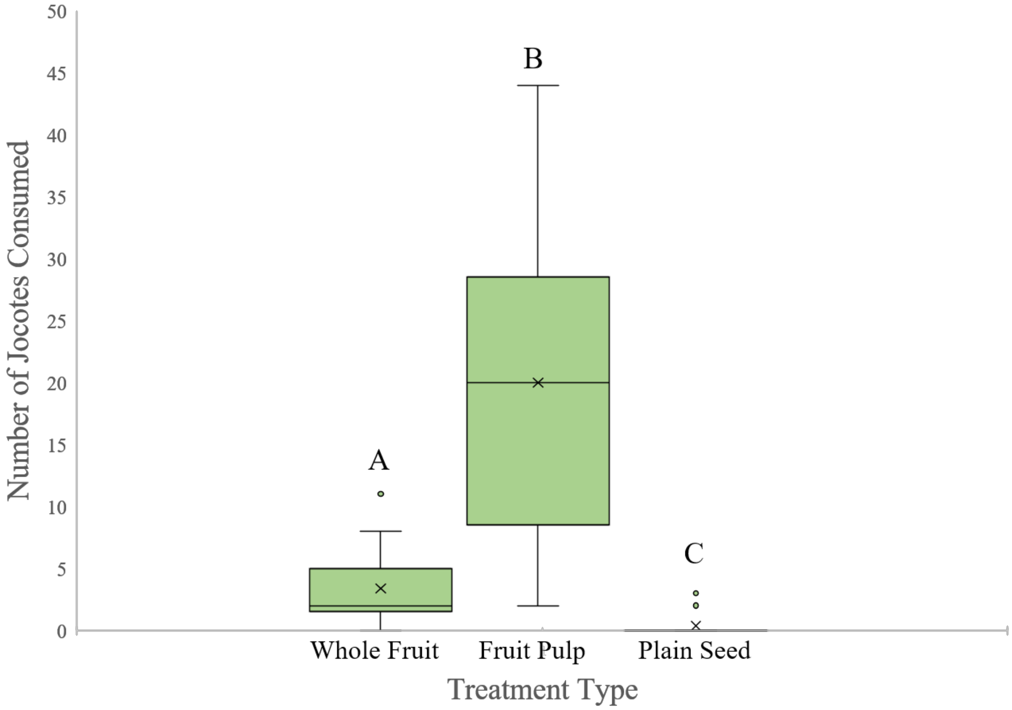

Figure 4. Number of jocotes (*Spondias purpurea*) consumed by agoutis with different outcomes. Whole Fruits = both fruit pulp and seed were consumed, Fruit Pulp = just fruit pulp consumed with seed left on ground, Plain Seed = seed with pulp previously removed were eaten. The trend indicated by this plot is that fruit pulps were eaten most by agoutis (*Dasyprocta punctata*) but leaving the seed behind. The different letters, A, B, C, above the plots signify that the treatments showed significant differences (Friedman post hoc tests at $p < 0.05$). Data were collected in closed canopy secondary growth forest near the Estación Biológica, Monteverde, Costa Rica.

Cached

Whole fruits were preferred for caching than the seeds from fruits whose pulps were eaten by the agoutis. Neither of those differed significantly from the caching preference of the bare seeds (Friedman chi-squared = 12.842, df = 2, p-value = 0.0016). Caching of whole fruits occurred most frequently ($\overline{x} = 2.6923$ out of 50 placed per day) than of seeds that had their fruit pulp eaten ($\overline{x} = 0.3846$), however, of the bare seeds placed per day, the amount they were cached per day ($\overline{x} = 0.6154$) did not differ statistically from the two other possible outcomes. A pairwise comparison test showed this differences and similarities of agouti preference (A-B p = 0.024, A-AB p = 0.057, B-AB p = 0.057; Figure 5). Labels of A and B in Figure 5 indicate significant differences between each other and label AB indicates a similarity between both A and B that those two do not share.

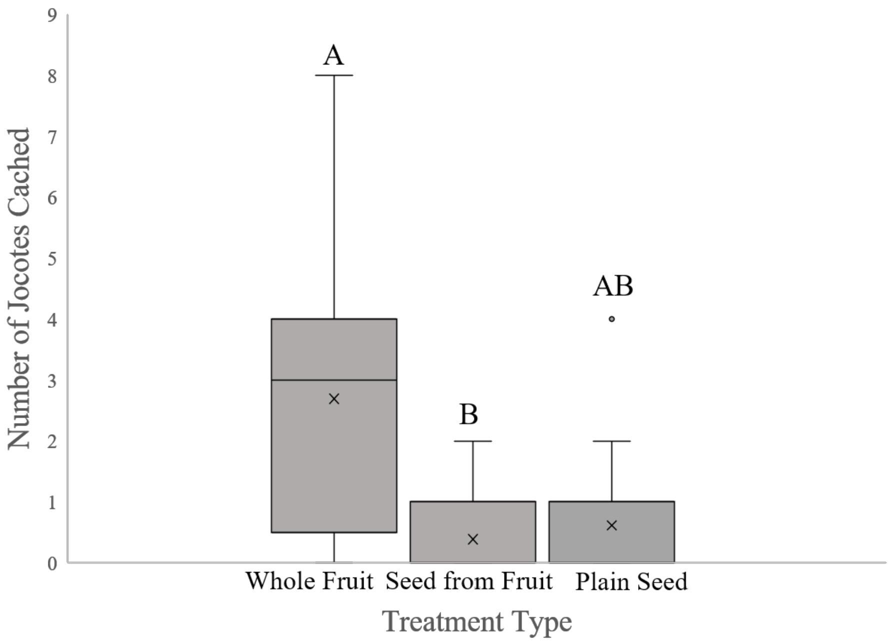

Figure 5. Number of jocotes (*Spondias purpurea*) cached as whole fruits, with pulp removed by the agouti or with pulp previously removed by researcher (Plain Seed). Whole fruits are preferred for caching by agoutis (*Dasyprocta punctata*) over seeds that have their fruit pulp eaten (Friedman test, $p < 0.05$). The different letters, A, B, AB, above the plots signify that treatments A and B showed differences among caching activity by the agoutis (*D. punctata*), but neither really differed from treatment AB. Caching, on a per day average, was done minimally in comparison to consuming the jocotes. Most activity was seen in the consumption of the whole fruits and fruit pulps and not to caching. Data were collected for this plot was collected in closed canopy, secondary growth forest near the Estación Biológica, Monteverde, Costa Rica.

Untouched

Agoutis were not always active at every site, on every day. Even when they were active, they would not consume or cache every whole fruit or seed in the sites. Here, agoutis preferred to leave seeds without fruit pulp untouched more than seeds surrounded by fruit pulp (Friedman chi-squared = 20.039, df = 2, p-value = <0.001). The pairwise comparison test proved whole fruits and seeds with eaten fruit pulp were similarly untouched, and bare seeds hardly received any interest from the agoutis as those with fruit pulp did. 50 bare seeds were placed out per day and as indicated by the graph, most remained at the end of the 3-4 hours (A(1)-A(2) p-value: 0.9060, A(1)-B p-value: 0.0032, A(2)-B p-value: 0.0007; Figure 6). Labels A(1) and A(2) indicate a statistically similarity between the whole fruits and their seeds after the pulp was eaten. Label B indicates a significant difference from A.

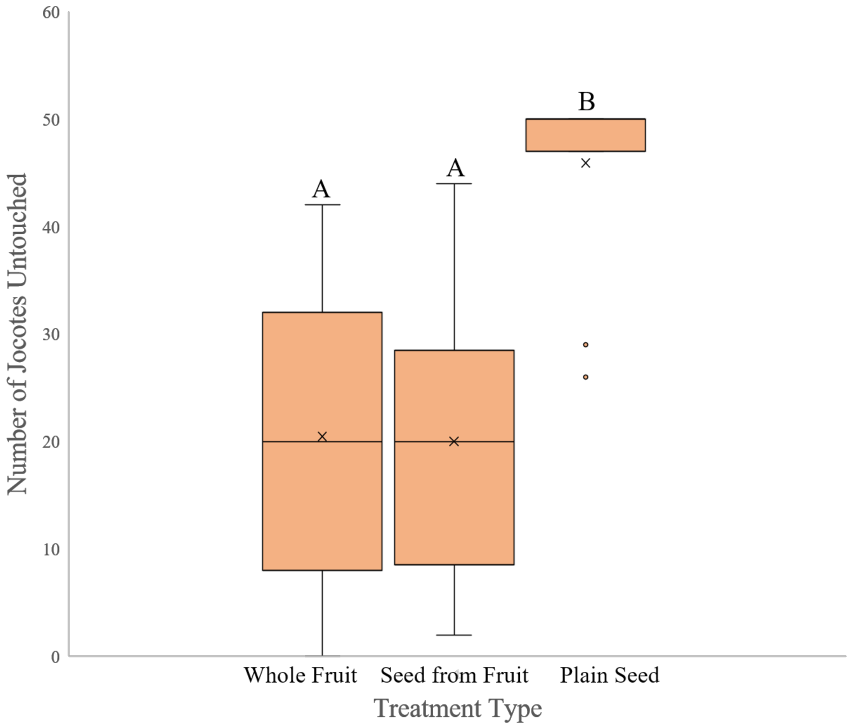

Figure 6. This box-and-whisker plot shows the amount of jocotes (*Spondias purpurea*) untouched with the various treatment types. The trend indicated by this plot is that manipulated seeds are significantly more likely to be left untouched by agoutis (*Dasyprocta punctata*) than seeds with consumed fruit pulps and whole fruits. The different letters, A and B, above the plots signify that both treatments of A and treatment B showed differences among activity by the agoutis (*D. punctata*). Data collected for this plot was collected in closed canopy, secondary growth forest near the Estación Biológica, Monteverde, Costa Rica.

DISCUSSION

Agoutis (*D. punctata*) are keystones of tropical forests due to their roles of both seed predators and seed dispersers (Guimarães Jr 2006). What favors seed caching over predation may depend on nutritional value (Jansen and Forget 2001), hunger, or other factors, such as abundance and density of the food (Gálvez *et al*. 2009). The Quick-Meal Hypothesis proposes fruit pulp may satiate seed predators like agoutis to prevent seed predation and favor caching (Guimarães Jr 2006).

Observations here clearly show that fruit pulp plays a role in the caching behavior of agoutis. Fruit pulp was eaten much more often than plain seeds, which also prevented the predation of the seed as well, to some extent. Most of the time only the fruit pulp was consumed, and the seeds were left. These findings agree with the Quick-Meal Hypothesis (Guimarães Jr 2006). A greater amount of caching of whole fruits was seen more often than the caching of their seeds after the fruit pulp was eaten, which goes against the Quick-Meal Hypothesis (Guimarães Jr 2006) but correlates with Jansen and Forget (2001) finding that rodents can determine the nutritional value of food source. The agoutis saw the fruits to be of greater value than manipulated seeds, for manipulated seeds saw greater rates of being ignored by their potential predators, and were left untouched most often (Figure 5). These results differ from the previous study conducted testing the Quick-Meal Hypothesis, for the agoutis showed a preference towards the fruit pulps, and in that study, there was not a preference toward either the fruit pulps or bare seeds. This study suggests something differ, for juicy fruit pulp is the preferable option here, which differs from their usual foraging preferences (Janzen 1983) and from that of dry pulp (Guimarães Jr 2006) or bare seeds, making it an advantageous adaptation.

These findings provide some insight in the advantageous evolutionary adaptations by seed-bearing species and what factors influenced them. For instance, there are two outcomes that impact seed germination, establishment and recruitment: if there are large numbers of fruits around, seeds are left alone and fruits are cached. Both decrease seed predation and caching whole fruits increases seed dispersal. As agoutis are density-dependent foragers, they maintain the diversity of tropical forests by reducing competition between tree species (Jansen and Forget 2001). Fruit pulp increases seed survival and dispersal by agoutis, so it should not be seen as merely an anachronism or having secondary importance to other seed dispersers, but as an evolutionary tactic. Fruit pulp may have evolved to increase seed survivorship and dispersal by caching animals, like agoutis, which should lead to viewing agoutis as frugivores or even seed dispersers rather than seed predators.

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Declining Herpetofauna in a Neotropical Cloud Forest

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ABSTRACT

Climate change is believed to be the number one cause of amphibian and reptile decline worldwide. In tropical montane Cloud Forest, climate change creates a lifting cloud bank, lowering historical mist frequency. This causes species to move up in elevation, but also brings new diseases, acid rain, and other changes that lead to defaunation. For example, in the late 1980's, climate change likely caused a chytrid fungus to become more virulent, leading to the rapid loss of nearly all amphibian populations on neotropical mountains. In Monteverde, Costa Rica, there were many local extinctions and one global extinction as a result. Nearly thirty years later, the impacts are still being felt. Using the Biological Station in Monteverde Costa Rica, over 100 hours of surveys were taken above 1400 meters elevation and compared to historic data embedded in past student research projects. Comparisons show that populations of every herpetofauna species with data are down from previous studies done in the same location. Comparisons with data 2004 and 2005 suggest *Norops* species have declined by 72%. This number can be extended to amphibian populations as well, although exact numbers could not be determined as previous population studies on most species are lacking. In addition to the defaunation of Monteverde's herpetofauna, current data show *Norops tropidolepis* moved its elevational range further up, in keeping with the concept of the lifting cloudbank. In fact, *N. tropidolepis* is nearly absent from its lower range. This agrees with studies showing other *Norops* species moving into the Monteverde Cloud Forest Reserve. Monteverde's many reserves and protected areas are insufficient to keep its herpetofauna from declining. The outcome is a new and emptier Cloud Forest ecosystem with novel species interactions whose populations will not recover without global changes.

RESUMEN

Se cree que el cambio climático es la principal causa de el decline de anfibios y reptiles a nivel mundial. En el bosque nuboso tropical montano, cambio climático crea que el banco de nubes se levante, disminuyendo la frecuencia histórica de neblina.Esto causa que las especies suban en elevación, pero también trae nuevas enfermedades, lluvia ácida y otros cambios que llevan a la defaunación. Por ejemplo, en los últimos años de la década de los 80s, el cambio climático causó que el hongo quitidrio se volviera más virulento, llevando a la rápida pérdida de cerca de todas las poblaciones de anfibios en las montañas neotropicales. En Monteverde, Costa Rica cerca de de 100 horas de muestreo fueron llevadas a cabo por encima de los 1400 metros de elevación y comparado con datos históricos incluidos en pasados proyectos de estudiantes. Comparaciones muestran que las poblaciones de cada especie de herpetofauna con datos son menores a previos estudios hechos en la misma locación. Comparaciones con datos del 2004 y 2005 sugieren que especies de *Norops* han declinado en un 72%. Este número puede ser extendido a poblaciones de anfibios también, aunque los números exactos no puedan ser determinados por falta de estudios previos de la población de la mayoría de especies. Además de la defaunación de la herpetofauna de Monteverde, datos actuales muestran que *Norops tropidolepis* movió su rango elevacional mucho más arriba, concordando con el efecto del levantamiento del banco de nubes. De hecho, *N.tropidolepis* es prácticamente ausente de su rango más bajo. Esto concuerda con estudios que muestran otras especies de *Norops s*e mueven hacia la Reserva del Bosque Nuboso de Monteverde. Muchas reservas de Monteverde y áreas protegidas son insuficientes para evitar el decline de herpetofauna. La consecuencia es un nuevo y vació ecosistema de bosque nuboso con nuevas interacciones de especies las cuales sus poblaciones no se recuperarán sin cambios globales.

Key words: Costa Rica; *Norops*; *Craugastor*; *Pristimantis*; climate change; declining populations; herpetofauna, frog, anoles, reptiles, amphibians

INTRODUCTION

Anthropogenic alterations to the natural world have led to massive defaunation, including the documented extinctions of 322 species since 1500, with the remaining species showing 25% declines in abundances (Dirzo *et al.* 2014). Although many suggest these changes to climate change, there are many factors including habitat transformation, introduced species, enrichment of nitrogen, carbon, and phosphorous, water usage, pollution, and many others (Malhi *et al.* 2014). Defaunation is a global driver of ecological change, resulting in a myriad of changes in species interactions (Jordano 2016). Defaunation is affecting all areas and taxa globally (Redford 1992). Discovering the species that are impacted the most and where allows scientists to understand the drivers of defaunation and how they impact ecosystem dynamics.

Defaunation is affecting nearly every taxon. The World Wildlife Fund suggests that total populations of more than 14,000 animals has decreased a total of 52% (Livingplanetindex.org 2017). Areas outside human protection may be worse, extending extinctions and population declines (Gardner 2001). Amphibian and reptile species are objectively hit the hardest. Reptile decline parallels amphibian decline on a global scale (Whitfield Gibbon *et al.* 2000). A 75% reduction in leaf litter reptile density over thirty five years was recorded at La Selva Biological Station, a lowland tropical wet forest, in Costa Rica (Whitfield *et al.* 2000). Similar numbers were found with anoline lizard declines in Monteverde, Costa Rica (Pounds *et al.* 1999). Amphibian species have gone extinct or declined in protected areas of Monteverde Cloud Forest, including the Golden toad (*Bufo periglenes*), Harlequin frog (*Atelopus varius*; Pound & Crump 1994), and Cascades Frog (*Rana cascadae*; Fellers & Drost 1993). These amphibians have declined due to a chytrid fungus, *Batrachochytrium dendrobatidis*, and its virulence has increased due to higher nighttime temperatures and lower daytime temperatures on neotropical mountains like Monteverde (Pounds & Crump 1994). This waterborne fungus impacts amphibian species related to aquatic tadpoles including *Rana warszewitschii* found in the Monteverde area (King & Piovia-Scott 2000).

Amphibians are not the only taxa to decline. Insects have declined as well on global scales (Potts *et al.* 2010). The declines in insect and pollinator abundances have profound effects on plants and animals as well, lowering their populations (Mahli *et al.* 2014). Anoline lizards have decreased their abundances as well in the cloud forests of Monteverde. *Norops tropidolepis* and *Norops altae* have historically been the most abundant species in Monteverde (Pounds *et al.* 1999). However, recent studies put *Norops humilis* as the most abundant species (Place 2005). Recent research has suggested the numbers of *Norops altae* are extremely depleted if not absent from the Monteverde Cloud Forest Reserve (Pounds personal communication). Along with changes in abundances, new *Norops* species are moving in from lower elevations due to changes in precipitation, cloud cover, and temperature, including *Norops intermedius* and *Norops cupreus* (Pounds & Masters 2017). The populations of *Norops* as compared to Place (2005) and Martin (2004) should be lower and show new species moving from lower elevations including *N. intermedius*.

As herpetofauna continue to decline, the rates and numbers of these species needs to be calculated. The rate of decline may be rapid such as that in La Selva. Each area will have a unique rate due to its unique climatic, pollution, and nutrition cycling attributes. Protected areas can protect these species from a myriad of human made destruction, however it is inadequate by itself to preserve species due to the nature of these various drivers. These drivers should push species such as *N. intermedius* onto the Biological Station's property in higher abundances than previously measured. The populations of many if not all the herpetofauna should be lower and follow patterns found in the Monteverde Cloud Forest Reserve.

MATERIALS AND METHODS

Sites

Starting at 1400 meters in elevation to 1780m, the Holdridge life zones surveyed were Tropical Premontane Moist, Tropical Lower Montane Wet, and Tropical Lower Montane Rain forests. The study location was zoned out into four zones which were determined by the ease of access to trails and their forest characteristics (*Appendix II*). Any trail below the Biological Station's property in Monteverde Costa Rica was deemed Zone A and consisted of heavily disturbed land, pasture, and residential areas. Any trail on the Biological Station property below the station was deemed Zone B and consisted mainly of a large stream and varying levels of primary and secondary forests. Trails starting above the station were deemed Zone C until the Biological Station property ended and was primary forest. Zone D and was primary elfin forest located after the Biological Station's property. Each zone also encompassed different altitudinal gradients. Zone A consisted of two trails, B consisted of two trails, C consisted of four trails, and D consisted of only one trail. Each trail was given four surveys lasting two hours. Each trail also had an hour and a half night survey. Each zone was surveyed off trail totaling four hours each. Miscellaneous surveys were any survey done outside of expected surveying hours which would include those herpetofauna found by chance, during travel, or outside of the intended survey area and consisted of roughly an hour and a half. The total surveying hours was exactly one hundred.

Organisms

Leaf litter and understory (sub-arboreal or those under two meters above the ground) species were the focus of this survey. Individuals were found within two meters of the. Off trail surveys were also made with a two meter wide strip starting at least ten meters off the path. The path off trail was determined by ease of access and followed natural ridges, streams, and animal trails. Night surveys were treated the same as day surveys and on trails. Every organism found was recorded.

Survey Method

Surveys start October 19th, 2017 and lasted until November 19th. Altitude and Global Positioning System (GPS) coordinates were taken using an altimeter and a Garmin eTrex 10 GPS unit. Every species was identified, and its location, map zone (*Appendix II*), and altitude were recorded. G tests were used to compare previous student research from the same areas to current survey data. A total of fifty nine specimens were found (*Appendix 1*). Scanning method consisted of a slow pace, between one and two kilometers an hour, with a swiveling head motion to capture both sides of the trail. Specimens were occasionally scared out from logs and clumps of leaf litter if needed.

RESULTS

The abundances of *Norops spp.* found was lower than the abundances found in the same forest over thirteen years ago (Martin 2004; Figure 1). Martin (2004) found a total of fifty four individual *Norops spp*. in twenty four hours compared to fourteen individuals here, despite larger transects, encompassing almost 250m more elevation and one hundred hours of this study. *N. intermedius* suffered the greatest loss, going from nineteen individuals from thirty hours surveying to two in one hundred hours of survey. This equates to a rate of *N. tropidolepis* found every seven hours and nine minutes while Martin (2004) found them at a rate of twenty six minutes. The same can be said for *N. intermedius*, although the current rate is one every fifty hours.

Except for *N. tropidolepis*, trends were similar when comparing data to summer of 2005 (Place 2005) (Figure 2). Only *Norops spp.* within twenty meters were taken for comparison with Place (2005).The summer of 2005 survey was made at an altitude of 1540 meters, effectively eliminating *N. tropidolepis.* Only one individual of *N. tropidolepis* was found below 1600 meters in elevation. The numbers were greatly lower than the one hundred and twenty eight found by Maximov (2003), in which forty seven were *N. intermedius,* seventy eight *N. tropidolepis,* and three *Norops woodi*.

Amphibian species were not surveyed previously on the Biological Stations property. A sample of thirty individuals of *Craugastor bransfordii* was collected in the same area in 2001 (Salonga 2001), while none were seen in 2017. Similarly, a study was done on *Hyalinobatrachium fleischmanni*, which found rates as high as thirty individuals an hour, or one every two minutes (Ward 2004), while only one was encountered one hundred hours during my 2017 surveys.

All herpetofauna was extremely rare, only 0.59 individuals encountered per hour, of one every hour and thirty six minutes. The most abundant species were *Pristimantis cruentus, N. tropidolepis, Craugastor underwoodi,* and *Sceloporous malachiticus*. *P. cruentus* had individuals outside of its lower literature range. The one *Craugastor crassidigitus* was found over one hundred meters lower than its lower range. Anomalies were extremely rare. The location of these individuals were near streams.

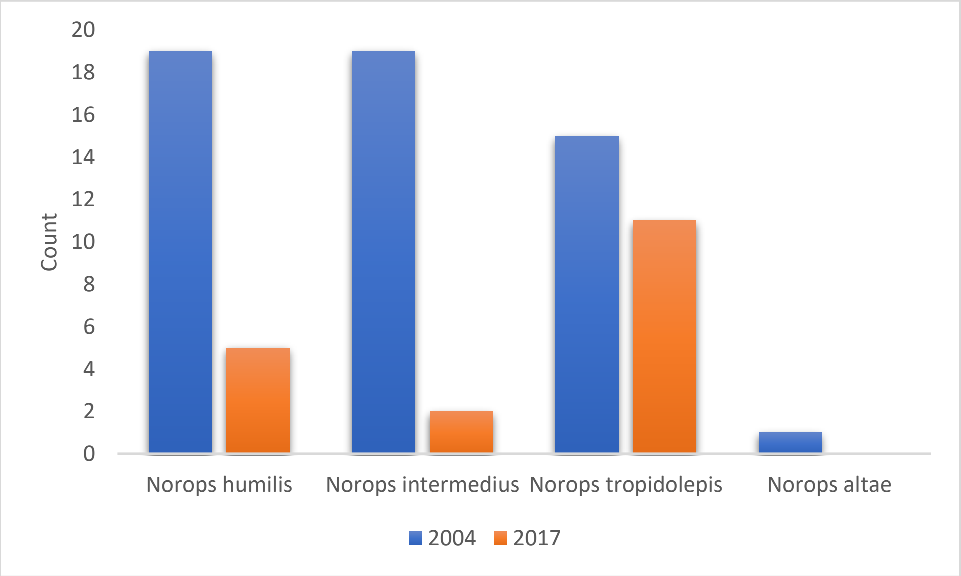

Figure 1: Comparing Martin's 2004 survey data of the same ridge, there was a decline in *Norops humilis*, *Norops intermedius*, and *Norops tropidolepis. Norops altae* did not show significant differences as only one

was found in Martin's 2004 survey. Average decline was 72.45%. Note, 2004 survey was between 1400m and 1600m for a total of twenty four hours on Biological Station property. G test was taken by shrinking the current data into a 24 hour equivalent (G=296.9; df=3; p<.0001).

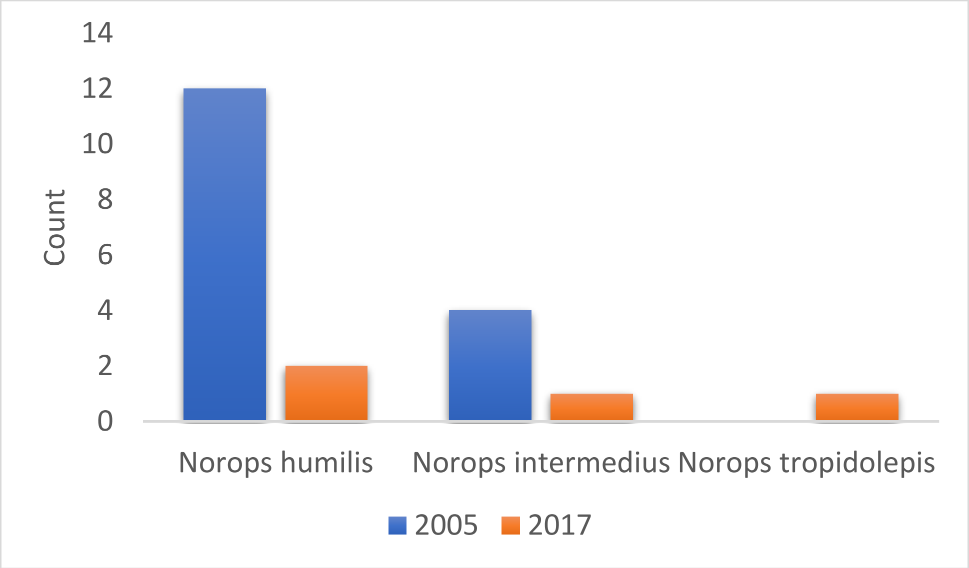

Figure 2: Comparing Place's 2005 survey data of the same ridge, there was a decline in *Norops humilis* and *Norops intermedius. Norops tropidolepis*'s range is generally higher than the 1540m altitude of Place's study. Note, a total of thirty hours on Biological Station property. Only individuals within 25 meters in elevation were chosen to compare (G=54.09; df=2; p<.0001).

DISCUSSION

Monteverde had a pronounced crash of amphibian species in 1987 (Pounds *et al* 1997). Yacoe (2009), repeated earlier transects (Pounds 1994; Laval personal communication) along streams and found similar post-crash low population abundance of *H. fleischmanni*. However, both numbers are staggeringly lower than pre-crash numbers that included hundreds of individuals nightly. Although not the focus of this survey, *H. fleischmanni* seems to have remained stable or slightly declined since 2009 (Yacoe 2009). The declining results suggest that the herpetofauna has continued to decline at a steady rate since 1987.

Reptile populations are just as low. Hundreds of *Norops spp.* were found before 1987 (Pounds & Crump 1994) along with dozens post-crash (Pounds *et al.* 1999). Unfortunately, the steady decrease is represented through the fifty four found by Martin (2004) and now the fourteen present in 2017. *N. tropidolepis* also has shown an increase in its lower altitudinal range. Only one individual of this species was found below 1700 meters in altitude. The population is more stable for *N. tropidolepis* at high elevations. This is also where most mist and cloud cover occur. The Holdridge zone changes to lower montane rain forest, a more suitable habitat for *N. tropidolepis.* There were also more perching areas above 1700 meters in altitude suitable for *N. tropidolepis.* There was a greater visual abundance of old tree stumps and short understory plants. As *N. tropidolepis* moves up in elevation, new species can invade the vacant lower elevations such as *N. intermedius* and *N. cupreus*. *N. cupreus* was absent while the population of *N. intermedius* was lower than Martin (2004).

Like *Norops spp.* in Monteverde, birds are well documented to move up mountains to improve climatic conditions (Sekercioglu 2007). Although possible, many terrestrial species do not move their geographic ranges to accommodate new climatic conditions, including many reptiles (Moritz and Agudo 2013). This would suggest that *N. tropidolepis* and other reptiles are being pinched off at the upper limits of the Monteverde ridge. On the Biological Station property, this would leave around one hundred and twenty meters left for their altitudinal range.

With populations of herpetofauna continuing their decreasing population trends in Monteverde, Costa Rica, the future is bleak without drastic human intervention. As many other herpetofauna have also declined in Central America and globally, global intervention of carbon and greenhouse emissions is the only solution to widespread population declines. Defaunation affects every taxa differently, making conservation hard, yet necessary.

ACKNOWLEDGMENTS

Many thanks to Katty Gonzalez for helping me correctly identify animals and for accompanying me on night hikes. Special thanks to Jose Carlos “Moncho” Calderon and Johel Chavez for support and assistance. Special thanks to Dr. Alan Masters for mentoring this project. Finalmente, muchas gracias a mi familia tica Haydee, Carlos, y Osita, y también a su familia extendida por todo el amor y cuidado que me dieron.

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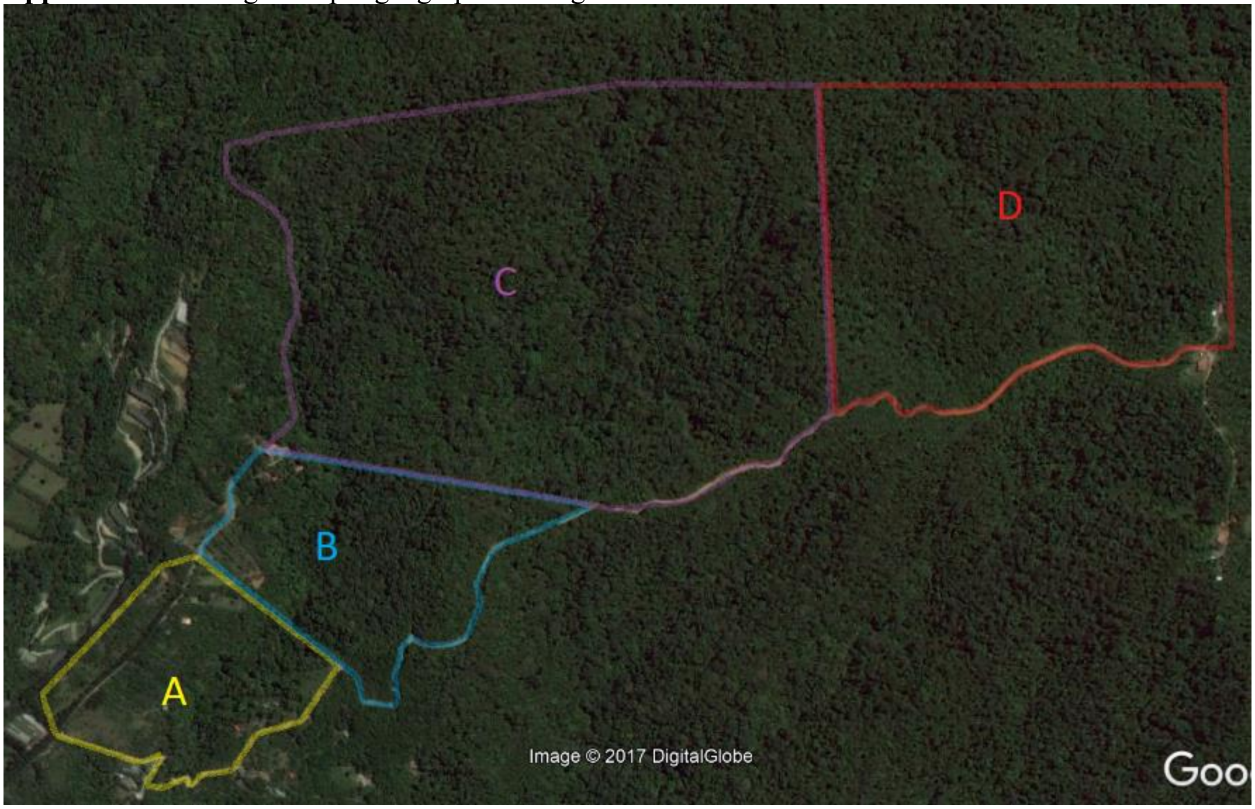
Appendix 1: Literature ranges were determined by *An Annotated List and Guide to the Amphibians and Reptiles of Monteverde Costa Rica*. Individuals marked with an (*) are outside of literature range.

Appendix 1: **Individuals and Altitude**

Species	Altitude (m) Altimeter	Altitude (m) GPS	Literature Range
Bothriechis lateralis	1465	1532	>650
*Craugastor crassidigitus**	1470	1452	>1600
Craugastor fitzingeri	1500	1519	1300-1600
Craugastor fitzingeri	1520	1504	1300-1600
Craugastor podiciferus	1485	1510	>1300
Craugastor podiciferus	1530	1517	>1300
Craugastor podiciferus	1545	1563	>1300
Craugastor podiciferus	1655	1664	>1300
Craugastor underwoodi	1490	1487	NA
Craugastor underwoodi	1505	1485	NA
Craugastor underwoodi	1535	1521	NA
Craugastor underwoodi	1525	1528	NA
Craugastor underwoodi	1520	1539	NA
Craugastor underwoodi	1705	1728	NA
Craugastor underwoodi	1570	1600	NA
Craugastor underwoodi	1530	1559	NA
Craugastor underwoodi	1540	NA	NA
Craugastor underwoodi	1585	1589	NA
Craugastor underwoodi	1560	1551	NA
Craugastor underwoodi	1560	1563	NA
Craugastor underwoodi	1770	1774	NA
Espadarana prosoblepon	1545	1528	>650
Hyalinobatrachium fleischmanni	1540	1536	<1600
Lithobates warszewitschii	1535	1521	>1300
Norops humilis	1540	1510	>650
Norops humilis	1530	1522	>650
Norops humilis	1790	1812	>650
Norops humilis	1790	1812	>650
Norops humilis	1770	1790	>650
Norops intermedius	1510	1494	1300-1600
Norops intermedius	1465	1465	1300-1600
Norops tropidolepis	1525	1527	>1300
Norops tropidolepis	1770	1795	>1300
Norops tropidolepis	1765	1795	>1300
Norops tropidolepis	1765	1791	>1300
Norops tropidolepis	1795	1810	>1300
Norops tropidolepis	1790	1795	>1300
Norops tropidolepis	1780	1788	>1300
Norops tropidolepis	1790	1789	>1300
Norops tropidolepis	1780	1783	>1300
Norops tropidolepis	1780	1783	>1300
Norops tropidolepis	1780	1779	>1300
Pristimantis cruentus	1525	1524	>1470
Pristimantis cruentus	1540	1536	>1470
Pristimantis cruentus	1580	1590	>1470
Pristimantis cruentus	1780	1780	>1470
Pristimantis cruentus	1780	1776	>1470
*Pristimantis cruentus**	1430	1427	>1470
*Pristimantis cruentus**	1465	1462	>1470
Pristimantis ridens	1705	1704	>1600
Rana warszewitschii	1445	1453	>1300
Sceloporous malachiticus	1435	1428	>650
Sceloporous malachiticus	1480	1453	>650
Sceloporous malachiticus	1530	1536	>650
Sceloporous malachiticus	1530	1536	>650
Sceloporous malachiticus	1530	1536	>650
Sceloporous malachiticus	1530	1536	>650
Sceloporous malachiticus	1530	1536	>650
Sceloporous malachiticus	1530	1536	>650

Appendix II: Zoning done per geographical range.



Behavioral acclimation of the Central American agouti (*Dasyprocta punctata)* to human presence

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ABSTRACT

Habitat transformation is most rapid in the Tropics, forcing biodiversity there to live in ever-closer proximity to humans. Even species capable of acclimating may increase their risk of predation and disease. Two sites within the Ecological Sanctuary in Monteverde, Costa Rica were chosen to determine behavior differences between Central American agoutis (*Dasyprocta* punctata) near human presence (within 50 m from reception office) compared to farther individuals (500 m from reception office). Unshelled peanuts near different stimuli were used to test responses to perceived threats near and far from humans: model snakes, aluminum foil, and wildcat urine. Model snakes ($\bar{x}_{Near =}$ 7.0 ± 0.68; $\bar{x}_{Far =}$ 5.7 ± 0.97) and wildcat urine ($\bar{x}_{Near =}$ 7.1± 0.62; $\bar{x}_{Far =}$ 7.6 ± 0.52) did not differ between near and far agoutis in average number of peanuts eaten, but foil led to a higher number of peanuts eaten Near (6.0 ± 0.59) than Far (3.7 ± 0.64). In addition, by approaching agoutis slowly, I recorded proximity as Alert Distance (AD) when agoutis took first notice and Flight Initiation Distance (FID) when agoutis moved away. Average FID and AD ($\bar{x}$ meters ± SE) were not different between Near and Far agoutis (FID_{Near} = 4.9 ± 0.42, FID_{Far} = 4.67 ± 0.34; AD_{Near} = 6.53 ± 0.34, AD_{Far} = 6.52 ± 0.42), but the distance between AD and FID was slightly greater for agoutis near human traffic (1.85 ± 0.37) than far (1.53 ± 0.61), but the data were not statistically significant. Additional behavioral observations suggest agoutis are more cautious farther from humans, as they were more hesitant to approach and eat peanuts regardless of treatment but especially with foil. Results suggest agoutis near humans are less cautious, so perhaps are also more susceptible to predators and pathogens.

RESUMEN

La transformación del hábitat es más rapida en los trópicos, forzando la biodiversidad ahí a vivir aún más cerca de la proximidad de los humanos. Aún las especies capaces de aclimatarse pueden aumentar el riesgo de depredación y de enfermedades. Dos sitios dentro del Santuario Ecológico en Monteverde, Costa Rica fueron escogidos para determinar las diferencias de comportamiento de la guatusa (Dasyprocta punctata) cerca de la presencia humana (a menos de 50 m de la oficina de recepción) comparado con individuos de más lejos (a 500 m de la oficina de recepción). Maníes con cascara cercanos a diferentes estímulos se usaron para probar las respuestas a posibles amenazas cerca y lejos de los humanos: modelos de culebras, papel aluminio, y orina de gato silvestre. Los modelos de culebras ($\bar{x}_{Cerca}$ = 7.0 ± 0.68; $\bar{x}_{Lejos}$ = 5.7 ± 0.97) y orina de gato ($\bar{x}_{Cerca}$ = 7.1± 0.62; $\bar{x}_{Lejos}$ = 7.6 ± 0.52) no difieren entre guatusas cercanas o lejanas en número de maníes consuimdos, pero el papel aluminio lleva a un mayor número de maníes consuimdos cerca (6.0 ± 0.59) que lejos (3.7 ± 0.64). Además, aproximándome lentamente a las guatusas, anoté la proximidad como Distancia de Alerta (AD por sus siglas en inglés) cuando las guatusas notaron mi presencia por primera vez y la Distancia de Despegue Inicial (FID por sus siglas en inglés) cuando las guatusas se alejaban. La distancia promedio de FID y AD ($\bar{x}$ meteros ± SE) no fueron diferentes entre las guatusas cercanas y lejanas (FID_{Cerca} = 4.9 ± 0.42, FID_{Lejos} = 4.67 ± 0.34; AD_{Cerca} = 6.53 ± 0.34, AD_{Lejos} = 6.52 ± 0.42), pero la distancia entre AD y FID fue levemnte mayor para las guatusas cercanas al tráfico humano (1.85 ± 0.37) que lejano (1.53 ± 0.61). Observaciones de comportamiento adicionales sugieren que las guatusas son más cuidadosas lejos de los humanos, al ser ellas vacilantes de acercarse a los maníes sin importar el tratamiento pero especialmente con el aluminio. Los resultados sugieren que las guatusas cerca de los humanos son menos cuidadosas, por lo tanto más suceptibles a depredadores y patógenos.

INTRODUCTION

Human land transformation is a major driver of global environmental change (Malhi et al. 2014). Land transformation leads to deforestation and fragmentation, which in turn forces remaining biodiversity to increasingly interact with humans. Forest degradation is increasing most rapidly in the tropics, which also host 50% of the world's biodiversity and most of its endemic species (Laurance 1994; Jha et al. 2006). Tropical species, therefore, have more contact with humans than previously as about one-third of the planets tropical evergreen woodlands were converted into used 'anthromes' (i.e. anthropogenic biome) (Ellis et al. 2010), likely resulting in changes to their ecology and behavior (Price et al. 2014; Lewis 2009).

Species most likely to persist in human-altered landscapes are those that are habitat generalists or adapted to natural disturbance (Laurance 2005; Woodroffe et al. 2005). Even here, species expanding their range with humans may still be harmed by their presence, including changes in behavior. Behaviors like foraging, mating, territoriality, predation, nesting, and awareness of predators can be altered in ways that reduce fitness (Lewis 2009). For example, bald eagles shift their distribution in response to human activity and desert bighorn sheep avoid construction activity by traveling to more-distant water sources (Stalmaster and Newman 1978; Campbell and Remington 1981 in Price et al. 2014). Similarly, wolves in Canada's Banff National Park tend to avoid certain parts with heavy human traffic, causing densities and survival of elk to be higher in those areas (Hebblewhite et al. 2005 in Price et al. 2014) which in turn leads to less food sources for wolves in those areas.

One method to assess changes such changes to humans is to provide feeding stations with different stimuli as a way to gauge a species willingness to approach, including with predator models or novel objects that can measure neophobia (i.e. caution with new stimuli; Coleman and Mellgren 1994). Additionally, alert distance (AD) and flight initiation distance (FID) are used to determine predator responses (Stankowich & Blumstein 2005). AD is the distance between the animal and a human approaching when the animal becomes alert to their presence, while FID is the distance between the animal and the approaching human when the animal begins to flee (Stankowich & Blumstein 2005). Lower AD and FID reflect a relaxation of predator awareness.

The Central American agouti (*Dasyprocta punctata*) is found from southern Mexico to Ecuador, from 0-2,400 m in elevation. It is common throughout its range and can be found in secondary forest and edge habitats, often putting it in close contact with humans (Wainwright 2007). These diurnal rodents are important seed dispersers by often neglecting to retrieve all of their cached fruits/seeds that are then perfectly prepared to germinate and grow (Wainwright 2007). With territories ranging between 1-3 hectares (Aliaga-Rossel et al., 2008), these mammals are territorial, and will defend against other individuals, more aggressively when food is scarce (Wainwright 2007). Agoutis are typically preyed on by larger mammals like cats and are a popular game animal in Costa Rica for their meat (Wainwright 2007).

Here, I measure how human presence has impacted predator awareness behaviors in agoutis around a private nature reserve frequented by tens of thousands of tourists annually. I present food with predator models and novel objects near and far from humans to assess agouti willingness to approach and degree of neophobia. In addition, I compare AD and FID for agoutis near the reception/Visitor Center and those farther away.

MATERIALS AND METHODS

Study Site

This study takes place in the Ecological Sanctuary in Monteverde, Puntarenas, Costa Rica. The Ecological Sanctuary is a private, 48-hectare wildlife refuge that contains four main trails. The sanctuary is considered a tropical pre-montane wet forest patch of 58-year-old regenerating secondary forest on what was once pastured. The property rests at roughly 1,300 m in elevation and includes a Visitor Center and a 30 m^2 parking lot connecting to the forest edge (Palm 2017). There were two main study areas located within the forest: within 50 m of the Visitor Center (Near) and over 500 m away (Far). The study area is shown in Figure 1. where each red circle represents a study site (Near and Far) roughly four hectares in size.

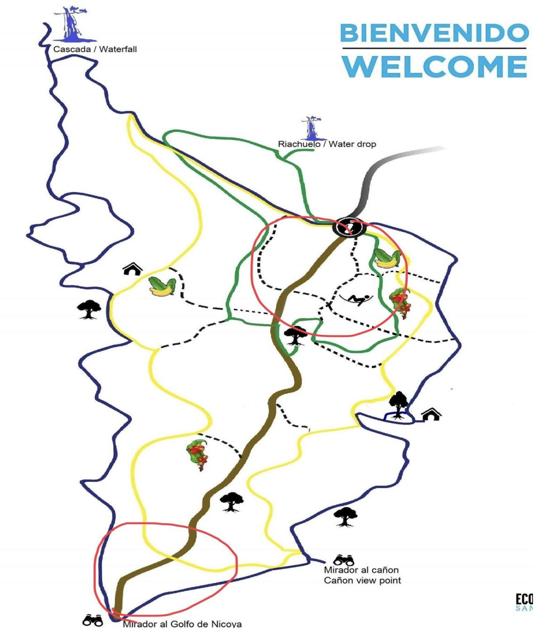

Figure 1. Map of trails in 48 Ha of protected secondary forest at the Ecological Sanctuary in Monteverde, Costa Rica. Here, agoutis were observed to see the impact of high (Near) and low (Far) human traffic on agouti antipredator behaviors. Two study sections are circled in red: Near is within 50 m of the information/Visitor Center at the apex in the map, while Far is the circle at the bottom in the map, approximately 500 m away from the information center.

Approach to Food with Predator Models

To determine willingness to approach potential threats, I placed unshelled peanuts near different models along with peanuts absent models as controls. For both Near and Far locations there were 10 sites spread roughly 20 m apart prepared with 10 peanuts each. All sites were in set up in the forest. Peanuts and models were placed between 8am and 9am starting with Near sites. Camera traps were used in each location to record visits and responses to treatments and controls: one camera on a treated site and the other at a control site for both Near and Far. Each day control and treatment sites were switched to remove location as a variable, while locations of peanut sites remained the same day to day.

Day one only peanuts without models were offered. Camera traps were set up roughly one meter from randomly chosen feeding sites on a nearby tree. Sites were left for two hours. At the end of two hours peanuts were counted. Numbers of empty, nearly whole shells were considered Eaten (Figure 2). I scanned the area within a one meter radius of where the peanuts were placed and if there were any peanuts left at the site untouched they were labeled Left and any remaining shells or peanuts that I could not locate were labeled Taken; Taken and Left were considered signs of perceived threat due to the fact that the individual seemed uncomfortable eating the peanut at the site.

Figure 2. Peanuts in shells were offered to the Central American agouti (*Dasyprocta punctata*) to test willingness to approach different predator models Near and Far from humans. When Agoutis ate the peanuts they left the empty shells nearly intact, as shown. These were considered Eaten in later analyses.

The first models tested were rubber snakes of 50 cm long garden hose and electrical tape (Williamson 2017). Control snake models were green (harmless) and treatment models had a coral snake pattern. Snakes were positioned in the center of the site with peanuts laid out around them (Figure 3). The second model offered was a novel object to assess neophobia, or fear of new things (Braveman 1978). Materials used for this treatment included 10 cans (roughly 14 cm in height and 7.5 cm in diameter) covered in aluminum foil with plastic straws sticking out (Figure 4). Each can was wrapped in aluminum foil, and then a knife was used to create six holes as wide as the knife used (3 by the top of the can and 3 by the bottom) by simply shoving the knife into the can. For each can, six straws were wrapped with aluminum foil and stuck in each of the holes as far as the straw would reach. The third model was wildcat urine. Thin cloth strips (cut to be roughly 12-13 cm in length and 5 cm in width) were dripped with drops of urine, and clothespins were used to hang up the strips at each site (Figure 5). Each strip had between 10-15 drops of urine, and was hung on a twig or leaf between 30-50 cm above the ground, depending on the available vegetation height. Peanuts were positioned within 50 cm around models or directly under urine cloth strips.

Figure 3. Test of agouti behavioral response to a perceived threat using peanuts with snake models. Snakes were garden hose and flagging tape: a harmless, green garden snake (left) used as the control, and the venomous coral snake (right) used as the treatment. Snakes were placed in the center of the site with ten whole peanuts scattered within 50 cm but not touching the model.

Figure 4. Test of agouti behavioral response to a perceived threat using peanuts at control sites versus treatment sites. Treatment on sites used was a hand-made aluminum foil creation made with aluminum cans, aluminum foil, and plastic straws. Control sites for this treatment were left empty of anything; used to test for neophobia. Models were placed in the center of the site with peanuts scattered within 50 cm.

Figure 5. Test of agouti behavioral response to a perceived threat using wildcat urine as a site treatment. Treatment on peanut sites used wildcat urine scent by putting drops of urine on cloth strips and hanging them between 30-50cm above the site on the nearest available vegetation. Control sites for this treatment were left empty of anything. Purpose was to provide a possible threat by a predator. Peanuts were placed both under and within 50 cm cloth strip.

After two hours of allowing sites to be approached by agoutis, models were removed and peanuts would be counted and labeled as Eaten, Taken, and Left, and any shells found would be removed.

Alert Distance & Flight Initiation Distance

Meanwhile, during the two hours between placement and collection of peanuts at sites, to test for variation in behavioral response to approach by humans, I collected data on alert distance (AD) and flight initiation distance (FID). I located agoutis Near (within 50 m) and Far (500 m) from the Visitor Center. As I slowly approached an agouti I noted AD and FID. To find agoutis, I walked up and down available trails within each section (Near and Far) at a pace of ½ to 1m/s occasionally stopping for 5-10 seconds depending on the density of the vegetation and the visibility of the trail.

Once I noticed an individual, I slowly and quietly placed a stick or rock to later measure the initial distance between me and the agouti when encountered, labeled as starting distance (SD). I then walked towards the individual at a speed of 1 m/s (Price, et al. 2014) wearing my black backpack with a typical upright walking posture until the individual reacted (became alert) of my presence and used a marker or a specific landmark like a tree to mark my location. To determine alertness of an individual I would watch for the individual to freeze in its position with its head tilted upright or turned towards me. I continued walking towards the individual until it fled to refuge in nearby vegetation, marking my and its location. I measured the distance between markers to determine AD and FID. Distances between AD and FID were also calculated to show the distance at which an individual would allow me to continue approaching from the time they became alert to the time they fled.

RESULTS

Model-free Day one peanuts had more peanuts Taken from Near sites and Left more peanuts from Far sites (ANOVA F = 3.53, df =1, p = 0.036; Figure 6). There was no real difference between locations for average number of peanuts Eaten ($\bar{x}$ ± SE), with average number of peanuts eaten for Near (0.6 ± 0.22) being only slightly greater than Far (0.5 ± 0.22). However, peanuts Taken from Near were much higher (6.3 ± 1.39) than those for Far (3.2 ± 1.33), and the average number of peanuts Left for Near were much lower (3.1 ± 1.51) than those Left for Far (6.3 ± 1.54).

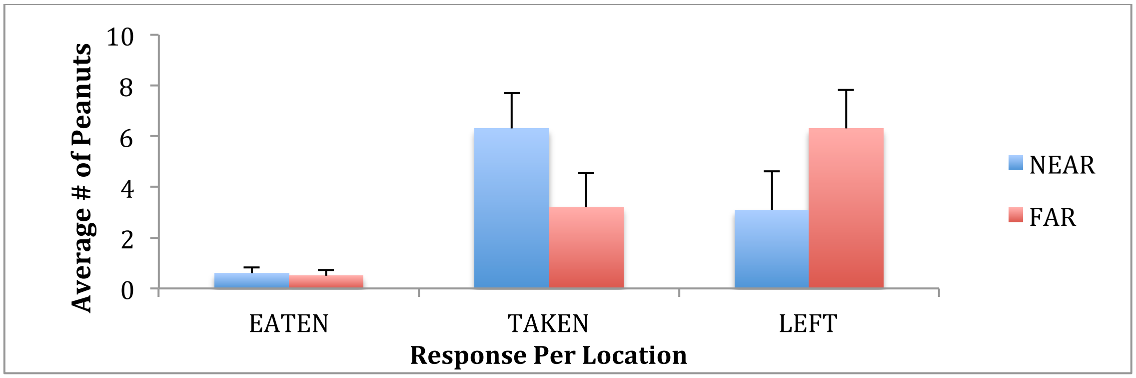

Figure 6. Peanuts Eaten, Taken, and Left by agoutis within 50 m of the Visitor Center (Near) and 500 m away (Far) at the Ecological Sanctuary in Monteverde, Costa Rica ($\bar{x}$ ± SE). Ten sites were placed in the near location, and 10 sites in the far location. All sites contained 10 whole peanuts and were left for two hours to determine initial differences in likelihood of predation with proximity to humans. Sites were set up between 8am and 9am. Eaten = whole shells with no peanuts found, Left = whole peanuts, and Taken = no shell found. Results were recorded after two hours.

Snake Models

Model snakes had no impact on foraging behavior ($F = 0.31$, $df = 2$, $p < 0.73$; Figure 7). Most peanuts were Eaten, nearly 70%, regardless of proximity to humans. Approximately 30-40% of peanuts were Taken and nearly no seeds were Left. In fact, at Near sites, no peanuts were Left for either treatment or control and just one was Left at Far sites, on average.

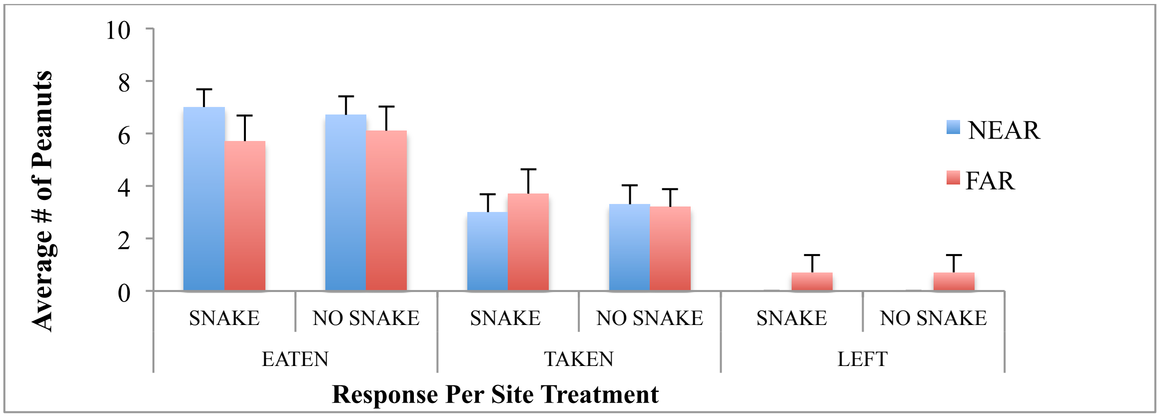

Figure 7. Peanuts Eaten, Taken, and Left by agoutis within 50 m of the Visitor Center (Near) and 500 m away (Far) at the Ecological Sanctuary in Monteverde, Costa Rica ($\bar{x}$ ± SE).15 sites located Near for both treatments and controls (30 sites total), and 15 sites located Far for both treatments and controls (30 sites total). Treatment for these sites was models of coral snakes and the control was models of a harmless green garden snake (See Figure 3). Eaten = whole shells with no peanuts found, Left = whole peanuts, and Taken = no shell found. Treatment was tested on three separate days with 10 peanuts put out in each site between 8am and 10am each day. Results for each site were recorded after two hours.

Novel Models

For the aluminum foil creations, distance impacted behavior ($F = 7.12$, $df = 2$, $p < 0.001$; Figure 8). Peanuts eaten Near were lower with models (6.0 ± 0.59) than control sites without models (7.8 ± 0.37). Near Taken peanuts were higher with models (4.0 ± 0.59) than without (2.2 ± 0.37), and no peanuts were Left at Near sites. Far sites had even fewer peanuts Eaten with models (3.7 ± 0.64) than without (8.2 ± 0.57) compared to Near. Peanuts taken at Far sites were much higher with models (5.6 ± 0.64) than without (1.8 ± 0.57), suggesting more caution. As with Near, most peanuts were eventually Eaten or Taken, with only one Left at a treatment site. To determine which groups were statistically significant, I performed a Fisher LSD test fort each. When comparing Eaten, Taken, and Left, each comparison was significant ($p < 0.0001$ for each). Treatment compared to control was also statistically significant in peanuts Eaten and Taken ($p < 0.006$ for both) but not for peanuts Left.

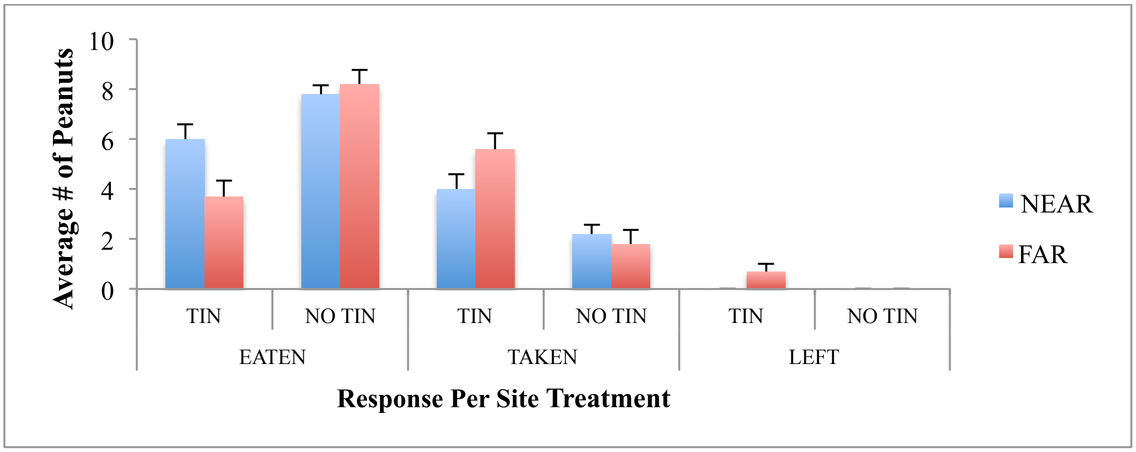

Figure 8. Peanuts Eaten, Taken, and Left by agoutis within 50 m of the Visitor Center (Near) and 500 m away (Far) at the Ecological Sanctuary in Monteverde, Costa Rica ($\bar{x}$ ± SE). 15 sites located near for both treatments and controls (30 sites total), and 15 sites located far for both treatments and controls (30 sites total). Treatment for these sites was a novel creation (TIN) made of aluminum cans, aluminum foil, and plastic straws (See figure 4) and the control was absent of a model. Eaten = whole shells with no peanuts found, Left = whole peanuts, and Taken = no shell found. Treatment was tested on three separate days with 10 peanuts put out in each site between 8am and 10am each day. Results for each site were recorded after two hours.

Wildcat Urine

Near and Far did not differ in how many peanuts were Eaten, Taken or Left (ANOVA, $F = 0.14$, $df = 2$, $p < 0.87$; Figure 9). Number of peanuts Eaten under wildcat urine was only slightly lower ($\bar{x}_{Near} = 7.1 \pm 0.62$; $\bar{x}_{Far} = 7.6 \pm 0.52$) than control sites (7.7 ± 0.56; 8.5 ± 0.43). Peanuts Taken were just slightly higher with cat urine ($\bar{x}_{Near} = 2.9 \pm 0.62$; $\bar{x}_{Far} = 2.5 \pm 0.52$) than in control sites (2.3 ± 0.56; 1.5 ± 0.43), and the average number of peanuts left were 0 for both treated and control sites.

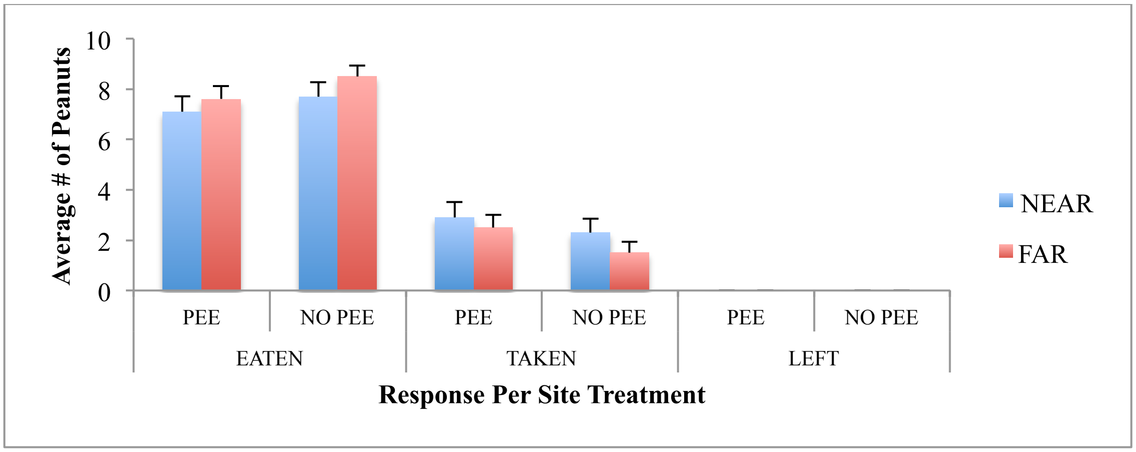

Figure 9. Peanuts Eaten, Taken, and Left by agoutis within 50 m of the Visitor Center (Near) and 500 m away (Far) at the Ecological Sanctuary in Monteverde, Costa Rica ($\overline{x}$ ± SE). 15 sites located near for both treatments and controls (30 sites total), and 15 sites located far for both treatments and controls (30 sites total). Treatment for these sites was a cloth strip dripped with wildcat urine and hung 30-50cm above the peanuts (See figure 5), and the control only had peanuts. Eaten = whole shells with no peanuts found, Left = whole peanuts, and Taken = no shell found. Treatment was tested on three separate days with 10 peanuts put out in each site between 8am and 10am each day. Results for each site were recorded after two hours.

Approach by Human

Alert Distance (AD) and Flight Initiation Distance (FID) did not differ statistically by location (ANOVA; F = 0.06, df = 1, $p < 0.8$; Figure 10). AD was nearly identical for Near (6.53 ± 0.34) and Far (6.52 ± 0.42), as was FID ($\overline{x}_{Near} = 4.9 \pm 0.42$; $\overline{x}_{Far} = 4.67 \pm 0.34$). The distance between AD and FID, as a measure of how far I was allowed to approach once noticed, was also not statistically different for Near and Far (ANOVA, F = 0.44, df = 2, $p < 0.65$). For Near, the average difference found between AD and FID was only slightly greater (1.85 ± 0.37) than Far (1.53 ± 0.61; Figure 11).

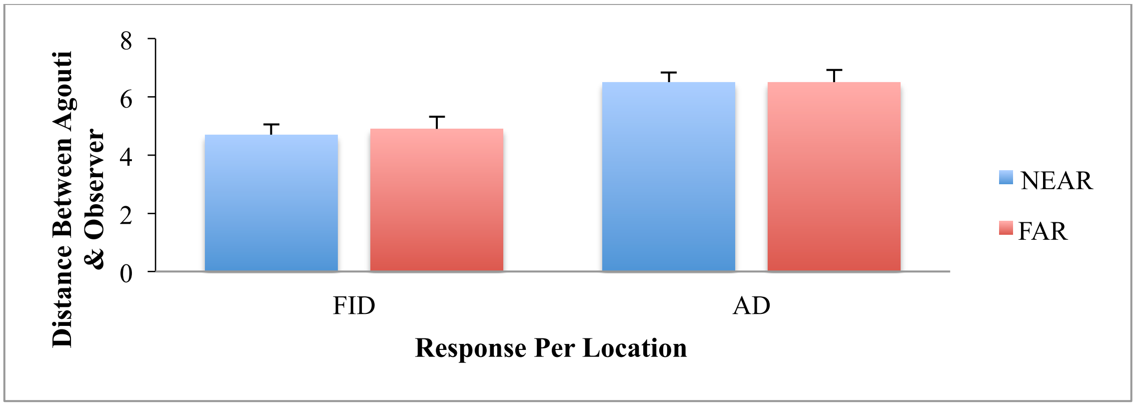

Figure 10. Flight initiation distance (FID) and alert distance (AD) for agoutis within 50m of the Visitors Center (Near) and 500m away (Far) at the Ecological Sanctuary in Monteverde, Costa Rica ($\overline{x}$ ± SE). Sample size included 26 approaches for the near location, and 17 approaches for the far location. Approach data were collected over a period of 28 days between 9am and 1pm.

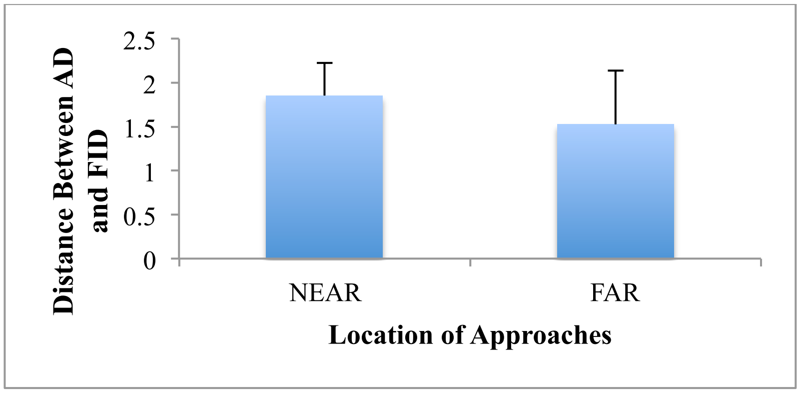

Figure 11. Distances between flight initiation distance (FID) and alert distance (AD) for agoutis within 50m of the Visitors Center (Near) and 500m away (Far) at the Ecological Sanctuary in Monteverde, Costa Rica ($\overline{x}$ ± SE). Sample size included 26 approaches for the near location, and 17 approaches for the far location. Approach data were collected over a period of 28 days between 9am and 1pm.

Additional Observations

Visual:

Near agoutis were initially found in a banana patch just off of the Visitor Center, but became more abundant in the forest after several days of peanut trials. In fact, even on the second day offering peanuts, agoutis would be found waiting at sites that had peanuts the previous day. Agoutis began to approach feeding stations before I left and some would follow me to the next feeding station. Many times when I returned to gather my results at sites that had the model coral snakes I would find that the snake had been moved or thrown, sometimes even one meter away from where the snake was originally placed.

Camera Traps:

The snake models fooled neither Near nor Far agoutis. While they would approach slowly and investigate, they would quickly continue on and even walk over it. For the aluminum foil treatment, Far agoutis were much more hesitant to approach and peanuts were even Left, which was rare with other models. Far agoutis would carefully approach, grab the peanut, and immediately dart away from the area. Though they were clearly hesitant and slightly afraid of the unknown object, for the most part it did not keep them from taking the peanuts if they wanted them. However, Near agoutis were not nearly as threatened by foil models, and would even bump into them or knock them over. As for the wildcat urine treatment, individuals in both locations would notice the scent but pay little to no attention to it, and would even walk right under the hanging strip and often just sit right next to it.

DISCUSSION

On the first day only, few peanuts were eaten on the spot, but that quickly changed. This could reflect hesitance at a novel situation or that the feeding sites had to be discovered. As most Near individuals were located in a banana/coffee plantation, peanuts in the forest may have been missed on the first day. Similarly, in the Far location there may not have previously been food sources where I placed the peanut sites, and they would therefore not have been visited due to knowledge of food elsewhere.

Predator models did not change agouti behavior, regardless of human contact. The novel aluminum foil model however was approached more cautiously, and more so by Far agoutis. Perhaps near agoutis have greater exposure to novel items from contact with humans. It is found that prior exposure to novel items or tastes in some studies reduce the aversiveness of subsequent exposure increasing their acceptability of novel items (Domjan 1976 in Braveman 1978), which is what appeared to happen in my study.

By the time I began with the wildcat urine treatment, expecting that to deter the agoutis from the sites, the actual results were unexpected. It's possible that after putting out so many peanuts each morning, it did not matter what type of treatment was used, the agoutis just knew that the peanuts were there and that considering nothing bad had happened from approaching the sites so far they were not worried anymore. Like captive animals that become domesticated gain dependence on humans to provide food on a relatively uniform basis, the result is a relaxation of natural selection on traits associated with food selection and predator avoidance (Price 1999). Another theory is that they could be somewhat used to that scent considering there is at least one puma that roams within the sanctuary and surrounding forest as well (Palm 2017) and therefore it could be a somewhat normal scent, and if they are exposed to it often it may not greatly impact their foraging behavior, similar to the effect of prolonged exposure to novel items.

AD and FID did not differ with degree of human contact. Perhaps humans are not perceived as a threat by agoutis until it is clear they are being stalked and approached. Flight distance can depend on compensation for increased or reduced risk (e.g. Greater distance to refuge may lead to greater FID) (Stankowich and Coss (2006). Agoutis near human activity typically benefit from reduced predator abundances as well as increased food sources (Jorge 2007 in Williamson 2017). Amount of food present (Cooper et al. 2006 in Stankowich and Coss 2006) and whether animals are approached by a human on or off trail (Miller et al. 2001; Taylor and Knight 2003b in Stankowich and Coss 2006) can also influence FID. All of my approaches were done on trails, which may discount for any individuals who avoid the trails in that area. It is typically accepted that animals who flee at shorter distances may be at greater risk (Stankowich and Coss 2007) to either predator encounters or human activities like vehicle collisions (Hebblewhite and Merril 2008).

It is likely that the Central American agouti is not very much harmed by human presence, and if provided with something such as a consistent food source, they will learn to accept it and take advantage of the resource availability. Agoutis cache fruits and seeds when resources are low, but more often than not forget to retrieve cached seeds, which germinate and grow, and they are therefore considered a valuable seed disperser (Wainwright 2007). However, when resources are abundant, which is a common association to human development (e.g. dumpsters and gardens) (Woodroffe et al. 2005), there would be less of a need to cache seeds, and dispersal of many plants may be severely impacted. Increasing human activity threatens wildlife conservation in many ways (e.g. mortality, fragmentation, human avoidance) (Sinclair and Byrom 2006 in Hebblewhite and Merril 2008), and habitat fragmentation is known to influence species interactions and ecosystem functions (Malhi et al. 2014; Price et al. 2014).

To minimize the impact that humans have on local animal species, it is important to provide them with sufficient habitat that allows them to behave naturally and perform various ecosystem functions. Even in protected parks, if organisms roam outside of the area they are subject to hunting (Woodroffe et al. 2005) or dangerous encounters with cars and possibly dogs. Conflicts between people and wildlife continues to impact ecosystems in many different ways from extinctions to ecosystem structure and habitat destruction (Woodroffe et al. 2005), and the best solution is to try and keep wildlife in natural habitat and away from human activity.

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Impacts of artificial light on foraging of aerial insectivorous bats in a Costa Rican cloud forest

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ABSTRACT

Studies within temperate zones have found that artificial lights can impact aerial insectivorous bats by disrupting their foraging patterns and increasing availability of prey in a concentrated location. Very few similar studies have been conducted in the tropics and the topic remains poorly understood at lower latitudes, where there is a much higher diversity of bats. Frank (2015) conducted the first comparison of bat calls in dark areas and adjacent areas illuminated by street lights in Costa Rica. He found similar species diversity but higher activity of bats under street lights. Some species were only detected in dark areas. Frank (2015) did not account for potential confounding variables in his study. In this study I also measured call activity in adjacent areas with and without street lights but also measured potential confounding factors known to affect bat activity: distance to forest edge, light type, lunar phase, order, time since sunset, and wind velocity. In total, echolocations were recorded at 20 pairs of light and dark sites in and around Monteverde, Costa Rica. There was a higher number of bat passes at light sites (mean ± standard error 35.9 ± 8.5 recorded echolocations per night) as opposed to dark sites (mean ± standard error 15.8 ± 3.3 recorded echolocations per night), with distance to forest edge, lunar phase, light type, and order of site recording having a significant effect on number of echolocations as well. There was also a higher number of species at light sites (mean ± standard error 2.2 ± 0.29) than at dark sites (mean ± standard error 1.7 ± 0.3), and while lunar phase and distance to forest edge did significantly affect the dependent variable, light/dark treatment was still the most significant factor. Furthermore, some species were only recorded in light sites, and some only in dark sites, suggesting that tropical bat species are differentially impacted by artificial lights. As such, increasing human occupation and development in the tropics could lead to shifts in local bat distributions and community compositions in the tropics, as it has been reported in the temperate zone.

RESUMEN

Estudios en la zona templada han encontrado que las luces artificiales pueden impactar los murciélagos insectivoros aéreos al interrumpir los patrones de forrajeo y aumentar la disponibilidad de presas en un área concentrada. Muy pocos estudios similares se han llevado a cabo en los tropicos y el tema permanece poco entendido a latitudes menores, donde hay una diversidad mayor de murciélagos. Frank (2015) llevó a cabo la primera comparación de llamadas de murciélagos en áreas oscuras y áreas adyacentes iluminadas por luces de calle en Costa Rica. Él encontró una diversidad de especies similar pero una mayor actividad de murciélagos bajo las luces. Algunas especies se detectaron únicamente en áreas oscuras. Frank (2015) no tomó en cuenta variables que pueden confundir en este estudio. En este estudio, también medí la actividad de llamadas en áreas adyacentes con y sin luces de calle pero también medí otros factores que pueden influir la actividad de murciélagos: distancia al borde de bosque, tipo de luz, fase lunar, orden, tiempo desde la puesta del sol, y velocidad del viento. En total, la ecolocalización se grabó para 20 pares de sitios con y sin luz alrededor de Monteverde, Costa Rica. Hay un mayor número de pases de murciélagos en los sitios con luz (promedio ± error estandarr 35.9 ± 8.5 grabaciones por noche) opuesto a los sitios oscuros (promedio ± error estandarr 15.8 ± 3.3 grabaciones por noche), con la distancia al borde de bosque, fase lunar, tipo de luz, y orden de los sitios muestreados teniendo un efecto significativo en el número de ecolocalizaciones también. También hay un mayor número de especies en los sitios con luz (promedio ± error estándar 2.2 ± 0.29) que en los sitios oscuros (promedio ± error estandar 1.7 ± 0.3), mientras la fase lunar y distancia al borde del bosque si afectaron significativamente las variables dependientes, los tratamientos de luz/oscuro fue aún así el factor más significativo. Además, algunas especies se grabaron solamente en los sitios con luz, y algunas otras solo en los sitios oscuros, sugiriendo que las especies de murciélagos tropicales se ven impactadas de manera diferente por las luces artificiales. Así, el aumento de la ocupación humana y el desarrollo en los tropicos puede llevar a un cambio en la distribución local de los murciélagos y la composición de la comunidad en los tropicos, ha como ha sido reportado en las zonas templadas.

INTRODUCTION

Human development and urbanization is increasing rapidly around the world, and the effects of this development are evident across many ecosystems and species, including bats (Chiroptera) (Jung and Kalko 2011). Bats are the second most species-rich mammalian order in the world (Wilson and Reeder 2005), as well as effective bioindicators of the health of an ecosystem (Jones et al. 2009). As such, the effects of development and urbanization on bats are important issues that may impact regional biodiversity and ecosystem health. One of the results of development and urbanization is an increased number of lights, which have been shown to impact bat diversity, distribution, and foraging behavior (Bat Conservation Trust 2009).

As with many orders, Chiroptera is most diverse in tropical zones. In Costa Rica, where bats represent almost 50% of the mammals in the country, 113 species have been recorded. Of these 113 species, many are aerial insectivorous bats that feed on nocturnal insects such as moths (LaVal and Rodriguez-H 2002). Most studies done on the impact of street lights on bats have been done in the temperate zone (Bat Conservation Trust 2009; Kuijper et al. 2008; Polak et al. 2011; Rydell 1992; Rydell and Racey 1995; Stone et al. 2015). Many temperate bat species do well around mercury vapor lights because these lights interfere with moths' defensive behavior, thus making them easy prey for some bats (Svensson and Rydell 1998). Species that do well under street lamps tend to be common species that can fly fast and have long-range echolocation systems; slower species and less common species tend to avoid street lights altogether (Polak et al. 2011; Rydell 1992). This suggests that the presence of street lights may alter bat diversity in temperate zones.

Despite higher levels of diversity and functionality in tropic zones (LaVal and Rodríguez-H 2002) the effect of street lights on tropical bats remains largely unknown. In a study measuring bat activity through bat echolocation, Frank (2015) found that foraging activity of aerial insectivorous bats in Monteverde, Costa Rica, was higher at artificially lighted sites as opposed to dark sites, but species richness was not statistically different between sites. Furthermore, he found that two species of bats (*Myotis pilosatibialis* and an unidentified bat) only occurred at dark sites, appearing to avoid lighted areas. The study by Frank (2015) did not account for potential confounding variables that could affect bat activity in general, weakening the conclusion of the study. Potential confounding factors include artificial light type, lunar phase, wind velocity, time since sunset, and distance to forest edge. Different types of lights have been shown to have differential impacts on the abundances and behaviors of certain insects, thus leading to differential abundances of bats foraging around these lights. For example, mercury vapor lamps decrease moths' defensive behaviors more so than other light types (Svensson and Rydell 1998), thus increasing the ease-of-capture for bats (Rydell and Racey 1995). For some tropical bat species, increased levels of moonlight can impact their foraging activity. Some species may decrease their foraging activity under increased moonlight (e.g. *Myotis riparius*) while others increase their foraging activity with more moonlight (e.g. *Pteronotus parnellii*, *Saccopteryx bilineata*) (Appel et al. 2017). Wind velocity may also impact bat abundance, by making foraging more difficult. Bat abundance also changes during the night, and some bats show peaks of activity in the first two hours since sunset (Brown 1968; LaVal 1970). Finally, distance to forest edge has been shown to decrease the abundance of some aerial insectivorous bat species, while increasing that of others (Kalko 1998).

The goal of this study is to compare bat calls between dark sites and sites with artificial light taking into all these potential confounding variables that could have potentially skewed the effect of artificial lights on bat richness and abundance found by Frank (2015). I measured all variables in 9 sites not included in Frank (2015), and all possible variables on the sites originally measured by Frank (2015). Some variables were inferred for the 2015 dataset when the information was available (i.e lunar phase) while other variables were impossible to measure (e.g. wind velocity).

METHODS

Study Site

This study was conducted in Monteverde, Costa Rica. Located on the western side of the Atlantic slope, Monteverde is classified as a lower montane wet forest. Monteverde rests at an elevation of about 1400 meters and has a mean annual temperature of 18° C. Annual rainfall averages around 3000 millimeters, and humidity varies between 74% and 97%. Monteverde is a suitable area for the study of bats because the local bat community has been relatively well-studied in the past (LaVal and Fitch 1977).

This study was conducted at 20 pairs of sites, each with a light and dark site. Of these paired sites, 11 were conducted by Frank in 2015, and 9 were new sites conducted by me. All studies were conducted on or around 18h, when the sun had completely set. Each light site was conducted 5-10 m from directly below a street light located along the road. Seventeen of the light sites were lit by sodium-vapor lamps, while the other three were lit by white lights that were likely high intensity discharge lamps or LEDs~~.~~ (Longcore and Rich 2004). Sites were chosen due to their proximity to forest edge and the availability of a corresponding dark site no more than 60 m from the light source.

Field Methods

Bat calls were recorded using the Echo Meter Touch (EMT) connected to an iPad Mini 2. The EMT's microphone was restricted from 15 to 100 kHz because all known bats from Monteverde fall into this range. I alternated between the light and dark sites every 30 minutes for two hours, resulting in a total of one hour of recording for both the light and dark sites. I alternated which site I would start at each night, beginning at the light site for one location and the dark site for the next location. No data was collected on nights when it rained, as this could affect the abundance of bats foraging. Calls were later converted using Kaleidoscope software and identified in the AnaLook program with the help of Richard K. LaVal.

Potential confounding variables were also recorded at each site except for wind velocity, which was only measured at new sites using an anemometer for each 30-minute recording period. Distance to forest edge was measured from the point of recording. Light type was visually assessed. Lunar phase and time since sunset for each data period were obtained from lunar phase and sunset tables (Timeanddate.com and Tides4Fishing.com).

Statistical Analyses

Bat activity was analyzed using a generalized linear mixed model (GLMM) with Poisson distribution that included all measured variables as fixed effects. Site identity was included as random effect. The number of species was analyzed using a linear mixed model (LMM) that included all measured variables as fixed effects and site identity as random effect. Non-significant factors were sequentially removed from the models in descending order by p-value until the model only included significant factors. P-values are presented from the final models. For each species recorded at more than 4 sites, a paired t-test was used to determine whether there was a significant difference in activity between light and dark sites.

RESULTS

Bat Activity

Most of the measured variables affected bat activity in the study. Distance to forest edge ($z = -6.428$, $p = 1.29e^{-10}$), lunar phase ($z = 3.527$, $p = 0.000421$, Figure 2), light type ($z = 2.469$, $p = 0.013553$), and the order ($z = -2.118$, $p = 0.034137$) in which the light and dark sites were sampled all impacted the variation in bat activity. Wind velocity ($z = 0.933$, $p = 0.350882$) and time since sunset ($z = -0.629$, $p = 0.529631$) did not significantly affect bat activity at each site. The light/dark treatment of interest significantly also explains variation in bat activity when all other significant factors are present in the model. More passes were recorded at light sites than at dark sites (GLMM, $z = 7.259$, $p = 3.91e^{-13}$), with mean activity at light sites more than twice that of dark sites (Figure 1).

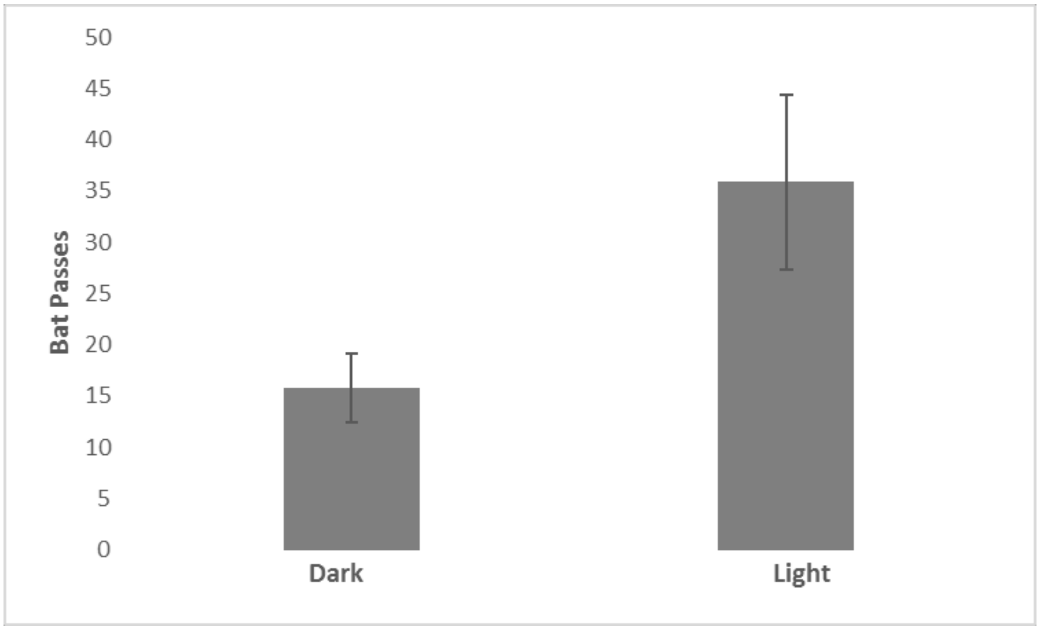

Figure 1: Number of bat passes identified in light and dark sites in Monteverde, Costa Rica. There is significantly more bat activity at light sites than at dark sites (GLMM, $z = 7.259$, $p = 3.91e^{-13}$). Distance to forest edge ($z = -6.428$, $p = 1.29e^{-10}$), lunar phase ($z = 3.527$, $p = 0.000421$), light type ($z = 2.469$, $p = 0.013553$), and the order ($z = -2.118$, $p = 0.034137$) in which the light and dark sites were sampled all impacted the variation in bat activity.

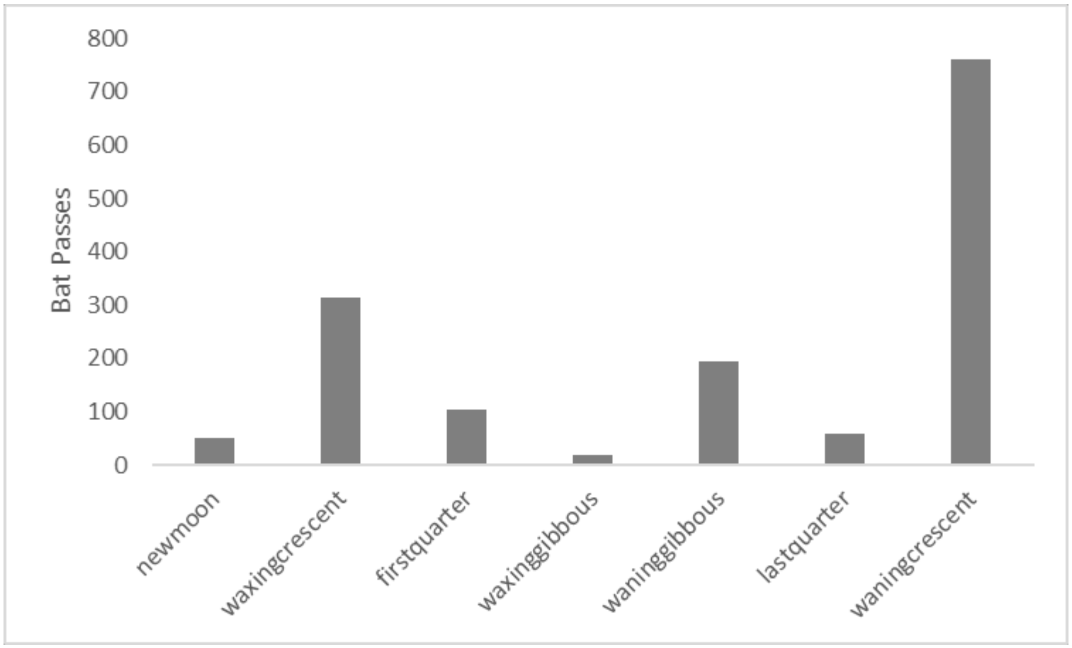

Figure 2: Number of bat passes per lunar phase as recorded in Monteverde, Costa Rica. The most activity was recorded during the waning crescent. Lunar phase was one of the most significant confounding variables in this study ($z = 3.527$, $p = 0.000421$), although light/dark treatment was still a significant factor.

Species

For the sampled sites, there are significantly more species located at light sites than at dark sites (LMM, $x^2 = 5.5597$, $p = 0.01838$; Figure 3). Of the potential confounding variables, only distance to forest edge ($x^2 = 3.7075$, $p = 0.05417$) and lunar phase ($x^2 = 9.5299$, $p = 0.04914$, Figure 4) had significant effects on the number of species at a site. Light type ($x^2 = 0.1890$, $p = 0.6638$), order ($x^2 = 0.4971$, $p = 0.9195$), time since sunset ($x^2 = 0.0$, $p = 0.9952$), and wind velocity ($x^2 = 0.3757$, $p = 0.5399$) were nonsignificant. Light/dark treatment remains the most significant factor and does impact the number of species.

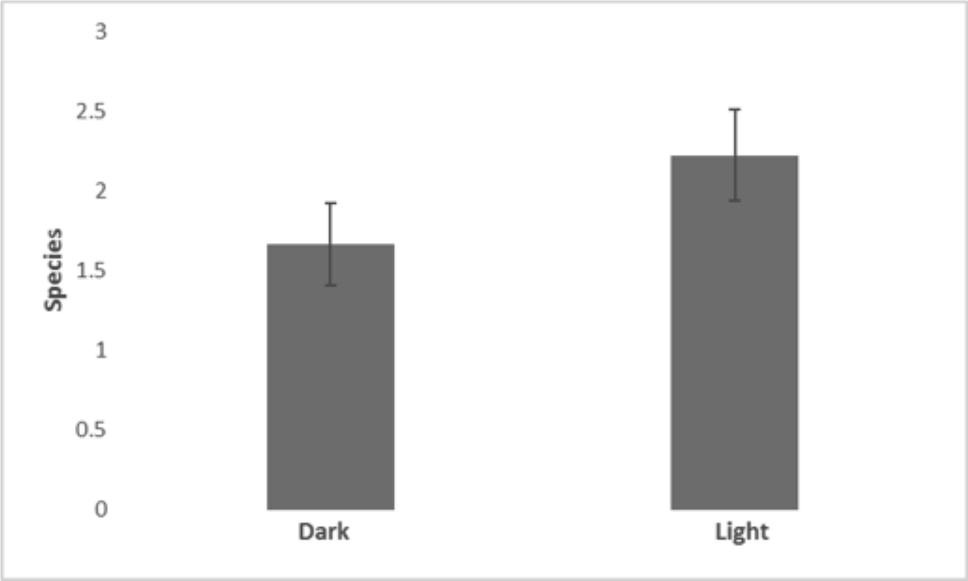

Figure 3: Number of bat species identified in light and dark sites in Monteverde, Costa Rica. There are significantly more bat species at light sites than at dark sites (LMM, $x^2 = 5.5597$, $p = 0.01838$). Of the potential confounding variables, only distance to forest edge ($x^2 = 3.7075$, $p = 0.05417$) and lunar phase ($x^2 = 9.5299$, $p = 0.04914$) had significant effects on the number of species at a site.

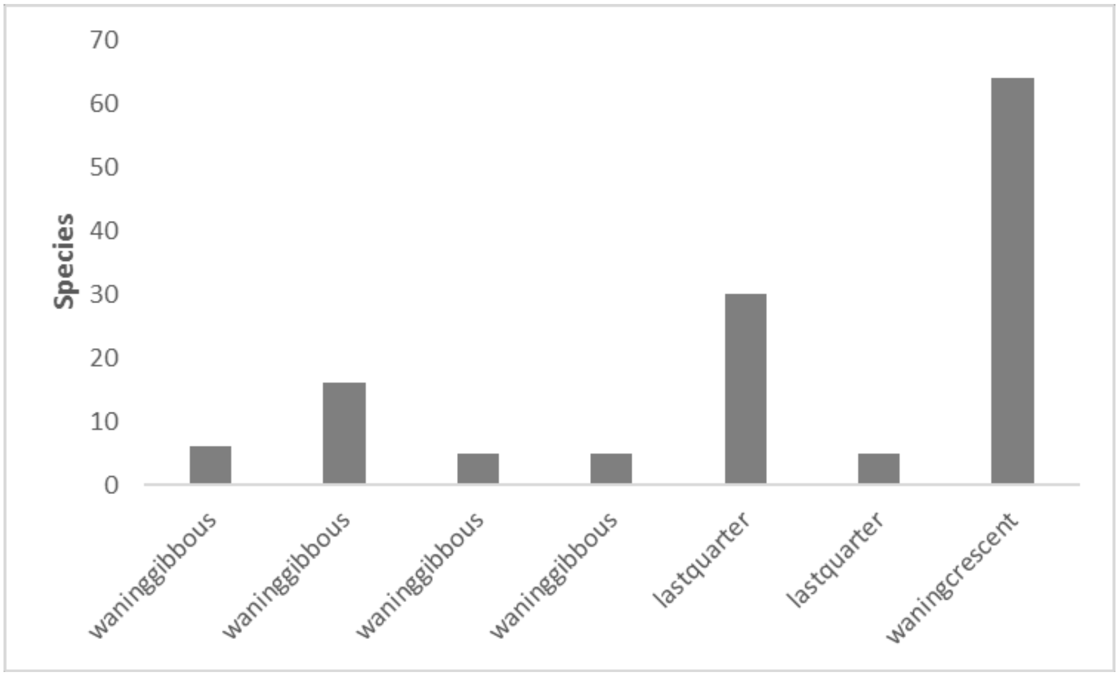

Figure 4: Number of bat species per lunar phase as recorded in Monteverde, Costa Rica. The most species were recorded during the waning crescent. Lunar phase was the most significant confounding variable in this study ($x^2 = 9.5299$, $p = 0.04914$), although light/dark treatment was still the most significant overall factor.

Of the species sampled, four species only occurred at one of the two pairs of sites. *Lasiurus intermedius* was only recorded at dark sites. *Molossus sinaloae*, *Peropteryx kappleri*, and *Peropteryx macrotis* were only recorded at light sites, although they were each only recorded at one light site. Eight species were found at more than four sites. There was no significant difference in recorded bat activity between light and dark sites for seven of the species, but *Myotis nigricans* did show a significant difference in activity between light and dark sites (t = -2.0956, p = 0.04529, Table 1).

Species	**t-value**	**df**	**p-value**
Diclidurus albus	-1.6054	28	0.1196
Eptesicus fuscus	-0.27328	28	0.7866
Eumops auripendulus	0.52214	28	0.6057
Molossus molossus	-1.9908	28	0.05635
Myotis nigricans	-2.0956	28	0.04529
Myotis oxyotis	-1.2111	28	0.236
Myotis pilosatibialis	1.285	28	0.2093
Myotis riparius	-0.42596	28	0.6734

Table 1: Results of paired t-tests between artificially lighted and dark sites for bat species that were recorded at more than four sites in Monteverde, Costa Rica. The only statistically significant difference in recorded activity was found for *Myotis nigricans*.

DISCUSSION

Artificial lights attracted significantly more bat activity than dark areas, as well as a significantly higher number of species. This is similar to the findings of a previous study in the temperate zone that found that artificial lights increase bat activity (Stone et al. 2015), but differs from older studies in the temperate zone (Kuijper et al. 2008; Stone et al. 2009). This is likely because in the past all lights were sodium vapor, but in recent years they have been progressively replaced with LEDs (Stone et al. 2015). As this study was mostly conducted around LED street lights, these findings seem to be in line with previous studies on the same light type.

In the 9 new sites, 10 species were found that were not recorded by Frank (2015) and two species were not recorded that had been recorded by Frank (2015). Furthermore, one species that was only recorded in dark sites during Frank's (2015) study, *Myotis pilosatibialis*, was found at one of the new light sites. Something to note is that five species were only recorded at one of the two pairs of sites. Of these, *Peropteryx kappleri* is not usually found in Monteverde, and was only recorded once. Thus, it may be that this was one individual foraging outside of its usual range for some reason. Another of these bats is an unidentified individual recorded by Frank (2015). While it is likely that the species only recorded at light sites, all classified as Least Concern by IUCN (2017), *Molossus sinaloae*, *Peropteryx kappleri*, and *Peropteryx macrotis*, would do fine in dark sites as well, the one species only recorded in dark sites (*Lasiurus intermedius*) may be indicative of a need for corridors to promote the foraging of select species. *L. intermedius* primarily feeds on leafhoppers, dragonflies, diving beetles, and mosquitoes (Allen 1997), which may not have the same level of phototaxis as moths and as such may not be as disproportionately abundant around artificial lights. If there are other species in addition to *L. intermedius* that specifically avoid lighted areas, it may benefit these species to keep unlighted corridors along roads at regular intervals. In this way, current richness and abundance of bats in the tropics may be able to be maintained in spite of human development.

It would seem that, on the surface, artificial lighting supports higher bat species richness and abundance. This may be due to increased availability of prey. In temperate zones, artificial lights have served as local patches of food for some bat species during critical times of the year (Rydell 1991) and the same may be true in the tropics. However, it is unknown if artificial lights provide benefits to a select group of bats while affecting other species, perhaps due to different insect behaviors (e.g. moths being more attracted to lights) and correspondingly different bat diets, or due to exploitation of resources under lights by certain species, perhaps due to an advantage such as flight speed. Studies in temperate zones have shown that faster-flying species tend to congregate around lights, while slower-flying species avoid lights altogether (Longcore and Rich 2004; Polak et al. 2011). This may also hold true in the tropics, giving some species a decisive advantage over others in urban areas.

Overall, artificial lighting as a result of urbanization and human development is altering the activity and local distributions of bat species in Monteverde, Costa Rica. Some species avoid street lights while others flock to them. In contrast to Franks's (2015) study, this study found a significant difference in both bat activity and species richness between light and dark sites. Furthermore, significant differences in species-specific activity between light and dark sites was not found for the same species, i.e. *Diclidurus albus*' activity is no longer significantly different, while that of *Myotis nigricans* is. These differences may be attributed to more sample sites and/or the inclusion of potential confounding variables. These confounding variables affected bat activity and species richness in a similar way to the only other study that has addressed the effect of urbanization on bats in the tropics (Jung and Kalko 2010). In both cases, light type, distance to forest edge, and lunar phase did affect microhabitat use (i.e. light and dark sites), and bat foraging activity was higher around artificial lights, though this pattern did not hold true for every species. These findings indicate urbanization and increased artificial lighting in the tropics may alter bat community composition and distribution, with some species benefitting from artificial lighting and others avoiding lighted areas altogether. Future research should focus on determining whether some species are being pushed out of urban areas due to the effects of artificial lighting. Furthermore, darkened corridors should be implemented so as to maintain populations of bat species that tend to avoid lights.

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Anthropogenic noise pollution on frugivorous and nectarivorous bats' foraging patterns in captivity

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ABSTRACT

The rapid growth of the human population means greater loss of biodiversity due to pollution. While many species are heavily impacted by noise pollution, bats may be the terrestrial organism most greatly affected due to their heavy reliance upon echolocation for spatial awareness and the discovery of food. The effect of traffic noise pollution on bats has only been studied in insectivorous species. In this study, I investigated the affects of traffic noise, ambient noise, and no noise on the eating patterns of captive frugivorous and nectarivorous bats at the Bat Jungle Exhibit in Monteverde, Costa Rica. During the bats' first feeding period of the day, on separate days, they were exposed to traffic noise, ambient noise, or no noise. The number of visitations to the fruit and nectar feeders were observed, and the amount of fruit consumed in 45 minutes was recorded. Frugivorous bats did not decrease the frequency of visitation to feeders in the presence of noise and did not decrease consumption. Nectarivorous bats tended to visit less the feeder with ambient noise and but did not eat significantly less in the presence of noise. Mean visits to feeder on traffic days: ambient 32.6, control 58.61, traffic 43.6. These results suggest that nectarivorous bats may be more affected by noise than frugivores in the wild because nectarivorous bats rely more upon echolocation to find the exact location of the nectar.

Resumen

El crecimiento rápido de la población humana significa una mayor perdida de diversidad debido a la contaminación. Mientras varias especies se ven altamente impactadas por la contaminación sónica, los murciélagos pueden ser el organismo terrestre más ampliamente afectado debido a la necesidad de la ecolocalización para el conocimiento espacial y el descubrimiento de comida. El efecto del sonido del tráfico en los murciélagos ha sido solo estudiado en murciélagos insectívoros. En este estudio, investigué los efectos del sonido del tráfico, sonido ambiente, y sin sonido en los patrones de alimentación de murciélagos frugívoros y nectarívoros en cautiverio en el Bat Jungle en Monteverde, Costa Rica. Durante el primer período de alimentación del día, en días separados, ellos fueron expuestos a sonido de tráfico, sonido ambiente y sin sonido. El número de visitas a las frutas y los comederos con néctar se anotaron, y la cantidad de fruta consumida en 45 minutos fueron tomados en cuenta. Los murciélagos frugívoros no disminuyen la frecuencia de visitas a comederos en la presencia del sonido y no disminuye el consume. Los murciélagos nectarívoros tienden a visitar menos los comederos con el sonido ambiente pero no difieren en la cantidad comida con la presencia del ruido. Las visitas promedio a los comederos: ambiente 32.6, control 58.61, tráfico 43.6. Estos resultados sugieren que los murciélagos nectarívoros pueden ser más afectados por el ruido que los frugívoros en el campo ya que los murciélagos nectarívoros dependen más en la ecolocalización para encontrar la ubicación exacta del néctar.

INTRODUCTION

As the human population continues to soar, urban areas will grow to accommodate more people, leading to a worldwide decline in biodiversity. The expansion of urban settings will lead to an increase in noise pollution as transportation networks, buildings, and infrastructure develop. Noise pollution affects both terrestrial and marine ecosystems through changing the availability and accuracy of the information provided through sound in the environment (Fuller et al. 2007). Unfortunately, much of the natural environmental sound overlaps with anthropogenic noise, thus inhibiting species and community interactions (Schaub et al. 2008).

In general, noise pollution affects the behavior of individuals with consequences that can expand at the level of populations and communities. For example individual aquatic hermit crabs may become less aware of approaching predators due to distractions caused by loud motor boards (Chan et al. 2009). At the population level, birds may also experience decreased foraging and higher predation rates due to reduced communication with conspecifics about food location and predator presence, respectively (Brumm et al. 2004). Some birds increase their call amplitude when singing in environments polluted by anthropogenic noise (Fuller et al. 2007). Birds that cannot be heard by conspecifics over the noise may suffer reduced reproductive success and may leave the area (Fuller et al. 2007). Furthermore, orcas also decrease their foraging efforts in louder environments (Lusseau et al. 2009). At the community level, noise pollution can increase pollination by hummingbirds but reduce seed dispersal by birds and increase seed predation by rodents, resulting in less seed recruitment (Francis et al. 2012). Hence, studying the effect of noise pollution on species with important ecosystem roles, such as pollinators or seed dispersers, is key to understanding how human expansion affects the health of ecosystems. Conducting this kind of study in the tropics is critical, because ecosystems are more diverse at nearly all facets of diversity (Francis et al. 2012).

Bats are very diverse in the tropics and function as pollinators and seed dispersers. They are arguably the terrestrial animal most greatly affected by noise pollution because they rely upon sound for communication and echolocation to locate food (Schnitzler and Kalko 2001). Echo locating animals emit high frequency signals and analyze the returning echoes to detect, characterize, and localize the reflected objects for spatial orientation (Schnitzler and Kalko 2001). The frequency of anthropogenic noise typically falls under 3 kHz (Goodwin and Shriver 2011). and the frequency of bat echoes is usually 20-200 kHz (Van Ryckegham 2017).

To my knowledge, the effects of noise pollution have only been studied on insectivorous bats. Insectivores hunt prey in the air, on stationary surfaces such as the ground, and in water, heavily relying upon echolocation to find food, and thus are negatively impacted by anthropogenic noise (Schnitzler and Kalko 2001). For example, great mouse eared bats in captivity avoid hunting in areas with anthropogenic noise recorded with an ordinary microphone (Schaub et al 2008).
Daubenton's bats, which find prey by echolocation, also reduced their foraging efficiency in the presence of traffic noise and avoided areas with anthropogenic noise (Luo et al 2015), whereas wild bats foraging for ground running arthropods have decreased foraging efficiency due to sound overlap (Siemers and Schaub 2010).

Frugivorous and nectarivorous bats may be also affected by anthropogenic noise pollution. These two types of bats use a combination of scent and echolocation to search and locate food (Thies et al. 1998, D. von Helverson and O. von Helverson 1999). Frugivorous bats exhibit scouting behavior where they fly to trees known to have their source of food using echolocation, and then search for ripe fruits through odor at very close distances (Fleming et al. 1977). The bats rely on these olfactory cues to find ripe fruits among leaves (Korine and Kalko 2005). Nectarivorous bats do the opposite; they rely on odor for long distance search and on echolocation at short distances, using acoustic guides to find the nectar in the flower (D. von Helverson and O. von Helverson 1999).

Hence, in noise-polluted environments frugivorous bats might be less effective at locating fruiting plants, but should have fewer problems taking fruits once the tree has been discovered. Nectarivorous bats should be less affected in their ability to find plants with fruits, but may have more problems finding the nectar in the flower. In this study, I tested for the first time if the foraging success of frugivorous and nectarivorous bats is affected by anthropogenic noise. I further tested if foraging is affected differentially in these two groups of bats given their natural differences in the use of olfaction versus echolocation to find food at short distances. Using artificial feeders in a bat exhibit with captive bats, I evaluated whether the bats decreased the number of visits to feeders and ate less when exposed to broadcasted traffic noise in relation to natural ambient noise and no noise.

MATERIALS AND METHODS

Study Site

The Bat Jungle, an exhibit located in Monteverde, Costa Rica, houses about 75 live bats. The bats eat and spend their active hours in a simulated forest environment that is 17m x 2-3 m x 2.5 m (Shelley 2011) (Figure 1). About 50 individual frugivorous bats live in the bat jungle: *Artibeus toltecus, Artiveus lituratus, Artibeus jamaicensis, Carollia brevicauda, and Platyrrhinus vittatus*. Also, about 20 nectarivorous bats live with the frugivores: *Anoura geofrayi, Hylonycteris underwoodii*. Each species is native to Monteverde. The day and night cycle is reversed so that bats can be studied during the real day, when the enclosure is dark inside. The bats are fed nectar and a variety of fruits and vegetables about eight times per day and receive an assortment of fruit that is hung up. Inside the exhibit, there are three stationary fruit feeders that hold 2-3 bowls of fruit and a nectar feeder that can be hung up in multiple locations. The experiment was conducted in the morning during the bats' first feeding period. The bats inside the bat jungle experience a variety of disturbances daily: vacuum cleaners, noise from the restaurant above the bat jungle, and flashlights.

Figure 1a (left): Picture of the bat jungle from the inside with full lights on. 1b (right) picture of the bat jungle exhibit from the observation area, picture taken by Guadalupe Quiroga Realizaciones.

General Approach

To determine the effects of noise pollution on the bats' eating patterns, the experiment consisted of three different treatments: ambient noise, traffic noise, and no noise (control). Each of these treatments took place on separate days because the area where the bats eat is not large enough to conduct the three treatments simultaneously without great acoustic interference between them. The sound played on ambient days was recorded at night behind the biological field station in Monteverde. The sounds of potential predators (owls) were edited out. The traffic noise was recorded at night in Santa Elena. Both recordings were made with an ordinary microphone and iPod touch.

The types of fruit used in the experiment were the same used by the bat jungle to feed their bats: a mixture of watermelon, papaya, apple, and banana. The bat jungle has three stationary fruit feeders inside the exhibit, each with room for two bowls. Thus, 6 bowls all of the same type were weighed and then each filled with 60 grams of watermelon, 47 grams of papaya, 40 grams of apple, and 25 grams of banana. The sizes of the fruit pieces were consistent with the size of the pieces already cut by employees of the bat jungle. The bowls with the fruit were each weighed separately. The nectar feeder was filled with anywhere between 210-310 mL (measured each day) of pre-made nectar and placed next to the one selected fruit feeder for the day. This feeder was selected randomly for the first two weeks and systematically on the final days to make sure that all feeders had roughly the same number of replicates.

Once the fruit and nectar were placed inside the exhibit, an ordinary speaker was hung up in the middle of the exhibit and connected to the iPod. The selected treatment of the day was played for the entire 45 minutes of observation. Both the traffic and ambient sound samples remained the same for the duration of the experiment, and played without pause. On control days, the speaker was hung up in the middle of the bat jungle, but remained off. The ambient and traffic treatments were run a total of five times. The control treatment was run 6 times. By the end of the experimentation period, a total of 16 days of data were collected.

Visits

As explained above, each day one of the three fruit feeders was visually observed to record the number of bat visitations to the two bowls of that feeder and to the nectar feeder located next to it. The number of visits to the feeders was counted over a total of 45 minutes, divided into three separate 15 minute time periods. Every 15 minutes, the count started again. Visits to the fruit feeder were counted when the bat hovered above the bowl, landed in the bowl, or took fruit from the bowl. Visits to the nectar feeder were counted when the bat hovered directly next to or above the feeder and drank from it.

Fruit and Nectar Consumption

After the 45 minute visit period ended for the day, the fruit bowls and nectar were removed from the exhibit. The fruit bowls were weighed according to which feeder they came from, and the remaining nectar was measured. The fruit and nectar were then returned to the bats.

To calculate the weight of just the fruit before the trial was ran, the weights of the fruit and the bowl were added together. Then, the weight of the six bowls when empty was subtracted from the weight of the bowls with the fruit.

Next, to calculate the weight of the fruit not consumed by the bats after 45 minutes, the weights of the bowls with the fruit inside were added together. Then, the same value for the weight of the six empty bowls was subtracted from the weight of the fruit and bowls after the 45 minutes. To calculate the amount of fruit consumed during the 45 minutes, the weight of fruit remaining after the 45 minutes was subtracted from the weight of fruit before the 45 minutes. The amount of nectar consumed was calculating by subtracting the volume of nectar after the 45 minute period from the starting amount of nectar.

RESULTS

Frugivorous Bats

Neither traffic noise nor ambient sound significantly impacted the amount of fruit consumed by the bats (LMM with feeder location as random effect, numDF:2, denDF: 13, F-value: 1.1, P-value: 0.36). While the average amount of fruit consumed on traffic and ambient days is less than that consumed on control days, the data is not significant (Figure 2).

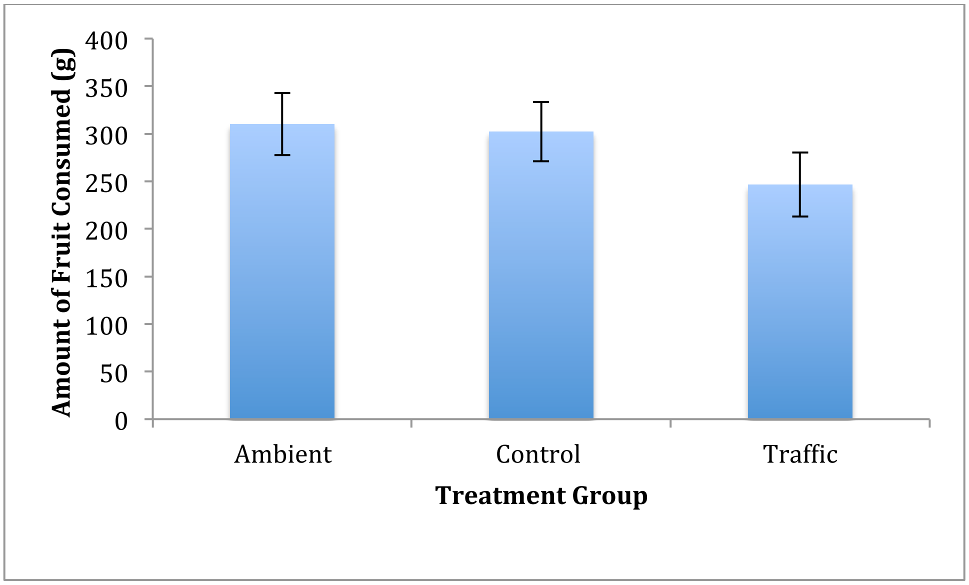

Figure 2: The mean amount of fruit consumed by the bats of the Bat Jungle exhibit in Monteverde, Costa Rica (+/- SE). The bats had access to the fruit for 45 minutes. The ambient noise is the natural sounds, excluding predators, of the forest recorded behind the biological field station at night. The traffic noise consists of cars and pedestrians from the streets of Monteverde at night. During the control treatment, no sound was played, but the speaker was hung up in the same location as in other treatment days.

During the entire 45 minute period, the bats, on average, visited the fruit feeders about the same number of times during the ambient, control, and traffic treatments (LMM with Day as random effect, F-value: 0.18, numDF:2, denDF:13, , p-value:0.84) (Figure 3). The bats visited the feeders less on ambient and traffic days than the did on control days, however, the data is not significant.

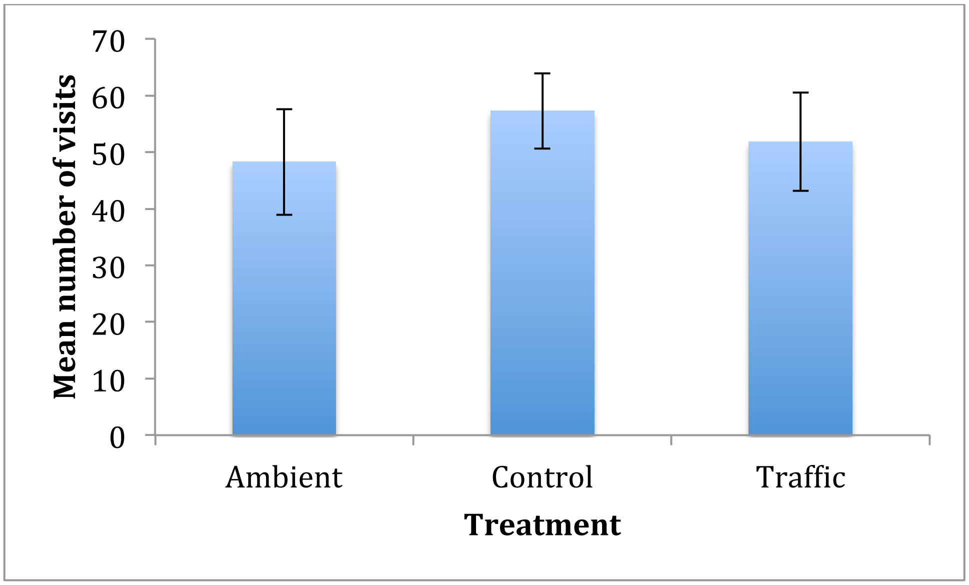

Figure 3: The average number of bat visits to a fruit feeder in the Bat Jungle exhibit in Monteverde, Costa Rica (+/- SE). The bats had access to the fruit feeder for 45 minutes. The ambient noise is the natural

sounds, excluding predators, of the forest recorded behind the biological field station at night. The traffic noise consists of cars and pedestrians from the streets of Monteverde at night. During the control treatment, no sound was played, but the speaker was hung up in the same location as in other treatment days.

Nectarivorous Bats

The bats did not consume a significantly different amount of nectar during any of the three treatments. (LMM with feeder location as random effect, numDF: 2, denDF: 11, F-value: 1.28, P-value: 0.32). It is worth noting that the bats appeared to have consumed, on average, less nectar on ambient and traffic days than they did on control days, but this potential trend is not significant (Figure 4).

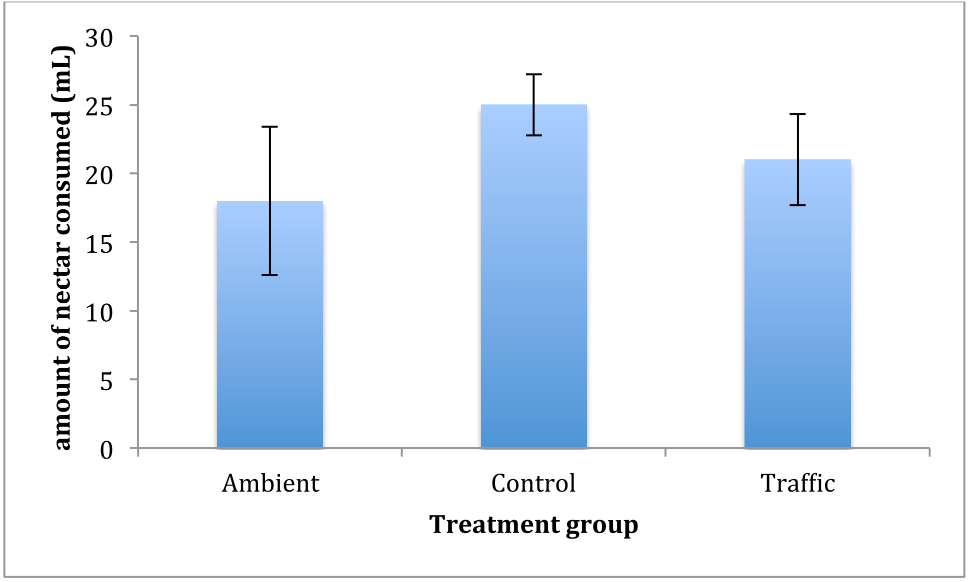

Figure 4: The average amount of nectar consumed by bats in the Bat Jungle exhibit in a 45 minute time period (+/- SE). The ambient noise is the natural sounds, excluding predators, of the forest recorded at night behind the biological field station. The traffic noise consists of cars and pedestrians from the streets of Monteverde at night. During the control treatment, no sound was played, but the speaker was hung up in the same location as in other treatment days.

During the entire 45 minute period, the bats tended to make fewer visits to the nectar feeder on the ambient days than they did on control days. (LMM with Day as random effect, treatment, numDF: 2, denDF: 13, F-value: 109.6, P-value: 0.081) (Figure 5). Furthermore, the visits to the nectar feeder during traffic treatments is less than that on control days, however, this pattern is not significant.

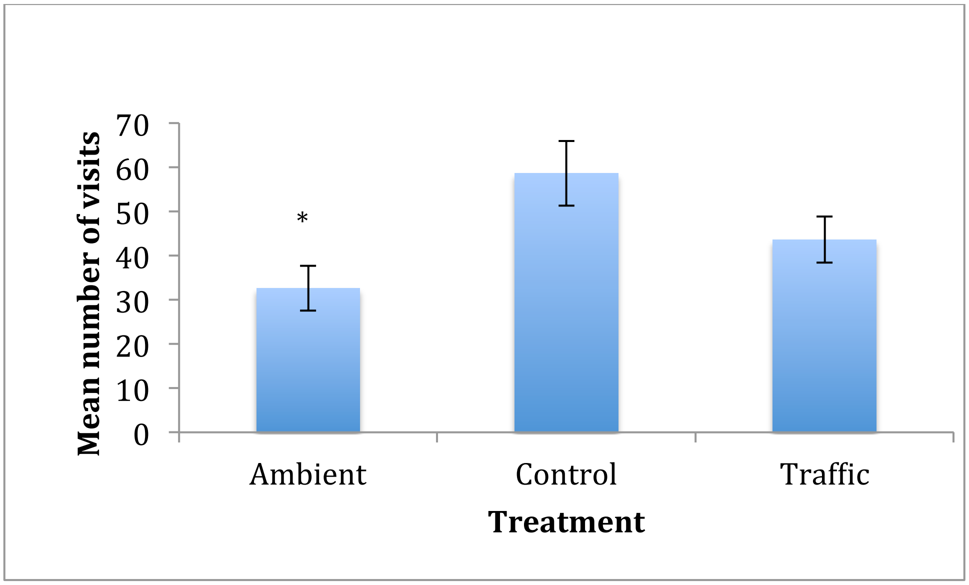

Figure 5: The average number of bat visits to the nectar feeder in the Bat Jungle Exhibit in Monteverde, Costa Rica (+/- SE). Post hoc comparisons show that the trend results from differences between ambient and control. The bats had access to the nectar for 45 minutes. The ambient noise is the natural sounds, excluding predators, of the forest recorded at night behind the biological field station. The traffic noise consists of cars and pedestrians from the streets of Monteverde at night. During the control treatment, no sound was played, but the speaker was hung up in the same location as in other treatment days.

Additional Observations

On the days with traffic or ambient treatments, the bats seemed to exhibit a decreased spatial awareness. On traffic days, the bats flew around more chaotically when initially released from the dormitory, and one day a bat flew into my head when I went inside to retrieve the bowls. On another ambient day, a bat flew into my hands that were carrying fruit bowls at waist level. And once again, on an ambient day, two bats collided with each other. When the sound was turned off for the day, the bats seemed to fly smoother and more gracefully. In addition, some species of nectarivorous bats would also visit the fruit feeders and drink the juice.

DISCUSSION

Both the traffic noise and the ambient noise had no significant impact on the foraging patterns of frugivorous bats. While the traffic noise had no significant impact on the nectarivorous bats, the ambient noise impacted their foraging habits.

Frugivorous bats are considered "pure searchers" because once their food is located, it need not be pursued or subdued (Schoener 1969). Frugivorous bats heavily rely upon odor at close distance to detect food instead of echolocation. *C. perspicillata* and *Glossophaga soricina*, for example, have developed a sensitive olfactory and visual search system for piper fruits (Fleming et al 1977). In fact, *Carollia* can find food by just its odor and even in the absence of other cues (Hessel and Schmidt 1994, Laska 1990). Some bats rely so heavily on odor detection that they do not echolocate (Gould 1978).

Data from the nectarivorous bats differs in terms of number of visitations throughout the 45-minute period. The nectarivorous bats visit the feeders less frequently under ambient conditions compared to under traffic and control conditions. Furthermore, the nectar consumption under ambient conditions follows this pattern, as the bats consumed less nectar under ambient conditions. A possible explanation is that some species of nectarivorous bats are bothered by the ambient noise. Many nectarivorous bats are known to also eat insects (LaVal and Rodiguez-H 2002). The ambient consists of many different insect calls, and thus, the ambient treatment could confuse some of the nectarivorous bats into searching for insects. In glossophagine bats, echolocation plays a major role in the detection of flowers when the bat is within 2-3 meters and all the way down to a few centimeters (D. von Helverson and O. von Helverson 2003). The bats likely had difficulty in precisely locating the holes in the nectar feeder because their short range echolocation may been disrupted. Therefore they could not consume as much nectar as they did under other conditions.

In addition, the nectarivorous bats also sometimes eat fruit from the bowl. During ambient days, they may possibly have an easier time locating the fruit feeders because they never change location and the fruit has a stronger odor. However, it is possible that once a nectarivorous bat located the nectar feeder, it drank more that it would under normal circumstances because it expended energy looking for food.

Furthermore, the frequency of frugivorous and nectarivorous bat calls likely does not overlap with those emitted by the traffic or ambient conditions. Frugivorous bats produce multiharmonic frequency modulated (FM) signals of broad bandwidth, with a high frequency, short duration, and low intensity (Thies et al 1998). Although the main energy of traffic noise is within the hearing range of humans (up to 5 kHz), it does have an ultrasonic component that could overlap with prey rustling sounds (3-30 kHz), but it is very unlikely that these sounds would overlap with echoes (Siemers and Schaub 2010).

Ultimately, seven different species of bats live within the bat jungle. Each species of bat uses different echolocation signals and have different hearing systems depending upon factors such as habitat type and foraging modes (Schnitzler and Kalko 2001). Thus, making it likely that only a couple of species of bat get disrupted by the ambient and traffic noises which impacts the data. The more species looked at, the more diluted the results will be. While frugivorous bats seem unaffected by both traffic and ambient sounds in captivity, nectarivorous and insectivorous bats seem to be impacted. Anthropogenic noise disrupts the feeding behavior of insectivorous bats (Schaub et al. 2008). My study shows that at least some types of noise disrupt the feeding behavior of nectarivorous bats as well.

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