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Human in the Tropics

Ant Caste and Antibiotic Resistance in Soil Bacteria Near Leaf-Cutter Ant Nests

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ABSTRACT:

Antibiotic resistance (ABR) in bacteria has been studied as a consequence of the abuse of clinical of antibiotics. However, bacteria can naturally obtain ABR. ABR in soil bacteria increases with proximity to *Atta cephalotes* nests, presumably due to secretion of antibiotics by actinomycete bacteria that are symbiotic with the ants. However, the true source of antibiotics, and therefore ABR, in soil bacteria near leaf cutter ant nests has never been directly studied. I examined ABR of soil bacteria from soil samples collected near seven *A. cephalotes* colonies in Monteverde, Costa Rica: from the undisturbed colony mount, from inside or close to the fungal chamber, and from 30m away from the mount. Soil samples were plated on petri dishes with an antibiotic treatment to measure how close soil bacteria could grow to the antibiotic, as proxy for ABR. Soil from under the colony was significantly more resistant (mean ± standard error = 2.53 ± 0.08µm) than soil from the mount (mean = 2.74 ± 0.05µm). In addition, actinomycete colonies were isolated from gardener and forager ants to test whether these bacteria inhibited the growth of soil bacteria. Forager-based actinomycetes were more repellant to soil bacteria (mean = 2.35 ± 0.20µm) than were gardener-based actinomycetes (mean = 1.45 ± 0.03µm). Based on these data, I find it likely that different ant caste-types in *A. cephalotes* are able to specialize on pathogens for colony defense. Overall, the results from this study demonstrate that the source of ABR in soil bacteria near *A. cephalotes* nests is very likely the symbiotic bacteria carried by leaf cutter ants.

Resumen:

La resistencia a antibióticos (RA) en bacterias ha estado estudiado como un resulto del abuso clínico de antibióticos. Sin embargo, las bacterias pueden obtener RA naturalmente. RA en bacterias de tierra aumenta con cercancía a colonias de *Atta cephalotes*, probablemente debido a la secreción de antibióticos por bacterias que se llaman actinomicetos cuales son simbióticos con las hormigas. Sin embargo, el origen cierto de antibióticos, y por lo tanto RA, en bacterias de tierra cercanas a las colonias de hormigas cortadoras de hojas nunca ha sido estudiado. Yo examiné RA de bacterias de muestras de tierra las cuales fueron colectadas cerca de siete colonias de *A. cephalotes* en Monteverde, Costa Rica: del nido de una colonia sin disturbio, de encima o cerca del jardín de hongos, y de 30 m lejos del nido. Muestras de tierra fueron colocados en platos de Petri con un tratamiento de antibióticos para medir la distancia entre las bacterias y el antibiótico, como un apróximado para RA. La tierra debajo de colonia era significativamente mas resistente (promedio ± error estándar = 2.53 ± 0.08µm) que la tierra del nido (promedio = 2.74 ± 0.05µm). Además, colonias de actinomicetos fueron aisladas de hormigas del jardín y de forrajeadoras para probar si estas bacterias inhibieron la expansión de bacterias de tierra. Actinomicetos de forrajeadores fueron más repelente a bacterias (promedio = 2.35 ± 0.20µm) de tierra que las de jardineras (promedio = 1.45 ± 0.03). A causa de estos datos, pienso que es probable que las hormigas de castas diferentes puedan especializarse en patógenos para defender su colonia. Por lo general, los resultados de esta investigación demuestran que el origen de RA en bacterias de tierra a cerca de colonias de *A. cephalotes* es probablemente bacterias simbióticas que están llevado por hormigas de cortados de hojas.

INTRODUCTION:

Many people, when they think of antibiotic resistance (ABR), picture a clinical scene. Indeed most publications on the subject are only concerned with how resistant bacteria will change how we use modern medicine (World Health Organization 2018, Gould & Bal 2013, US Department of Health and Human Services 2013). Interestingly, we are not the only species to use antibiotics (Sengupta *et al.* 2013), meaning some ABR can result from non-human activity. Indeed, low doses of antibiotic compounds, enough to keep competing strains at bay (Gullberg *et al.* 2011), have been in existence well before the 20th century (Waksman 1961). For example, a mutualist mycorrhizae on wheat plants secretes antibiotics against the root pathogen Take-all (Thomashow & Weller 1988). Additionally, bacteria strains like *Salmonella typhimurium* have been known to use antibiotic molecules outside of human influence (Goh *et al*. 2002). Likewise, resistance to such antibiotics long predates modern medicine, existing outside of human intervention. In fact, phylogenetic studies have concluded that the very first ABR genes originated in a natural environment (Aminov & Mackee 2007). Specifically, there is strong evidence that supports at least eight clusters of ribosomal protection proteins in the genus *Streptomyces* that predate the 'antibiotic era' (Aminov *et al.* 2001).

An example of natural antibiotic use that leads to ABR occurs near the nests of fungus-cultivating ants in the tribe Attini. Such ants are widespread throughout the neotropics, some of which cut down leaves to feed to a specific fungus (family Lepiotaceae), such as the genera *Atta* and *Acromyrmex*, (Currie *et al.* 1999, Hölldobler & Wilson 1990). The fungus only exists inside leaf cutter ants nests and constitutes the only food for the colony (Hubbel *et al.* 1980). It was only recently discovered that aside from the symbiosis between the ants and the fungus they eat, there are two more relationships of note. First, a parasitic fungus specific to leaf-cutter colonies (Muchovej & Della Lucia 1990), called *Escovopsis*, which tries to infiltrate ant colonies to feed off of the cultivated fungus. Second, a mutualistic actinomycete bacteria, in the genus *Streptomyces* (there is some disagreement as to what genera the mutualist bacteria belong to: *Pseudonocardia* or *Streptomyces*. However, the consensus seems to be that while both genera exist in the ant nest and produce antibiotics, those from *Streptomyces* are more specific to *Escovopsis* and those produced by *Pseudonocardia* are more broad-range antibiotics (Haeder *et al*. 2009, Mueller *et al.* 2008, Sen *et al*. 2009)) that is specialized to keep the *Escovopsis* at bay, and in return the ants provide the bacteria with food (Currie *et al.* 2003). In fact, Haeder *et al*. (2009) found that the main antibiotic produced was candicidin, which was effective at inhibiting *Escovopsis* growth while not damaging the fungal garden. Candicidins are known to induce cell death in fungi (Hammond & Kliger 1976).

Leaf cutter ants seem to carry other bacterial strains not specific to *Escovopsis*, which produce more broad-spectrum antibiotics (Seipke *et al.* 2011, Barke *et al.* 2010, Haeder *et al.* 2009). If this is true, as the ants dig colonies and move throughout the soil, the antibiotics would seep into the soil as well, potentially causing ABR near the ant nest. In support of this hypothesis, Simon (2008) reported ABR levels in soil bacteria closer to colonies of *Atta cephalotes* are significantly higher than ABR levels further away from the colony. Simon (2018) found ABR in soil bacteria in reference to commonly used and broad-range antibiotics (World Health Organization 2017): Ceftiofur, Gentamicin, Oxytetracycline, and Penicillin (also referred to as Dihydrostreptomycin). Hence, the potential relationship between ABR and ant colony proximity was inferred and not directly tested. If the source of ABR in soil bacteria is truly symbiotic bacteria that live with *A. cephalotes,* then the results of Simon (2018) would demonstrate that at least some of the antibiotic(s) present in ant colonies are not specific to *Escovopsis*.

To evaluate whether ABR in soil bacteria near leaf cutter ant nests comes from the actual nests, it is necessary to test whether the ants themselves are inducing ABR in the many strains of soil bacteria surrounding their nests. Leaf-cutting ants have a metapleural gland (MG), which contains bacteria capable of producing broad-spectrum antibiotics (Poulsen *et al.* 2001, Yek & Mueller 2010). They also house the specialist actinomycetes on their cuticle (Currie *et al.* 1999b, Ortius-Lechner *et al.* 2000). *A. cephalotes* also use a caste system to divide labor, which includes a strict separation between ants that forage great distances for leaves, ants that tend to the fungal garden, and ants that remove harmful waste (Hart & Ratnieks 2001). Only the ants constantly in contact with the fungal garden (hereafter called gardeners) seem to produce antibiotics and proceed to coat their bodies in it (Fernández-Martin et. al. 2006). Gardeners also have a proportionally larger MG than other castes (Yek *et al.* 2012)

In this study, I conducted a two-part experiment to evaluate whether the origin of ABR in soil bacteria is the gardener ants inside the nest. In the first part, soil samples taken near or inside the fungal garden were examined for ABR and compared to samples from the nest mount and far from the nest. The goal of this experiment was to test whether ABR is truly higher inside the nest, where gardener ants are confined. In the second experiment, actinomycetes isolated from both gardener and forager ants were put in contact with soil bacteria to test whether they truly inhibit soil bacteria growth.

MATERIALS AND METHODS:

Study site:

All samples were collected in the Caballeriza al Rodeo in Santa Elena, Costa Rica (**Figure 1**) from October 18th to November 10th, 2018. This property is located in the premontane moist life zone, and it contains open areas as well as forest fragments. *A. cephalotes* nests were identified by dark mounds, often in clusters. The nests were also identified as mature due to the presence of large soldiers (Powell & Clark 2004). The best mounds to begin digging in were the ones with the most ant activity. Mounds with ants exiting and carrying soil pellets from excavation were considered exit holes. I dug around the exit hole.

Figure 1. Arial view of Caballeriza al Rodeo area in Santa Elena, Costa Rica. Soil and ant samples taken in order to investigate the true origin of ABR in *Atta cephalotes* nests were taken from deforested areas denoted by a dashed green line. Coordinates: 10.310676, -84.834585.

Experiment 1- ABR in response to soil depth:

Once the fungal garden was reached (**Figure 2**), sterilized spoons were used to scoop and place soil samples into new Ziploc bags. Soil samples were collected from the fungal garden, from undisturbed soil on the nest mount, and from 30m away. Samples from 30m away were taken under the assumption that soil collected would not be from another nest mount (researcher

Figure 2. Fungal chamber of *A. cephalotes* nest in Caballerizo al Rodeo in Santa Elena, Costa Rica. Most of the ants pictured are soldier ants; the smaller ones are gardener ants.

judgement was used to ensure no other ant nests were in the vicinity). The nest was carefully covered up with the excavated soil after all samples were collected. This invasive methodology does render the excavated exit path useless, at least for four weeks. However, excavated colonies remained active during the entire length of the project.

Soil samples were then made into a solution following the procedure of Ghosh and LaPara (2007), where 0.5g of each soil type was mixed with 9ml of phosphate-buffered saline (PBS). Following the procedure of Simon (2018), 1ml of the soil-PBS solution was further diluted in 9ml of 1X PBS. PBS was prepared following Cold Spring Harbor protocol (Cold Spring Harbor Laboratory Press 2006). A small volume of 0.1ml of the solution was dropped onto an agar (Bacteriological Agar, 1.5% volume working solution) plate treated with an antifungal (Nyastin, 20mL/L) and spread using the spread-plate method (Herigstad et al. 2001, Hoben & Somasegaran 1982). Following the protocol of Simon (2018), filter disks were soaked in either Oxytetracycline or PBS, the later acting as a control. Three plates for each soil sample were made, each with one PBS-soaked disk, and one Oxytetracycline-soaked disk. In total, nine plates were made per colony, and seven colonies were sampled in total. Bacterial cultures were left to grow in a cardboard box inside of a dehumidified room with a constant temperature of approximately 25 °C for four days. Many of the agar plates became mildly melted under these conditions, likely due to the presence of the antifungal solution. I attempted to mitigate this error by keeping plates upside-down the entire time to keep the disks out of reach of the liquid.

The closest bacterial colony to each disk was visually identified under a dissecting microscope, and its distance to the disk was measured in µm using a micrometer installed on the dissecting scope, following the protocol of Simon (2018) at a constant magnification (**Figure 3**).

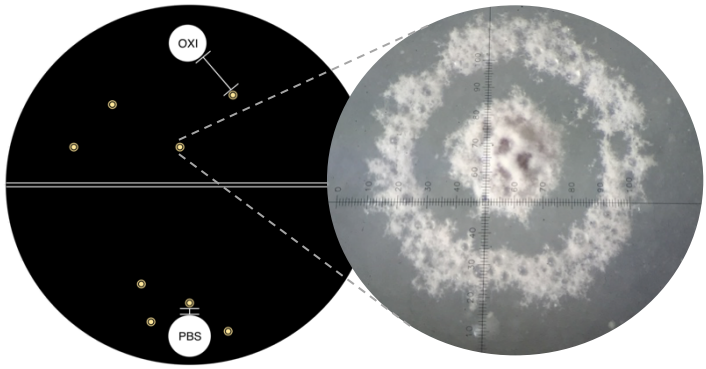

Figure 3. (From left to right): 1) a diagram of the experimental setup where labeled white circles are the treated filter disks and golden concentric circles represent soil bacterial growths. Crossbars show measuring distance between the filter disks and the closest colony. 2) An example of soil bacterial growth seen through a microscope, overlayed with a micrometer. Magnification was 1.9x.

Experiment 2 – ABR in response to ant caste:

At the seventh excavated colony, individual ants were collected using autoclaved tweezers and placed into autoclaved Eppendorf tubes. Six foragers and six gardeners of equal size were taken, ground up, and mixed for about five minutes with 0.5ml PBS before being plated on prepared chitin-based plates (15g Agar, 3g Chitin, 0.575g K_2HPO_4, 0.375g $MgSO_4$ x $7H_2O$, 0.275g KH_2PO_4, 0.0075g (two grains) $FeSO_4$ x $7H_2O$, 0.00075g (one grain) $MnCl_2$ x $4H_2O$, 0.00075g (one grain) $ZnSO_4$ x $7H_2O$, 750mL H_2O) with antibiotics and grown in the same conditions as above for five days. Each plate was plated with a crushed ant, either a gardener or forager, in the center. This selective media allowed for only actinomycetes to be grown (Poulsen *et al.* 2007). After five days, a sample was taken from each plate as close to the ant as possible, and dropped on a prepared yeast-malt extract plates (4g Yeast Extract, 10g Malt Extract, 4g Dextrose, 20g Agar, 1000mL H_2O) for fast growth. Immediately after, a soil-PBS solution prepared (as described for Experiment 1) with a soil sample collected 30m away from this ant colony was spread in a ring around the yeast-plate drop, at a constant distance from the drop, about 2mm. This was done for seven plates in total, 3 gardener plates and 4 forager plates. After four days stored in the same conditions as above, each plate was measured in the same manner described above, using a microscope and micrometer to find the distance from the closest soil bacteria colony to the edge of the actinomycete colony.

Statistical Analysis

Linear mixed models (LMMs) were used to compare distance to disks between treatments while keeping the dependency between observations that came from the same colony, soil sample, or petri dish (e.g. colony, soil sample and petri dish identity were included as a random effects in the model). Variables were log transformed to meet the assumption of normality. P-values correspond to analyses of deviance conducted with the function “Anova” of the R package “car”. Pairwise post-hoc comparisons of means were conducted using Tukey tests with the R package “emmeans”. All analyses were conducted in R 3.4.3.

RESULTS:

Experiment 1 – ABR response to soil depth:

Oxytetracycline-soaked disks (mean = 2.86 ± 0.03µm) repelled soil bacteria, on average, about 17.2% more than did control PBS-soaked disks (mean =2.44 ± 0.05µm) (*LMM: chi-square=56.0023, df = 1, p<0.0001*), as depicted in (**Figure 4**).

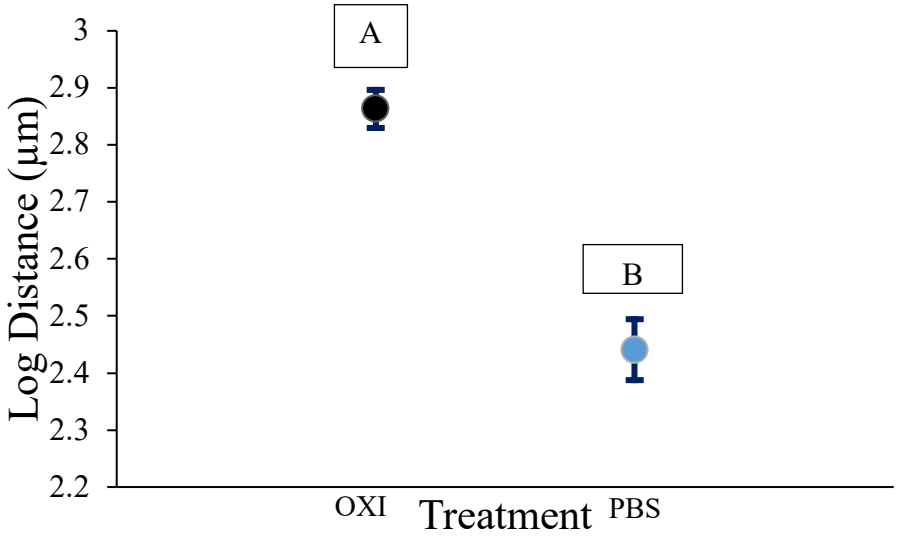

Figure 4. Antibiotic Resistance in soil bacteria measured as the distance from the closest bacteria on a petri dish to a filter paper disks soaked in either the antibiotic Oxytetracycline or a 1x PBS solution (control). Soil samples are averaged between three petri dishes from three soil samples collected from seven *A. cephalotes* colonies found in Santa Elena, Costa Rica. Bacteria were grown for four days in a dehumidified room at a constant temperature. Means are presented with ± 1 standard error. Different letters denote a statistically significant difference in distance according to a linear mixed model.

ABR distances varied in according to the origin of the soil sample in relation to the ant nest (LMM: *chi-square=7.099, df =2 , p=0.029*) The only significant difference between inhibition distance at the Oxytetracycline disk of nest locales was between soil from under the colony (mean=2.53 ± 0.08) and from soil at the mount (mean =2.74 ± 0.05). In fact, the mount soil was, on average, inhibited over 8.3% more than soil from under the nest. (**Figure 5**).

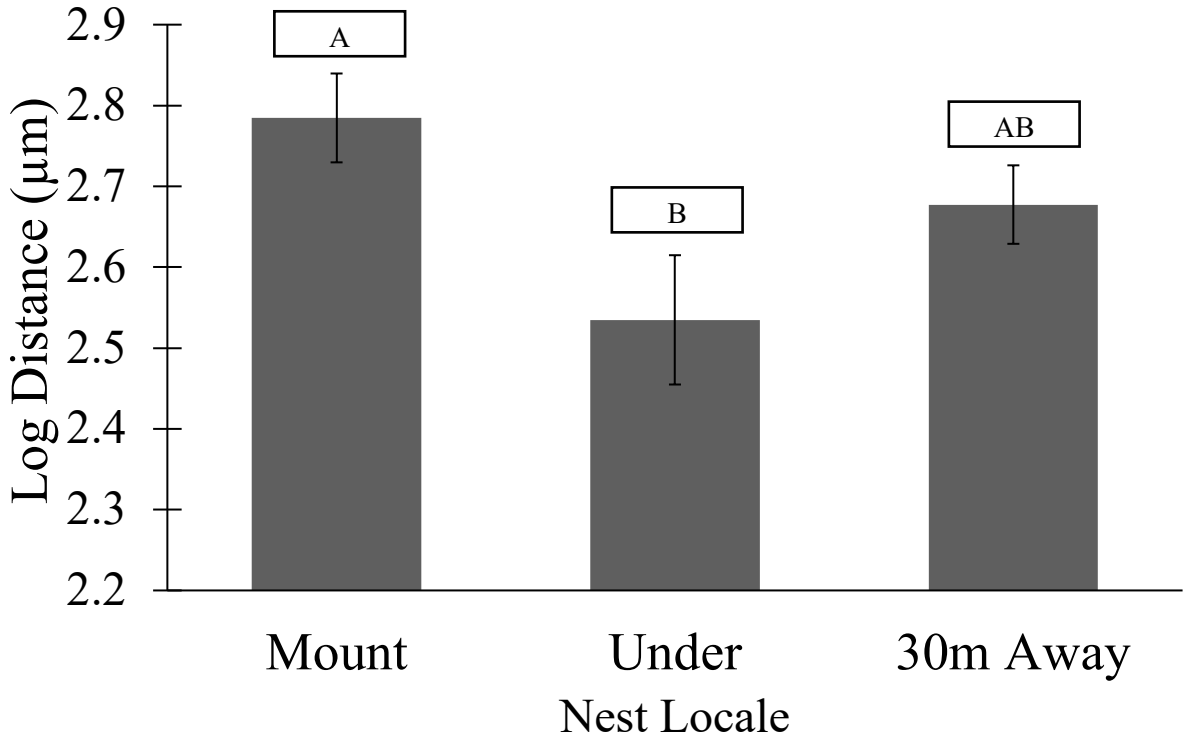

Figure 5. Antibiotic resistance in soil bacteria measured on a petri dish as the distance from the closest bacterial colony to the Oxytetracycline-treated filter disk. Distances are averaged between petri dishes from the same locale of seven different *Atta cephalotes* colonies found in Santa Elena, Costa Rica. Plates were grown in a dehumidified room at a constant temperature for five days. Error bars represent ± 1 standard error. Different letters indicate significant differences in post-hoc pairwise comparisons between means according to Tukey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model.

Experiment 2 – ABR in response to ant caste:

The average inhibition distance for forager-based actinomycete colonies (mean = 2.35 ± 0.20µm) was significantly greater than that for gardener-based actinomycete colonies (mean = 1.45 ± 0.03µm) (*Man-Whitney U test, H=4.58. d.f.=1, p=0.03*). On average, forager-based actinomycetes repelled soil bacteria almost 40% more than did gardener-based actinomycetes. (**Figure 6**).

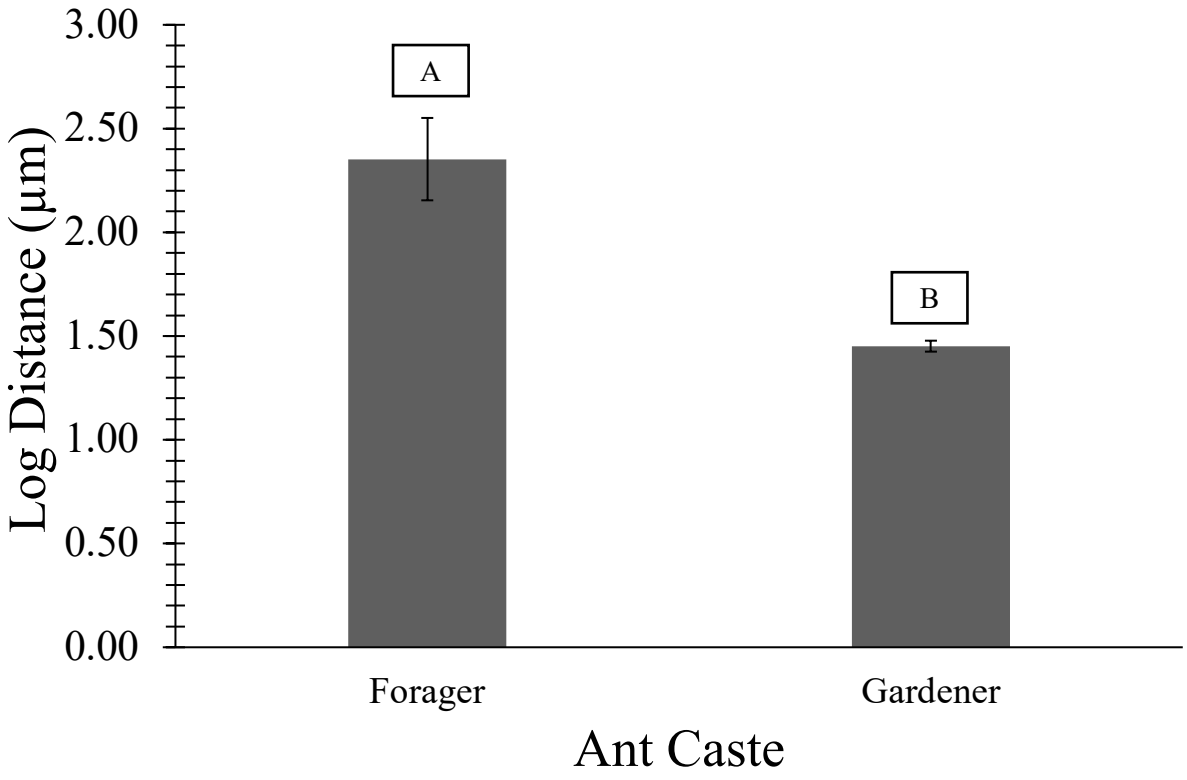

Figure 6. Antibiotic resistance in soil bacteria measured on a petri dish as the distance from the closest bacterial colony to either forager- or gardener-based actinomycetes. Distances are averaged between petri dishes from the same ant caste-type of four foragers and three gardeners from an *Atta cephalotes* colony found in Santa Elena, Costa Rica. Plates were grown in a dehumidified room at a constant temperature for five days. Error bars represent ± 1 standard error. Different letters denote a statistically significant difference in distance according to a Mann-Whitney U test.

DISCUSSION:

Antibiotics like Oxytetracycline are successful in inhibiting growth of bacterial colonies, especially in comparison to a control treatment. I can then use this information to assess ABR of soil bacteria in three different types of soil: near or inside the fungal garden of *A. cephalotes* nests, from the nest mount, and 30m away from the nest. While only a comparison between under the nest and the nest mount was significant, the comparison between soil from under the nest and soil from 30m away is suggestive. The greater amount of ABR under the nest could be telling of greater antibiotic activity under the nest. One explanation for this is simply a greater density of ant activity close to or inside the fungal garden than at the mount (personal observation, Fernández-Martin *et al.* 2006). Not only do MGs secrete actinomycetes (Schildknecht & Koob 1971, Mackintosh *et al.* 1995; do Nascimento *et al.* 1996), but secretion is also increased as ants come into contact with one another (Fernández-Martin *et al.* 2006). Therefore, a simple abundance of ants in a small space could mean that more antibiotics are excreted per unit area of soil. Furthermore, there have been findings that MGs of attine ants contain not only the specialized actinomycetes, but an array of generalist bactericidal compounds from other actinomycetes (do Nascimento *et al.* 1996, Barke *et al.* 2010). So, as *A. cephalotes* colonies grow and develop, they expand into the soil surrounding the nest, using the fungal garden as a food source, and therefore, the centerpoint of the expansion. Thus, secreted antibiotics can leak into the soil, and over time, soil bacteria become resistant (Read & Woods 2014). Where there is more antibacterial activity, more ABR will arise (Sengupta *et al.* 2013).

A second explanation for the increased ABR under the nest could be that antibiotics from under the nest are either more targeted or less potent, or a combination thereof, than those secreted at the nest mount, and therefore allow for a greater buildup of ABR. Aside from the type of ants (which is what was tested in the second experiment) the main difference between the mount soil and the fungal chamber soil is the type of soil. It has been found that nesting queens of *Atta* species will alter their MG secretions based on the soil type (Viera *et al.* 2015). However, there is no evidence that ants of the same species will adjust MG secretions solely based on the minute differences in soil type. What is left to consider is the difference in ant caste. While nest mounts and soil 30m away are frequented by foragers and, to a lesser extent, soldiers (personal observation, Wilson 1979, Hubbel *et al.* 1980), the fungal gardens are exclusively visited by soldiers and gardeners (Observational, Hölldobler & Wilson 1990). It is possible that while all leaf-cutter ant caste-types have MGs and produce the resultant antibiotics (do Nascimiento *et al.* 1996), different types of ants specialize in their production of different compounds. While differences in MG secretion is yet to be definitely shown, mandibular glands of several *Atta* species differ significantly between different castes in terms of secretion level and the gland's reservoir size (Pavon & Mathias 2005, Francelino *et al.* 2006). Perhaps if mandibular gland secretion can be altered between caste-types, so can MG secretion.

My data support this antibiotic specialization of castes by definitively demonstrating that actinomycetes from gardeners are weaker at repelling soil bacteria than actinomycetes from foragers. One evolutionary explanation for this difference could be that the two ant castes carry bacteria that produce antibiotics for different targets. Consider the difference between a forager and a gardener. Each time the forager goes out to collect leaves, it is exposed to different microhabitats, to different elements, and to an array of pathogens. An adaptation to favor this exposure would be to put more energy towards housing more *Psuedonocardia* perhaps, and

therefore, produce more broad-spectrum antibiotics. Likewise, a gardener is exposed to the same stable, isolated environment and only to the pathogens that are able to infiltrate, like *Escovopsis.* Perhaps gardeners have adapted to produce more *Streptomyces,* and therefore, more specific antibiotics. Already it is clear that gardener ants are specialized to recognize the *Escovopsis* parasite and have specific behaviors when finding it (Currie & Stuart 2001, Little *et al.* 2005) and have been doing so for millions of years (Chapella *et al.* 2004, Currie *et al.* 2003). Further research may be able to test ant-based actinomycetes against pure *Escovopsis* and definitively show whether the gardener actinomycetes are specialized towards the parasite.

Other alternative explanations include interference from the black yeast symbiont (which is closely related to the genus *Phialophora* in the phylum Ascomycota (Little & Currie 2008)) which feeds off of antibiotic-producing actinomycetes, thereby weakening *A. cephalotes* defense against pathogens such as *Escovopsis* (Little & Currie 2008). It is therefore possible that there are differing amounts of black yeast at different places in the ant nest. However, there is not enough evidence as of yet to determine how great of a factor this may play.

Simon (2018) established that ABR in soil bacteria increases in proximity to *A. cephalotes* nests. Following that procedure, I gathered samples from 30m away, however, they presented different results from those analyzed in Simon (2018). One reason why this could be is that Simon (2018) used ant colonies inside a forest, while mine were along the forest edge, or well outside the forest. Per unit area, there are considerably more *A. cephalotes* nests in disturbed or cleared areas than there are within an undisturbed forest (Jaffe & Vilela 1988). It is possible that my samples from 30m away were much closer to another *A. cephalotes* nest than were the samples of Simon (2018). This would explain the lack of significance in the difference in ABR between the nest mount and the 30m away samples.

In conclusion, this study provides evidence that symbiont bacteria in *A. cephalotes* ants produce antibiotics that inhibit the growth of soil bacteria. This inhibition seems to trigger a reaction of ABR from the surrounding soil bacteria, and different ant castes do so in different ways. These findings have two important inferences: first, the symbiosis between ants, *Escovopsis*, and antibiotic-producing bacteria exists in all species of Attine ants (Currie *et al*. 1999). Meaning, the caste specialization of antibiotics triggering differing levels of ABR could apply to more species than just *A. cephalotes*. Second, fungus-growing ants prefer to nest in clearings (Jaffe & Vilela 1988). As tropical deforestation increases in the new world (World Resources Institute 2017), more fungus-growing ants can be expected to colonize those areas. All of this boils down to the fact that natural ABR is largely ignored, which has serious implications. Already it is seen that the natural microbiota contains far more ABR genes than the number of ABR genes found in a clinical setting (Wright, 2007; Davies & Davies, 2010). In addition, it has been found that different ecosystems will contain different ABR genes (Martínez 2012), so understanding how systems like ant nests fit in is another piece of knowledge where studies have been lacking. To give an example that more directly affects human populations, there is increasing certainty that as global temperatures rise, bacterial agents trapped in polar permafrost will be released (Gilichinsky *et al.* 2008, D'Costa *et al.* 2011). These bacterial agents have never been in contact with the human immune system, let alone modern medicine (Gilichinsky *et al.* 2008), and understanding ABR beyond the clinical sense will prove invaluable.

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Avian Uropygial Oil on Rectrices, Migration Status, and Inhibition of Keratin-Degrading Bacteria

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ABSTRACT

Preening is a potential way birds inhibit the growth of keratin-degrading bacteria and prevent feather degradation as uropygial oil contains microbial properties and is spread throughout the feathers. Further, it is possible that the oil does not actually inhibit the growth of keratinase-producing bacteria but simply creates a physical barrier that isolates feather-degrading bacteria from feathers (Verea *et al.* 2017). By ranking the levels of degradation different rectrix types exhibit after being in the presence of keratinolytic bacterium *Staphylococcus aureus,* the antibacterial properties of 36 birds from Monteverde, Costa Rica were analyzed in relation to bird diet, migration status, and species. This was measured in the lab with three tests: change in feather weight after being plated in the presence of *S. aureus* for 14 +/- 3 days, the level of feather degradation after being plated in the presence of *S. aureus* using a scale of 0-9, and the distance from the edge of the rectrix to the nearest *S. aureus* colony after being plated together for 14 +/- 1 days. Rectrices found far from the uropygial gland degrade more in the presence of *S. aureus* than near rectrices. Additionally, the rectrices of migratory birds degrade more in the presence of *S. aureus*. The results suggest that the uropygial oil of migratory birds is less capable of inhibiting feather degradation by *S. aureus* than that of resident birds, and rectrices near the uropygial gland receive a better uropygial oil coating and protection against degradation than distant rectrices.

RESUMEN

El acicalamiento es una manera potencial en que los pájaros inhiben el crecimiento de bacterias que deterioran queratina y previenen la degradación, al contener el aceite uropigial propiedades microbianas y ser untado en las plumas. Además, es posible que en realidad el aceite no inhiba el crecimiento de bacterias que producen la enzima, queratinasa, pero que simplemente cree una frontera física que aísla a la bacteria que deteriora las plumas de las plumas. Para clasificar los niveles de deterioro de tipos diferentes que plumas de la cola exhiben después de estar en la presencia de la bacteria queratinolítica Staphylococcus aureus, las propiedades antibacterianas de 36 pájaros de Monteverde, Costa Rica fueron analizadas en relación a la dieta de pájaros, el estatus de migración, y las especies. Esto fue medido en el laboratorio con tres experimentos: el cambio en el peso de la pluma después de estar en un plato de agar con S. aureus por 14 +/- 3 días, el nivel de deterioro que la pluma experimentó después de estar en un plato en la presencia de S. aureus usando una escala de 0 a 9, la distancia del borde de la pluma a la colonia de S. aureus más cerca después de estar en un plato juntos por 14 +/- 1 día. Las plumas que fueron

encontradas lejos de la glándula uropigial se deterioran más en la presencia de S. aureus que plumas cercanas. Adicionalmente, las plumas de los pájaros migratorios se deterioran más en la presencia de S. aureus. Los resultados sugieren que el aceite uropigial de los pájaros migratorios es menos capaz de inhibir el deterioro de plumas por S. aureus que los pájaros residentes, y que las plumas cerca de la glándula reciban una mejor capa del aceite uropigial y protección contra el deterioro que las plumas lejos.

INTRODUCTION

Birds have developed a multitude of behaviors to combat bacterial parasitism. Preening is one such established behavior used to straighten feathers for improved flight, to increase waterproofing, and to inhibit pathogenic bacterial growth (Moreno-Rueda 2017). Birds gain their antibiotic resistance through the uropygial oil that is spread over the birds' feathers when preening (Jacob & Ziswiler 1982). Stored in the uropygial gland located near the tail feathers of the bird, the oil is made of mono- or diester fatty acids and alcohols, hydrocarbons and sterols (Martín-Vivaldi *et al*. 2010). These chemical functional groups have been previously studied and proven to inhibit the growth of bacteria (Jacob & Ziswiler 1982). For this reason, it has been hypothesized that without the feathers' coating of uropygial oil from preening, birds would have a weaker line of defense against bacteria that contain keratinase, an enzyme that digests the keratin found in the feathers of birds (Shawkey *et al.* 2003). As feathers are imperative for flight, their protection is essential, and they need a substantial coating of the antibacterial uropygial oil to prevent degradation (Fitzpatrick 1997).

There are many keratinase-bearing bacteria that degrade feathers and do so easily as bacteria attach to feathers at even the slightest contact (Verea *et al.* 2014). Some keratinolytic strains include *Bacillus licheniformis*, *Bacillus pumilis*, *Streptomyces fradiae*, *S. pactum*, *Staphylococcus epidermis*, and various fungi (Rubaiee *et al.* 2018, and references therein). When bacteria such as these are picked up by birds, feather degradation occurs. The edges of feathers become dented, the barbules lose their function as they become detached and cause holes in the feathers, therefore decreasing bird fitness (Rubaiee *et al.* 2018).

In a seminal study, Shawkey *et al.* (2003) first tested the hypothesis that uropygial oil inhibits the growth of keratinase-producing bacteria. They found that the oil extracted directly from house sparrow glands inhibits the growth of some, but not all, feather-degrading bacteria on agar plates. Similar studies conducted on other bird species have found mixed results (Moreno-Rueda 2017, Verea et al. 2017). It is still unclear what traits cause between-bird species variation in bacterial inhibition. Migratory status and diet may explain variation in inhibition among bird taxa. Given that there are a variety of species of keratin-degrading bacteria among different habitats (Burtt Jr & Ichida 2004, Lucas et al. 2003, Peele et al. 2009), temperate migratory birds may have a lesser tolerance to tropical bacteria than tropical resident birds due to different antibacterial specificity against local bacteria in the uropygial oil. The uropygial oil would have less antimicrobial specificity to foreign bacteria not found in their breeding grounds than if they were common, local bacteria. Diet might also help explain inter-specific variation because certain foods have antimicrobial properties (Ao *et al.* 2008, Baydar *et al.* 2004). Hence, different feeding guilds may influence the antimicrobial properties of the uropygial oil. Additionally, the

type of feather tested may be important because some feathers may get oil more often than others given their physical distance to the uropygial gland. To my knowledge, there is no previous research about the effects of feather distance to the uropygial gland on bacterial inhibition.

Additional studies that have tested whether that uropygial oil inhibits the growth of keratinase-producing bacteria have produced mixed results, and there is yet no evidence that the uropygial oil acts against bacteria on live birds (Moreno-Rueda 2017). Furthermore, it is possible that the oil does not actually inhibit the growth of keratinase-producing bacteria but simply creates a physical barrier that isolates feather-degrading bacteria from feathers (Verea *et al.* 2017). Here, I provide a step forward in the evaluation of this hypothesis by testing 1) whether a feather-degrading bacterium actually degrades feathers removed from wild birds on agar plates, and 2) whether feathers inhibit the growth of this bacteria to some extent. I also tested whether diet, migratory status, and feather position affect degradation rates.

MATERIALS AND METHODS

Study bacteria

Staphylococcus aureus. This easily obtainable bacterium was chosen for study as it belongs to the same genus as *Staphylococcus epidermis*, one keratin-degrading bacterium whose growth was found to be inhibited by uropygial oil (Shawkey *et al.* 2003). Similar to *S. epidermis* and other keratinolytic bacteria naturally found in plumage, *S. aureus* can degrade keratin in human fingernails (Cheng *et al.* 2011).

Study Site

Mist nets were set up at the Crandell Reserve, an old growth reserve owned by the Monteverde Institute, and at Bajo del Tigre, a protected second growth reserve of the Children's Eternal Rainforest. Both reserves are located in the premontane tropical wet forest of Monteverde, Costa Rica. Birds were caught from October 24 to November 4, 2018. Two rectrix feathers, equidistant to the uropygial gland, were collected from each caught bird. In total, the rectrices of 36 birds were of varying distance to the uropygial gland (Figure 1). The feathers were put into individual plastic bags and stored in a fridge at 4 degrees Celsius. Hand sanitizer was used in between captures to avoid sample contamination.

General laboratory procedures

Agar plates were made at a concentration of 1.5% mass volume using Bacteriological Agar. The agar was combined with water and heated with a hot plate until the powder was completely dissolved. Next, the liquid agar was poured into the sterilized petri dishes which were then left to solidify. When the plates were solid, they were wrapped in parafilm and stored in a fridge at 4 degrees Celsius until they were tested. All lab equipment was sterilized in boiling water for 10 minutes before use.

FIGURE 1. *Rectrices are displayed after catching a bird with mist nets at the Bajo del Tigre in Monteverde, Costa Rica. There are 12 rectrices to choose from on all birds but hummingbirds, which only have 10 rectrices. Two rectrices of equal distance from the uropygial gland were taken from every bird. The two type 6 rectrices are the outside feathers and the two type 1 rectrices are the inner two feathers, as pictured above.*

Experiment 1: Changes in Weight

The rectrices of 12 birds were cut with sterilized scissors until they had a weight of 0.01g (Appendix 1). Feathers from the same individual were tested in pairs. Two fragments, corresponding to two feathers from the same individual, were put on two different agar plates. One of the plates was inoculated with one to two colonies of *S. aureus,* which were swabbed on the agar using a metallic loop that was sterilized with fire. The other plate was left as control without *S. aureus*. The plates were wrapped in parafilm and placed in a dark container at about 25 degrees Celsius. After 14 +/- 3 days, the feather pairs (treatment and control) were either lifted or scraped off the plate, depending on their level of degradation, and placed on the scale to measure change in weight. The scale was cleaned off after each feather was weighed.

Experiment 2: Visual degradation

After the rectrices of the 12 birds were weighed, the amount of degradation was quantified using visual Feather Degradation Index (FDI) (Appendix 1). A score of 0 signifies an undegraded feather that is completely undamaged (Figure 2). A score of 1 represents a feather whose barbule tips are broken off, and a score of 2 stands for a feather who has full chunks of barbs broken off. A score of 3 signifies a feather that has a localized break of the rachis due to mold or bacteria, but the rest of the feather stays intact. A score of 4 represents a localized break of rachis due to mold or bacteria with unrelated barb breakage elsewhere. A score of 5 stands for a broken rachis on the upper half of the feather, a score of 6 represents a broken rachis on the upper half of the feather with barb breakage. A score of 7 signifies a broken rachis on the bottom half of the feather, and a score of 8 stands for a broken rachis on the bottom half of the feather with barb breakage. A score of 9 represents a fully degraded feather that does not stay intact at all.

FIGURE 2. *Examples of rectrices are shown after 14 +/- 3 days in the presence of keratin-degrading* S. *aureus and are ranked on their degradation based on the FDI scale in Table 1. Scoring from left to right: 1, 5, 6, 8, 9.*

Experiment 3: Inhibition of bacterial growth

One rectrix from 24 birds were cut into roughly 0.75 +/- 0.25cm^2 squares and put onto agar plates (Appendix 2). The agar plates were inoculated with two colonies of *S. aureus*, which were swabbed on the agar using a metallic loop that was sterilized with fire. As a control, filter paper discs of 6 +/- 0.5mm^2 were cut uniformly with a sterile hole punch and placed about 1 +/- 0.5cm away from the paired feather. The plates were then wrapped with parafilm and placed in a dark container at about 25 degrees Celsius.

After 14 +/- 1 days, bacterial growth was identified on the plates using a dissecting scope. Further, a micrometer set at a constant magnification was used to measure the distance in mm from the closest *S. aureus* colony to the edge of the filter paper disc or feather (Figure 3). Any fungal growth was ignored and could be recognized by the presence of filamentous hyphae. If the uropygial oil were to inhibit the growth of *S. aureas*, a longer distance from the closest *S. aureus* colony to the feather would indicate a greater antibiotic resistance (Shawkey *et al.* 2003). Additionally, the number of *S. aureus* colonies on the feather were counted.

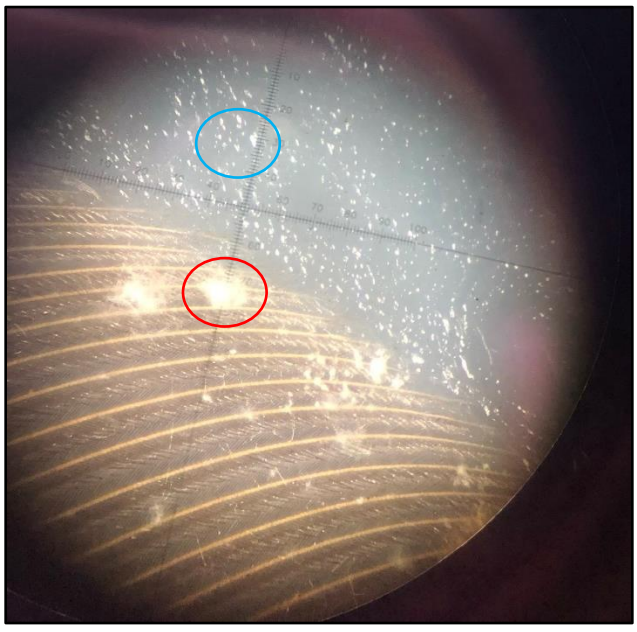

FIGURE 3. *Inhibition is recognized as the empty space between the edge of the rectrix and the closest bacterial colony. Some rectrices had larger* S. aureus *inhibition distances than others, and all were*

measured in mm. In this picture, fungi were beginning to grow on the feather and are circled in red, while examples of bacterial growth are circled in blue.

Data Analysis

All birds caught were classified by migratory status (resident or latitudinal migrant), diet (omnivorous, frugivorous, insectivorous, or nectivorous), and rectrix number removed (2, 3, or 5).

Feathers weighed more by the end of the experiment due to either the presence of bacteria and fungi growing on them, due to the impossibility of removing agar particles from very degraded and small feather fragments before weighing them, or both. For this reason, only the final weights and ranks were used in the comparison between treatment and control feathers.

Linear mixed models (LMMs) were used to compare weight and rank (individually) between migratory status, rectrix type and combination of these variables, while keeping the paired nature of the experimental design between individuals (i.e. treatment was compared to respective controls, so individual was included as a random effect in the model). Distance was analyzed using a regular ANOVA using the base function "lm" in R. Variables were log transformed, when necessary, to meet the assumption of normality. P-values correspond to an analysis of deviance conducted with the function "Anova" of the R package "car". Pairwise post-hoc comparisons of means were conducted using Tukey tests with the R package "emmeans." All analyses were conducted in R 3.4.3.

RESULTS

Experiment 1: Changes in weight

The influence of rectrix type on weight changes between feathers exposed to *S. aureus* and feathers that were not was marginally nonsignificant (Table 3). The nonsignificant trend comes from the fact that rectrices from the 5-position weighed less on average than the other two types (Figure 4, Table 1).

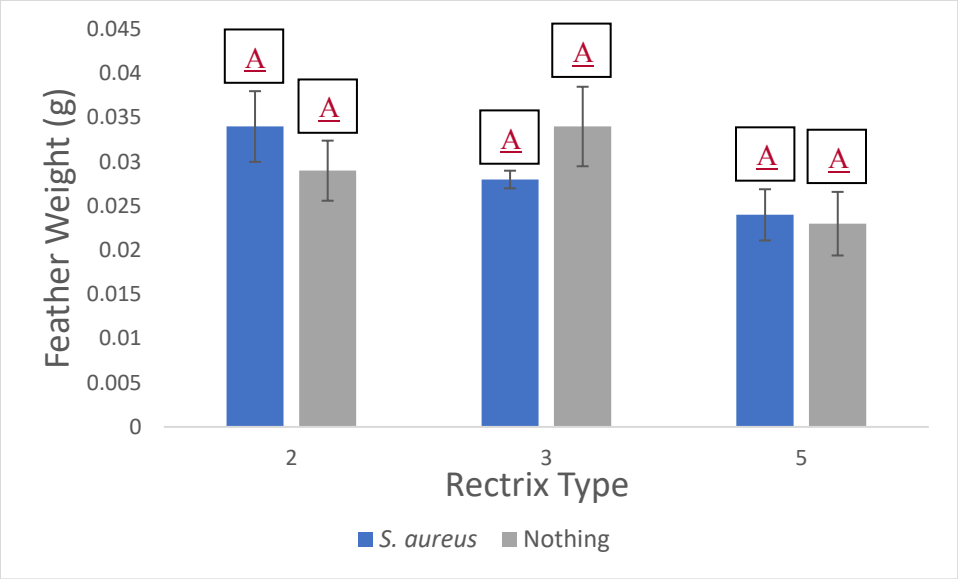

FIGURE 4. *Rectrix feather weight after 14 ± 3 days in the presence and absence of the keratin-degrading bacteria* Staphylococcus aureus*. Feathers were taken from 12 birds caught in the premontane tropical wet forest of Monteverde, Costa Rica. Means are presented with ± 1 standard error. Bars with differing letters indicate a significant difference in post-hoc pairwise comparisons between means according to Tukey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model. This shows a difference in weight between a presence and absence of* S. aureus *and a control for each rectrix type. Rectrix type 2 n=6, rectrix type 3 n=2, rectrix type 5 n=4.*

TABLE 1. *Results of a LMM* on *rectrix feather weight was tested in relation to* Staphylococcus aureus *presence and rectrix position. The weight of rectrices were tested after 14 ± 3 days with and without the presence of the keratin-degrading bacteria* S. aureus*. Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica.*

Bacteria	Rectrix	Mean (g)	SE
No	2	0.0288	0.0034
No	3	0.0335	0.0045
No	5	0.0225	0.0036
Yes	2	0.0342	0.0040
Yes	3	0.0280	0.0010
Yes	5	0.0235	0.0029

Migratory status did not influence feather weight significantly, either by itself or in combination with S. aureus presence/absence (Table 3). Overall weights were very similar between migratory and resident birds in either the presence or absence of *S. aureus* (Table 2).

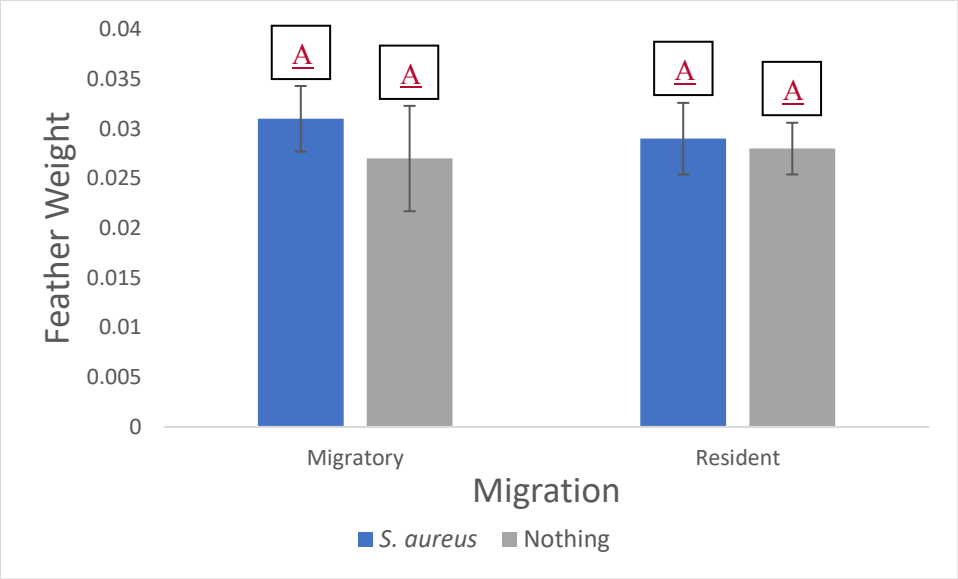

FIGURE 4. *Rectrix weight was tested after 14 ± 3 days in the presence of* Staphylococcus aureus *and without the presence of bacteria. All rectrices came from a variety of birds; some are migrants to Costa Rica, and others are residents. The migrants are traveling from North America to Costa Rica where it is warmer. Feathers were taken from 12 birds caught in the premontane tropical wet forest of Monteverde, Costa Rica. Bars with differing letters indicate a significant difference in post-hoc pairwise comparisons between means according to Tukey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model. This shows a difference in weight between a presence and absence of* S. aureus *and a control for each rectrix type. Means are presented with ± 1 standard error. Migratory birds n=4, resident birds n=8.*

TABLE 2. *Results of a LMM* on *rectrix feather weight was tested in relation to* Staphylococcus aureus *presence and migration status. The weight of rectrices were tested after 14 ± 3 days with and without the presence of the keratin-degrading bacteria* S. aureus*. Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica.*

Bacteria	Migration	Mean	SE
No	Resident	0.0280	0.0026
No	Migratory	0.0265	0.0053
Yes	Resident	0.0291	0.0036
Yes	Migratory	0.0305	0.0033

TABLE 3. *Results of a LMM on rectrix feather weight was tested in relation to* Staphylococcus aureus *presence, migration status, rectrix type, and two-way interactions variables. The weight of rectrices were tested after 14 ± 3 days with and without the presence of the keratin-degrading bacteria* S. aureus*. Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica.*

Weight	Chi-Squared	DF	P-value
Bacteria	0.618	1	0.432
Migration	0.322	1	0.570
Rectrix	4.529	2	0.104

Bacteria:Migration	1.021	1	0.312
Bacteria:Rectrix	2.933	2	0.231

Experiment 2: Visual degradation

Migration had a significant impact on the ranking of feather degradation in response to *S. aureus* presence (Table 6). Migratory birds in the presence of *S. aureus* has the greatest average feather degradation index (FDI) (Figure 5, Table 4).

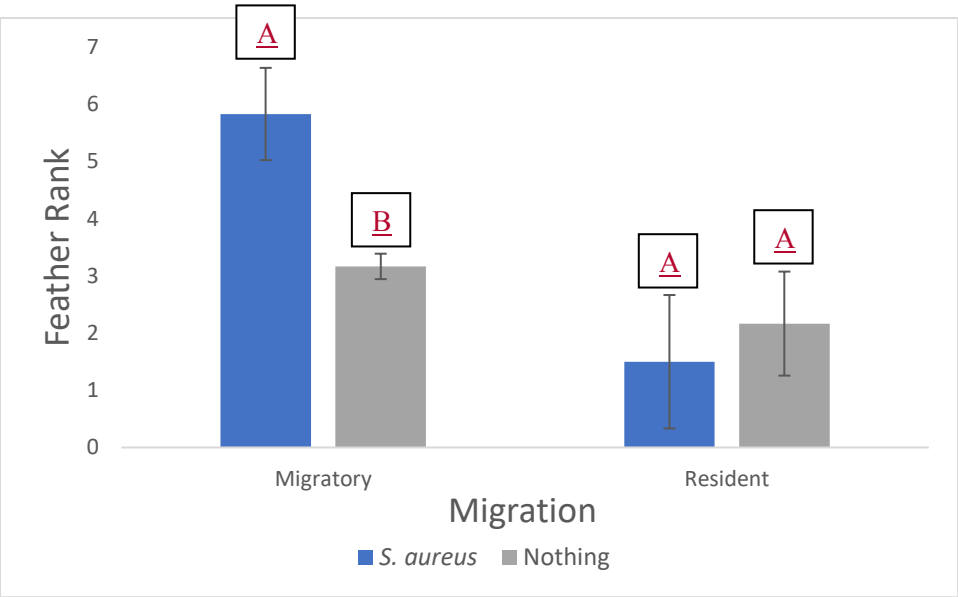

FIGURE 5. *Rectrix degradation ranking with the feather degradation index (FDI) was tested after 14 ± 3 days in the presence and absence of* Staphylococcus aureus. *Feathers were taken from 12 birds caught in the premontane tropical wet forest of Monteverde, Costa Rica. Means are presented with ± 1 standard error. Bars with differing letters indicate a significant difference in post-hoc pairwise comparisons between means according to Tukey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model. This shows a difference between feather degradation in the presence and absence of* S. aureus *and a control for resident and migratory birds. Rectrix type 2 n=6, rectrix type 3 n=2, rectrix type 5 n=4.*

TABLE 4. *Results of a LMM on visual degradation of rectrices was tested in relation to* Staphylococcus aureus *presence and migration status. The level of rectrix degradation was tested after 14 ± 3 days with and without the presence of the keratin-degrading bacteria* S. aureus. *Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica.*

Bacteria	Migration	Mean	SE
No	Resident	2.1666	0.9098
No	Migratory	3.1666	1.2225
Yes	Resident	1.5000	0.8062
Yes	Migratory	5.8333	1.6667

Rectrix type influenced degradation due to presence of *S. aureus* significantly (Table 6). Rectrix type 5 had the greatest amount of feather degradation in the presence of *S. aureus,* but there was no difference in degradation in the other rectrix types without and without the presence of *S. aureus* (Figure 6, Table 5).

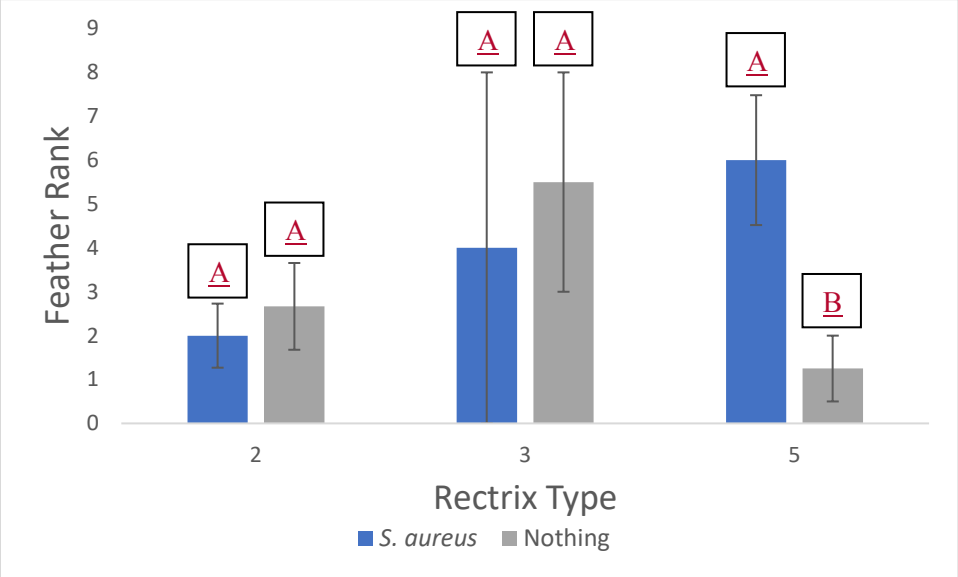

FIGURE 6. *Rectrix degradation ranking with the feather degradation index (FDI) after 14 ± 3 days in the presence and absence of* Staphylococcus aureus. *Feathers were taken from 12 birds caught in the premontane tropical wet forest of Monteverde, Costa Rica. Means are presented with ± 1 standard error. Bars with differing letters indicate a significant difference in post-hoc pairwise comparisons between means according to Tukey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model. Migratory birds n =4, resident birds n=8.*

TABLE 5. *Results of a LMM on visual degradation of rectrices was tested in relation to* Staphylococcus aureus *presence and rectrix type. The level of degradation in different rectrix types was tested after 14 ± 3 days with and without the presence of the keratin-degrading bacteria* S. aureus. *Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica.*

Bacteria	Rectrix	Mean	SE
No	2	2.6667	0.9888
No	3	5.5000	2.5000
No	5	1.2500	0.7500
Yes	2	2.0000	0.7303
Yes	3	4.0000	4.0000
Yes	5	6.0000	1.5811

TABLE 6. *Results of a LMM on visual degradation of rectrix feather was tested in relation to* Staphylococcus aureus *presence, migration status, rectrix type, and two-way interaction variables. The level of degradation rectrices underwent were tested after 14 ± 3 days with and without the presence of the keratin-degrading bacteria* S. aureus. *Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica.*

Rank	Chi-Squared	DF	Pr
Bacteria	1.167	1	0.28
Migration	4.352	1	0.037
Rectrice	1.179	2	0.555
Bacteria:Migration	5.41	1	0.02
Bacteria:Rectrice	12.754	2	0.001

Ranking of feather degradation of different rectrices under different variables were scored using the FDI and was seen to be statistically significant in the interaction between bacteria and migration, and bacteria and rectrix type. However, the other variables tested for their effect on feather degradation did not yield significant results.

Experiment 3: Inhibition of bacterial growth

Distance from closest *S. aureus* colony to the edge of rectrix did not demonstrate statistically significant results in any of the variables tested or in the interactions between them (Table 7). The interaction between rectrix type and *S. aureus* inhibition, however, has a marginal nonsignificant interaction as type 2 rectrices had a wider ring of inhibition (Table 8). Diet did not yield significant results regarding *S. aureus* inhibition (Table 9). Migration status did not yield significant results regarding *S. aureus* inhibition (Table 10).

TABLE 7. *Results of a LMM on rectrix inhibition of bacterial growth was tested in relation to* Staphylococcus aureus *presence, rectrix type, migration status, two-way interaction variables, and diet. The distances between rectrix edge and the closest* S. aureus *colony were tested after 14 ± 1 days in the presence of the keratin-degrading bacteria* S. aureus*. Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica.*

Distance (mm)	Sum Squared	DF	F-value	P-value
Diet	0.0714	3	0.5337	0.668
Migration	0.0562	1	1.2599	0.287
Rectrix	0.2204	2	2.4705	0.126
Diet:Migration	0.2157	2	2.4178	0.131
Diet:Rectrix	0.0055	2	0.0620	0.940
Migration:Rectrix	0.0122	1	0.2739	0.610

TABLE 8. *Results of a LMM on rectrix inhibition of bacterial growth was tested in relation to* Staphylococcus aureus *presence and rectrix type. The distance between rectrix edge and the closest* S. aureus *colony were tested after 14 ± 1 days in the presence of the keratin-degrading bacteria* S. aureus*. Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica. Rectrix type 2 n=6, rectrix type 4 n=2, rectrix type 5 n=16.*

Rectrix	Mean (mm)	SE
2	1.5708	0.8197

4	0.0750	0.0750
5	0.5094	0.2371

TABLE 9. *Results of a LMM* on *rectrix inhibition of bacterial growth was tested in relation to* Staphylococcus aureus *presence and diet type. The distance between rectrix edge and the closest* S. aureus *colony were tested after 14 ± 1 days with and without the presence of the keratin-degrading bacteria* S. aureus*. Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica. Frugivores n=5, insectivores n=13, nectivores n=3, omnivores n=3.*

Diet	Mean (mm)	SE
Frugivore	1.2250	0.7942
Insectivore	0.7539	0.3941
Nectivore	0.0833	0.0833
Omnivore	0.5167	0.0583

TABLE 10. *Results of a LMM* on *rectrix inhibition of bacterial growth was tested in relation to* Staphylococcus aureus *presence and migration status. The distance between rectrix edge and the closest* S. aureus *colony were tested after 14 ± 1 days with and without the presence of the keratin-degrading bacteria* S. aureus*. Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica. Resident birds n=16, migratory birds n=8.*

Migration	Mean (mm)	SE
Resident	0.8141	0.3785
Migratory	0.5875	0.2949

Additional Observations

Six days after initial plating, many feathers were seen growing filamentation that extended from the feather onto the plate. Not only this, but other bacterial colonies were growing from the feather at the same time as the filamentation and continued to do so until they were tested for weight and FDI. Mold was also seen growing on all of the feathers of White-eared Ground Sparrows, the Orange-billed Nightingale Thrush, and the Yellowish Flycatcher.

DISCUSSION

The results of this study demonstrate two main findings: (i) rectrices closer to the uropygial gland do not experience degradation to the same extent as distant rectrices, and (ii) rectrices of migratory birds degrade with exposure to keratinolytic *S. aureus*. In the case of position in relation to the gland, rectrices located farther from the uropygial gland undergo greater degradation than inner rectrices, which is also consistent with the nonsignficant trend that inner rectrices seem to inhibit bacterial growth more than outer ones. This pattern supports the hypothesis that proximity to the uropygial gland leads to greater bacterial inhibition as there is a higher probability of oil reach. Uropygial oil is secreted through a hormonally mediated process

and then is distributed throughout the rest of the feathers through preening (Blanco & Frías 2001). Feathers near the gland would likely receive a full coating of antibacterial oil whereas the others would depend on the bird's preening to avoid bacterial degradation. The efficiency of preening in distribution of oil is, in turn, questioned as there is variation in oil amount among feathers.

In the case of difference between migratory and resident birds, the rectrices of migratory birds sustain significantly more degradation in the presence of *S. aureus* than without its presence. This result was not matched by weight changes due to the methodological problems mentioned above. Bacterial plumage communities vary in geographic locations due to humidity, temperature, and other environmental conditions (Bisson *et al.* 2007). Therefore, birds encounter new bacteria as they migrate from a temperate area to a tropical zone like Monteverde in the winter. They come into contact with many new bacteria as they must make multiple rest stops on the way to their final destination and because each site contains a different variety of keratinolytic bacteria (Bisson *et al.* 2007). As uropygial oil has antibacterial properties, this suggests the oil may be specific in protection against native bacteria with which it is accustomed and less so with foreign bacteria that birds pick up on the way to their final destination. Because migratory birds underwent large amounts of degradation in the presence of *S. aureus,* it can be assumed that the birds are unfamiliar with this particular bacterium and do not have specific protection against it. Variation in plumage microbiota and the means for birds to protect themselves against it may result from differences in uropygial oil composition caused by migration-associated geographic changes (Bisson *et al.* 2009).

Tropical birds have a low basal metabolic rate which contributes to their long lifespan (Tieleman *et al.* 2005, Wiersma *et al.* 2007). They do not need to expend energy into migration as they live at a slow pace in the tropics their entire life and instead use their energy to invest in immunity against keratin-degrading bacteria to secure future opportunities to reproduce (Tieleman *et al.* 2005, Wiersma *et al.* 2007). In contrast, migratory birds have a faster pace of life, moreover a shorter life, and do not put as much emphasis on bacterial immunity (Wiersma *et al.* 2007). Therefore, migratory birds are at greater risk of feather degradation by bacteria as they frequently forage on the ground where many keratinolytic bacteria are commonly found and against which the birds do not have specific immunities (Holmgren & Hedenström 1995, Verea *et al.* 2014).

Not only this, but degradation begets further degradation and may be related to birds' physical effort. As migratory birds travel long distances and have an increased locomotor activity, their feathers become worn down (Ruiz-de-Castañeda *et al.* 2015). Feathers that are already damaged are more likely to become increasingly degraded when they come into contact with keratin-degrading bacteria (Ruiz-de-Castañeda *et al.* 2015). This further reinforces the feather degradation of migratory birds, which poses a problem as they initially are without immunity and are more susceptible to bacteria. The differences between migratory bird rectrices degradation and resident rectrices degradation suggests that there are differences in the properties of uropygial oil in different bacterial environments. In addition, knowing that migratory birds are more at risk of feather degradation from foreign bacteria may lead to a better understanding of

how birds may need more protection in the future as climate change will force birds to migrate to new locations. These new locations will have a different array of bacteria that the birds' uropygial oil is unaccustomed to, and therefore, the birds may have a less effective line of protection against feather degradation (Bisson *et al.* 2007).

Antibacterial specificity poses a problem as many birds molt annually or biannually before migration to ensure the best feather quality (Holmgren & Hedenström 1995). Regarding the activity of keratinase-bearing bacteria, it is possible that substantial feather degradation could decrease the fitness of migratory birds as they expend a substantial amount of energy into molting before leaving for their destination (Holmgren & Hedenström 1995). If feathers are more prone to degradation from the bacteria encountered throughout migration, then the birds would have to survive the rest of the year with incompetent feathers as there would be no other chance to regrow the feathers until the following year (Holmgren & Hedenström 1995). However, it is unknown if uropygial oil affects fitness as current studies have found that there are varying effects on bird reproduction and survival in response to the presence and absence of uropygial oil (Moreno-Rueda 2017). The impacts of uropygial oil on bird fitness demand further study in a natural context to see if oil does, in fact, directly affect flight through the reduction of feather degradation.

The level of degradation from *S. aureus* was tested in relation to bird migration status and rectrix type, while the inhibition of *S. aureus* also tested for bird diet. Diet did not have a significant impact on either of the two, which is surprising as some fruits and seeds that birds consume have antimicrobial properties (Ao *et al.* 2008, Baydar *et al.* 2004). There may be a minimum amount of antimicrobial food that birds must eat before exhibiting any signs of bacterial inhibition, which could explain diet's lack of effect on *S. aureus.* As many birds with different natural histories were mixed together to test for diet, more specific tests would be needed to isolate the effect of diet from other confounding factors. For instance, samples from multiple species would be needed to separate the effect of migration from feather type from diet.

The newfound knowledge gained from this study can yield itself towards benefitting the bird population at large and applying itself in future medicines. As previously mentioned, *S. aureus* has the ability to deteriorate the keratin in human fingernails and cause skin infections (Gong *et al.* 2006). Methicillin-resistant *S. aureus* (MRSA) was studied as it was the leading cause of skin and soft tissue infections in the United States (Klevens *et al.* 2007). The antimicrobial properties of uropygial oil could be applied in pharmaceutical lotions against MRSA and other bacteria-induced illness as it has already been used in topical lotions used to treat some skin disorders (Evers *et al.* 2014).

It was unknown before what factors influenced birds' ability to inhibit degradation caused by keratinolytic bacteria, and it was discovered that migratory status and rectrix type have significant roles in reducing feather degradation. Rectrices that are near the uropygial gland degrade less than rectrices further from the gland in the presence of *S. aureus*. Additionally, rectrices of migratory birds degrade more in the presence of *S. aureus* than resident birds. However, the ongoing debate on whether uropygial oil is an active inhibitor of keratin-degrading bacteria like *S. aureus* or a physical barrier that prevents feather degradation still persists. As this

study demonstrated in support of past studies, uropygial oil on the feathers prevents feather degradation. As results from the inhibition of bacterial growth test were not conclusive, it remains unknown in what ways uropygial oil prevents feather degradation.

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APPENDIX 1.

Rectrices from 12 birds sampled in the changes in weight test and the visual degradation test are categorized into species, family, migration status, and rectrix type. Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica.

#	Species	Family	Migration	Rectrix
1	BlackheadedNightingaleThrush	Thrush	No	5
2	OlivaceousWoodcreeper	Woodcreeper	No	2
3	OliveSidedFlycatcher	Flycatcher	Yes	6
4	OrangeBilledNightingaleThrush	Thrush	No	2
5	RuddyWoodcreeper	Woodcreeper	No	5
6	SwainsonsThrush1	Thrush	Yes	2
7	SwainsonsThrush2	Thrush	Yes	5
8	SwainsonsThrush3	Thrush	Yes	3
9	VioletSabrewing	Hummingbird	No	3
10	WhiteEaredGroundSparrow1	Sparrow	No	2
11	WhiteEaredGroundSparrow2	Sparrow	No	2
12	WhiteThroatedThrush	Thrush	No	2

APPENDIX 2.

Rectrices from 24 birds sampled in the bacterial inhibition test are categorized into species, family, migration status, rectrix type, and diet. Feathers were taken from birds caught in the premontane tropical wet forest of Monteverde, Costa Rica.

#	Species	Family	Material	Migration	Rectrix	Diet
1	BlackHeadedNightingaleThrush2	Thrush	Feather	Resident	5	omnivore
2	CopperyHeadedEmerald	Hummingbird	Feather	Resident	4	nectivore
3	GoldenCrownedWarbler	Warbler	Feather	Resident	4	insectivore
4	GoldenWingedWarbler	Warbler	Feather	Migratory	2	insectivore
5	GrayBreastedWoodWren	Wren	Feather	Resident	5	insectivore
6	GreyCheekedThrush1	Thrush	Feather	Migratory	5	frugivore
7	KentuckyWarbler	Warbler	Feather	Migratory	5	insectivore
8	LongTailedManakin	Manakin	Feather	Resident	5	frugivore
9	Ovenbird	Warbler	Feather	Migratory	5	insectivore
10	PurpleThroatedMountainGem	Hummingbird	Feather	Resident	5	nectivore
11	RuddyWoodcreeper2	Woodcreeper	Feather	Resident	5	insectivore
12	RufousAndWhiteWren	Wren	Feather	Resident	2	insectivore
13	SlateThroatedRedstart	Warbler	Feather	Resident	2	insectivore
14	SlatyAntwren	Antwren	Feather	Resident	2	insectivore
15	SootyFacedFinch	Finch	Feather	Resident	5	omnivore
16	StripedTailHummingbird	Hummingbird	Feather	Resident	5	nectivore
17	SwainsonsThrush4	Thrush	Feather	Migratory	2	frugivore
18	SwainsonsThrush5	Thrush	Feather	Migratory	2	frugivore
19	SwainsonsThrush6	Thrush	Feather	Migratory	5	frugivore
20	WhiteBreastedWoodWren	Wren	Feather	Resident	5	insectivore
21	WhiteEaredGroundSparrow3	Sparrow	Feather	Resident	5	insectivore
22	WhiteThroatedSpadebill	Flycatcher	Feather	Resident	5	insectivore
23	Woodthrush	Thrush	Feather	Migratory	5	omnivore
24	YellowishFlycatcher	Flycatcher	Feather	Resident	5	insectivore

Antibiotic resistant enterohemorrhagic *Escherichia coli* bacteria in rural Costa Rican cloud forest stream systems.

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Abstract

There has been a rise in antibiotic resistant (ABR) bacterial strains in recent decades due to overuse of medically important antibiotics. This poses a threat to public and environmental health as bacterial infections become increasingly difficult to treat and ABR genes enter natural systems. However, there is little research detailing how regular antibiotic pollution from agriculture correlates with ABR of water born pathogenic bacteria in natural systems. Enterohemorrhagic *Escherichia coli* (EHEC) are among the most common enteropathogenic bacteria, the most common serotype being O157:H7. I tested for ABR O157:H7 in farm runoff water (FRW) and farm adjacent streams (FAS) in the Monteverde region of Costa Rica, identifying the drug types and the number of drugs resisted. Antibiotic use in agriculture is common in this rural area, and previous studies have identified increased ABR in the local aquatic bacterial populations. I was able to identify ABR O157:H7 in the farm runoff and stream water samples collected and found a significant difference between the drug types resisted by bacteria from different collection sites (Cattle/pig farm vs Pig farm, $p=0.0147$) and between the number of drugs resisted in samples from FRW and FAS ($p=0.0280$). No significant difference was observed between the drug types resisted between FRW and FAS, or between the number of drugs resisted between sites (Cattle/pig farm vs Pig farm). These results show that drug types resisted remain consistent between farm runoff and adjacent streams, suggesting a direct causal relationship. However, multi drug resistance is comparatively reduced in stream populations.

Resumen

La resistencia antibiótica de sepas bacterianas ha aumentado en las décadas recientes, debido al sobreuso de antibióticos médicamente importantes. Esto resulta un peligro para la salud pública y ambiental ya que las infecciones bacterianas se vuelven cada vez más difíciles de tratar y los genes ABR entran en los sistemas naturales. Sin embargo, existen pocos estudios que detallen como la contaminación por antibióticos provenientes de la agricultura se correlaciona con el ABR de bacterias patógenas que nacen en el agua de los sistemas naturales. La enterohemorraica *Escherichia coli* (EHEC) es una de las bacterias enteropatógenas más comunes, siendo el serotipo más común O157:H7. Realicé la prueba de ABR O157:H7 en las aguas de escorrentía de granjas (FRW) y en los ríos cercanos a estas granjas (FAS) en la región de Monteverde en Costa Rica, identificando los tipos de droga y el número de drogas resistentes. El uso de antibióticos en la agricultura es común en áreas rurales y estudios previos han identificado un incremento en el ABR de las poblaciones bacterianas acuáticas locales. Pude identificar ABR O157:H7 en las muestras de aguas de escorrentía y en las de los ríos cercanos a las granjas y encontré una diferencia significativa entre los tipos de drogas que resisten las bacterias en los diferentes sitios de recolecta (ganado/porqueriza vs porqueriza, $p=0.0147$) y entre el número de drogas resistentes en las muestras de FRW y FAS ($p=0.0280$). No obtuve diferencias significativas entre los tipos de drogas resistentes entre FRW y FAS, o entre el número de drogas resistentes entre sitios (ganado/porqueriza vs porqueriza). Estos resultados muestran que los tipos de drogas resistentes permanecen consistentes entre las aguas de escorrentía de las granjas y los ríos adyacentes, sugiriendo una relación causal directa. Sin embargo, la resistencia a múltiples drogas es comparativamente reducida en las poblaciones de los ríos.

Introduction

Antibiotic overuse has caused a recent emergence and proliferation of antibiotic resistant bacteria (ABR) strains (Witte 1998). This poses a threat to public and environmental health. Infectious bacterial diseases are becoming increasingly difficult to treat and ABR genes are entering natural systems. In the United States, nearly 70% of the total volume of medically important antibiotics are sold for agricultural purposes (US Food and Drug Administration 2015). Global antibiotic use is projected to increase 67% by 2030, largely due to the spread of US meat production practices to middle income nations (Thomas et al 2015). These drugs are administered primarily for animal growth promotion and disease prevention (Graham et. Al 2007), livestock excreting 25 to 75% of the drugs they consume (Borghi and Palma 2014). If handled improperly, ARB infected animal waste can enter aquatic systems where it contaminates natural water sources (Zhang et al. 2009).

Pathogenic bacteria species that easily accumulate ABR genes and persist outside of a host present a unique risk. When ingested, antibiotics exert selective pressure on endogenous bacteria, thus pushing the evolution of resistant strains (Drusano et al. 1993). Increased ABR in agricultural waste can cause increased ABR of pathogenic bacteria in several ways (Chang et al. 2014). Direct disease transmission from livestock can occur if a pathogenic bacterial species infects multiple host species (Armand-Lefevre et al. 2005). Bacterial species can "jump" from livestock host to a human one through mutation (Spoor et al. 2013). Lastly, ABR bacteria often transfer resistance genes to more virulent strains through lateral gene transfer (*Kobashi et. al, 2007*). This presents a unique risk as large reservoirs of ABR bacteria can theoretically transfer ABR genes to pathogenic strains for years (*Kobashi et. al, 2007*).

After rotavirus, pathogenic *Escherichia coli* is the largest contributor to diuretic illness (Perez & Gomez-Duarte 2010). The majority of *E. coli* strains live symbiotically within the lower guts of birds and mammals; however, some serotypes have evolved to be pathogenic (*Nataro et. al, 2011*). O157:H7 is the most common strain responsible for intestinal disease amongst humans. While adapted to feed on sugars within herbivore digestive tracts, colonies can survive and proliferate in water sources contaminated with livestock waste (*Baquero et. al, 2008*). This serotype is enterohemorrhagic in humans, causing legions to form on the lower intestine of infected hosts (*Nataro et. al, 2011*).

The identification of ABR pathogenic bacteria also has major ecological implications. Previous research has identified antibiotic resistant genes in seemingly pristine lake systems in the tropics, where pathogenic and symbiotic bacterial species may live (Pontes et al. 2009). One study conducted near a pig farm in Ontario identified ABR bacteria in the digestive tracts of nearly all of the surrounding wildlife (Kozak et al. 2008). This should be of growing concern, as the implications of ABR coliform and pathogenic bacteria entering natural systems are poorly understood.

My study tests for ABR in the O157:H7 enterohemorrhagic *E. coli* (EHEC) in the Monteverde cloud forest region of Costa Rica. I will then identify the drug types resisted and the number of drugs resisted by cultured strains. Similar studies have identified multidrug resistant pathogenic *E. coli* living in stream systems throughout the tropics (Ramirez et al 2013, Kabiru et al 2015, Dhawde et al. 2018), however few have been conducted in Costa Rica. The National

Children's Hospital of Costa Rica in San Jose reports over 5,000 diarrheal cases per year caused by *E. coli*, yet there have been no studies to date identifying the sources of these infections. In this agricultural community, regular antibiotic use on livestock has resulted in antibiotic resistant bacteria near runoff streams (De la Cruz et. al 2014, Ordemann 2017,). Coliform bacteria have also been identified in the streams of Monteverde (Paliwoda 2015). There are several studies documenting ABR of bacteria in the Monteverde cloud forest, however the pathology of the cultured bacteria has not been explored. This is of special concern given the at-risk population and the unique ecological diversity of the area.

Methods

Study Sites

Three sample sites were used in this study. This first is property of Erick Rockwell, a pig/cattle farm located in Monteverde Costa Rica near the Cheese Factory (Cattle/pig farm). Farm water samples were taken from the water runoff that drained from the barn after the pens were washed (FRW). Stream water samples were taken from a stream adjacent to the farm. All stream water samples were collected downstream from the farm (FAS). Antibiotic resistant bacteria were previously identified along multiple points of this stream (Nixon 2018). The second site sampled was the property of Jose Louis, a small pig farm several km outside of the town of Monteverde (Pig farm). FRW samples were taken from the water runoff that drained after the pens were washed and from the biodigester below the pens that treats said runoff. FAS samples were taken from a stream directly below the farm. The third site sampled was a control stream in primary forest above the Monteverde Biological Station. This stream does not receive runoff from any human development. Samples were collected in October of 2018, near the end of the dry season.

Procedure

Ten mL of water were collected per sample in sterilized vials. Seven samples were collected from the barn runoff and seven samples were collected from the adjacent stream at both farms. Thirteen water samples were collected from the unpolluted stream at the Biological Station.

Waterworks 481197-1 Hydrogen sulfide strips were chosen to detect coliform bacteria. Test strips measuring sulfide contents in ppm were used. Two 10 mL samples were tested from each site. A sample was tested from the barn runoff of each farm site and from each farm adjacent stream site. At the control site, one water sample was tested. Ten drops of peptone and a testing strip were added to each sample. Strips were allowed to sit in the peptone mixture for 24 hours. Strips positive for H2S turn from white to a darker brown. All strips that change color were recorded (Paliwoda 2015).

The remaining 36 samples were plated on standard 100 mm x 15mm petri dishes. A Sorbitol MacConkey agar (SMAC) was used as the medium for culture growth. SMAC is a selective medium for *E. coli*. Sorbitol metabolizing strains of *E. coli* increase the pH of their environment, causing and pH indicator in the dye to turn the colony pink. O157:H7 are unable to metabolize sorbitol, thus grow in opaque non-colored colonies (March & Ratnam, 1986).

Each of the remaining 36 water sample were plated on 2 petri dishes to test for antibiotic

resistance. The three antibiotics tested in this experiment were Penicillin, Gentamicin, and Oxytetracycline. These three antibiotics were chosen because of their use in previous ABR studies in Monteverde (Nixon 2018, Ordemann 2017). The first of the two plates was a negative control with no antibiotics. The second was divided into thirds. Each third was covered with 3 ul of one antibiotic after the culture was plated (1 ul per 8.80 cm2). Plates were allowed to sit for 24 to 36 hours in a container varying from 18 to 30ºC. After that period, I recorded the presence of O157:H7 *E. coli* on each plate, the drug types resisted by each sample, and number of drugs resisted by each sample.

Results

Each H2S strip tested positive for coliform bacteria, including the one testing stream water from the undisturbed primary wet forest. EHEC was identified at both the Cattle/pig and the Pig farm (**Table 1**). While *E. coli* was cultured from the undisturbed primary forest stream samples, No EHEC was identified. None of the Unpolluted stream cultures displayed any level of antibiotic resistance (**Table 1**). My data was analyzed using Fisher Exact Tests. I first tested for variation in the drug types resisted by cultured O157H7 between sites (Pig Farm FRW + FASW vs Cattle/Pig farm FRW+ FASW) X (Penicillin vs Gentamicin vs Oxytetracycline) (p= 0.0147) (**Table 1**). Secondly, I tested for variation between the drug types resisted by cultured O157:H7 between FRW and FASW (Pig farm FRW+ Cattle/Pig farm FRW vs Pig farm FASW + Cattle/Pig farm FASW) X (Penicillin vs Gentamicin vs Oxytetracycline) (p= 0.132) (**Table 1**).

Table 1. Antibiotics types resisted by each sample type. Rows represent the different sample collection sites. Columns represent results for each site: 1. The number of water samples collected from each site. 2. The number of those water samples which tested positive for E. coli. 3. The number of water samples from which O157:H7 were cultured. 4. The number of water samples from which ABR O157:H7 were cultured. 5. The number of water samples from which Penicillin resistant O157:H7 were cultured. 6. The number of water samples from which Gentamicin resistant O157:H7 were cultured. 7. The number of water samples from which Oxytetracycline resistant O157:H7 were cultured.

Sample site and Type	# samples tested	# samples containing *E. coli*	# samples containing O157:H7	# samples with ABR EHEC	# samples resisting Penicillin	# samples resisting Gentamicin	# samples resisting Oxytetracycline
Cattle/Pig FRW	6	6	6	6	6	2	0
Cattle/Pig FAS	6	6	5	3	3	3	0
Pig FRW	6	6	6	6	5	5	6
Pig FAS	6	6	5	5	5	0	4
Undisturbed primary stream	12	12	0	0	0	0	0

Third, I tested for variation between the number of drugs resisted by cultured O157:H7 between sites (Pig Farm FRW + FASW vs Cattle/Pig farm FRW+ FASW) X (0 drugs vs 1 drug vs 2 drugs vs 3 drugs) (p= 0.332) (**Table 2**). Lastly, I tested for variation between the number of drugs resisted by cultured O157:H7 between FRW and FASW (Pig farm FRW+ Cattle/Pig farm FRW vs Pig farm FASW + Cattle/Pig farm FASW) X (0 drugs vs 1 drug vs 2 drugs vs 3 drugs) (p= 0.0280) (**Table 2**).

Table 2. Number of Antibiotics resisted by each sample type. Rows represent the different sample collection sites. Columns represent results for each site: 1. The number of water samples collected from each site. 2. The number of those water samples which tested positive for E. coli. 3. The number of water samples from which O157:H7 were cultured. 4. The number of water samples from which ABR O157:H7 were cultured. 5. The number of water samples from which O157:H7 resistant to one antibiotic were cultured. 6. The number of water samples from which O157:H7 resistant to two antibiotics were cultured. 7. The number of water samples from which O157:H7 resistant to three antibiotics were cultured.

Sample site and Type	**# samples tested**	**# containing *E. coli***	**# containing O157:H7**	**# ABR EHEC**	**# resisting 1 antibiotic**	**# resisting 2 antibiotics**	**# resisting 3 antibiotics**
Cattle/Pig FRW	6	6	6	6	4	2	0
Cattle/Pig FAS	6	6	5	3	0	3	0
Pig FRW	6	6	6	6	0	2	4
Pig FAS	6	6	5	5	1	4	0
Undisturbed primary stream	12	12	0	0	0	0	0

Discussion

My results for my H2S test would suggest that animal waste was entering the undisturbed primary forest stream systems. I also cultured non-pathogenic E. coli from this site, despite there being no agricultural runoff entering this site. It is important to note that fecal coliforms are commonly found in pristine environments and are often regarded as inadequate for determining the presence of coliform bacteria pollution (Hanzen 1988). Despite this, my results provide further evidence that antibiotic overuse and improperly managed agricultural runoff contributes to the development of ABR pathogenic bacteria in adjacent water systems. ABR O157:H7 colonies were identified in both of the farm sites tested (**Table 1**). ABR bacteria have been found to leach into the environment from agricultural installments in past studies (Zhang et. Al 2009), largely to the detriment of public and ecosystem health.

Of the antibiotics tested, Penicillin was the most resisted, possibly due to widespread use. These results are consistent with previous research into ABR in the Monteverde region (Nixon 2018). However, there was significant difference between drug types resisted by EHEC between sites (Cattle/pig farm vs Pig farm, **Table 1**). This is also consistent with previous Monteverde ABR research (Nixon 2018). There was significant variation between the number of drugs

resisted farm runoff cultures and adjacent stream cultures, FRW colonies having higher multidrug resistance. (**Table 2**). There is likely reduced selective pressure for ABR in streams due to lower comparative concentration of antibiotics. ABR genes often reduce bacterium fitness and are selected against in antibiotic depleted environments (Melnyk et al 2014). Despite being deleterious, ABR genes will persist if an environment receives continued antibiotic exposure (Maisnier-Patin & Anderson 2004).

Other ABR EHEC studies verify the presence of pathogenic *E. coli* through Shiga toxin gene PCR (Paton & Paton 1998). Shiga toxins are selectively produced by pathogenic serotypes of *E. coli*, including the EHEC serotype O157:H7. Shiga toxin immunoassays are also often used, however are generally less accurate than PCR verification (Kabiru et al 2015). PCR could also be used to confirm whether O157:H7 released into streams are developing ABR inside their livestock host vectors or in the streams they enter due to subsequent antibiotic pollution. The streams tested in this study likely receive runoff from farms throughout the area, and likely receive *E. coli* strains and antibiotics from multiple source points. It is also possible that the EHEC in the polluted streams systems are receiving ABR genes from benign *E. coli* serotypes via horizontal gene transfer (*Kobashi et. al, 2007*)., although this is more difficult to determine through phylogenetic analysis.

There is currently little research into how ABR coliform and pathogenic bacteria affect the ecosystems they enter. Multiple studies have documented ABR genes and ABR coliform bacteria entering tropical water systems and food webs (Kozak et al. 2008, Pontes et al. 2009). ABR resistant bacteria strains have even been identified in tropical bat species in Brazil (Sens-Junior et al. 2018). However, ss increased antibiotic resistance becomes the reality, we must prepare for any and all complications that may arise, may they in human or ecosystem health.

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Compost and tropical soil organic carbon storage

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Abstract

Increasing Carbon (C) stored in soil could mitigate the changing climate. This can be done by increasing Soil Organic Carbon (SOC) through the use of compost, if compost C is captured and stored in soil. In this study, three soils were compared at the same farm from: compost-treated coffee, inorganically fertilized coffee, and a secondary forest that had never been clearcut. All were sampled at 10, 20, 30, 40, and 50 cm depth for percent SOC, bulk density (BD), and SOC/ Ha. Results showed significant differences in percent SOC and BD with depth and site. The mean SOC percentage of the compost-treated plot was 2.38% +/- 0.09, while that for inorganic fertilizer was 2.45% +/- 0.10, and 2° Forest plots had a mean of 2.91% +/- 0.13. The mean SOC percentage of 10 cm = 3.00 % +/- 0.16, 20 cm =2.79% +/- 0.13, 30 cm =2.64% +/- 0.12, 40 cm= 2.34% +/- 0.09, and 50 cm = 2.13% +/- 0.09). The mean BD (in g/cm^3) of compost = 0.74 +/- 0.03, inorganic = 0.82 +/ - 0.02, and 2° Forest = 0.66 =/- 0/03. The mean BD (in g/cm^3) were 10 cm= 0.70 +/- 0.04, 20 cm = 0.67 +/- 0.05, 30 cm= 0.81 +/- 0.02,40 cm = 00.82 +/- 0.03, and 50 cm = 0.69 +/- 0.04. SOC/Ha showed significant results only in terms of depth with means (in tons) of 10= 20.64 +/- 1.25, 20= 18.27 +/- 0.89, 30= 21.46 +/- 1.12, 40= 19.10 +/- 0.53, 50= 14.65 +/- 0.83. Although this study did not find that compost affected the storage of SOC, the similarities between inorganic and compost treated fields suggest that knowledge of the duration of application, soil composition, leaching rates, and past management strategies are essential in understanding the movement of SOC in the soil column, and its storage. Extended research could lead to an increase in C credits for belowground C.

Resumen

Aumentar el Carbon (C) guardado en el suelo puede mitigar el cambio climático. Esto se puede lograr aumentando el Carbon Orgánico en el Suelo (SOC por sus siglas en ingles) a través del compostaje, si el C compostado es capturado y guardado en el suelo. En este estudio, tres tipos de suelos fueron comparados de la misma finca: café tratado con compostaje, café fertilizado inorganicamente, y bosque secundario que nunca ha sido cortado completamente. Todos fueron muestreados a 10, 20, 30, 40, y 50 cm de profundidad por el porcentaje de SOC, la densidad aparente (BD), y SOC por hectarea. Los resultados muestran diferencias significativas en el porcentaje de SOC y la BD con la profundidad y el sitio. El porcentaje de SOC promedio de la parcela con compostaje fue 2.38% +/- 0.09, mientras que con fertilizante inorgánico fue 2.45% +/- 0.10, y el bosque secundario 2.91% +/- 0.13. El promedio de SOC a 10 cm = 3.00 % +/- 0.16, 20 cm =2.79% +/- 0.13, 30 cm =2.64% +/- 0.12, 40 cm= 2.34% +/- 0.09, y 50 cm = 2.13% +/- 0.09). El promedio de BD (en g/cm^3) para compostaje = 0.74 +/- 0.03, inorgánico = 0.82 +/ - 0.02, y bosque secundario = 0.66 =/- 0/03. El promedio (en g/cm^3) fue para 10 cm= 0.70 +/- 0.04, 20 cm = 0.67 +/- 0.05, 30 cm= 0.81 +/- 0.02,40 cm = 00.82 +/- 0.03, y

50 cm = 0.69 +/- 0.04. El SOC/Ha muestra resultados significativos en terminos de profundidad con promedios de (en tons) de 10= 20.64 +/- 1.25, 20= 18.27 +/- 0.89, 30= 21.46 +/- 1.12, 40= 19.10 +/- 0.53, 50= 14.65 +/- 0.83. Aunque este estudio no muestra que el compostaje afecta la acumulación de SOC, las similitudes entre los campos tratados inorgánico y con compostajen sugieren que el conocimiento de la duración de la aplicación, la composición del suelo, la tasa de drenaje, y las estrategias pasadas de mantenimiento son esenciales para entender el moviemiento del SOC en la columna del suelo, y su almacenamiento. Una investigación más extendida puede llevar a un aumento en los créditos por carbono para el C subterraneo.

Introduction

Carbon sequestration is a top priority in climate mitigation, however not all carbon stores are equal. Carbon (C) stored in soil (~2344 Gt in the top 3 m of soil) is second only to that stored in the ocean (38,400 Gt), and is over four times that stored above ground in terrestrial ecosystems (~560 Gt, Stockmann *et al.* 2012). As we approach the critical point in our world's atmosphere of a 2° C increase and the use of 1 trillion tons of C (i.e., burning of fossil fuels)(World Resources Institute 2018), it is important to draw on below-ground processes to increase soil C storage. Soil management can increase carbon stored in soil (West & Post 2002). For example, compost has been shown to increase soil carbon storage, at least in temperate grasslands (Ryals *et al.* 2014). However, agriculture has interrupted this.

Most human land use lowers carbon stored belowground (Guo & Gifford 2002). Much of this results from agriculture and other forms of land transformation. The conversion of forest to cropland results in a 22% loss of soil C through loss of top soil, as well as a decrease in bulk density (BD) (Murty *et al.* 2002). Cultivation of crops leads to additional losses of top soil and 20-40% of soil C (Davidson & Ackerman 1993). Losses of C in the soil ultimately results in more being emitted back to the atmosphere or ocean (Lal 2004). C can be stored when it is not being used by plants or washed away by water. This is accomplished if it is locked inside soil aggregates, it is chemically bound by microbes to soil minerals (Ryals *et al.* 2004), or decomposition is slowed (Elliott 1986; Jastrow 1996; Six *et al.* 1998, 2000).

Reduced aggregate stability is the result of disturbances (like tilling) that reduce or breakdown the amount of organic matter present. Agriculture leads to reduced aggregate stability with increased exposure to wind and rain, and pesticides can further exasperate these effects with the removal of microorganisms (Andrews & Wander 2011). Reduced aggregate stability further increases below-ground decomposition (Ogle *et al.* 2004), and increasing cultivation reduces the number of C rich macro aggregates and increases the number of micro aggregates depleted of their C content (Six *et al.* 2000).

If C is not stored, it can be emitted into the atmosphere as CO_2 through mineralization, or as CH_4 through methanogenesis (Lal 2004). As a result, soil organic C (SOC) pools have been depleted by up to 60% in temperate soils, and by more than 75% in cultivated tropical soils (Lal 2004). This loss of SOC can be responsible for decreased soil quality, reduced plant and biomass productivity, and negative effects on the water quality as well as adding to atmospheric C enrichment. This makes the Tropics a good place for this study, as they have a greater SOC depletion, and thus greater potential for C storage. In addition, the soil quality is low in the Tropics, and increasing SOC storage may result in increased crop yield (Lal 2004).

In this study, I investigate if compost increases SOC in soils at different depths. A similar study found compost increased soil carbon (Ryals *et al.* 2014), as organically-bound nitrogen from compost slows release of C and thus C is more firmly attached to the soil particle.

In one study it was found that the application of organic fertilizers and charcoal reduced nutrient leaching (Steiner *et al.* 2007). Typically, compost application increases soil aggregation and slows below-ground decomposition (Elliott 1986; Jastrow 1996; Six *et al.* 1998, 2000), further retaining that C belowground. However, organic amendments have also been shown to reduce BD (Lynch *et al.* 2005), or the weight of fiber per unit volume (Sreerama *et al.* 2009), suggesting a disconnect between BD and SOC storage.

High-input application of compost to cropland increases SOC over 20 years in the top 30 cm of soil (Ogle *et al.* 2004), and the mean residence time of SOC increases with depth as decomposition slows with depth (Fontaine *et al.* 2007). Therefore, compost can increase SOC by slowing decomposition (Elliott 1986; Jastrow 1996; Six *et al.* 1998, 2000), allowing more time for leaching to bring SOC to greater depths where it may be stored even longer as it is hidden from the effects of cultivation and the resulting reduced aggregate stability (Andrews & Wander 2011). In addition, there are the aboveground effects of compost application as well, including a direct organic C input (Eghball and Power 1999; Ryals and Silver 2013), as well as increased plant production which results in more photosynthesis and C sequestration. This increased biomass will eventually fall to the ground, and return to the soil layer.

If it was determined that the application of compost increased the storage of SOC, as well as increased the amount stored with depth, regulations and adjustments in the awarding of carbon credits could be made to equally include SOC (Lal 2004). Carbon credits are very low for SOC, only resulting in $1 per ton of CO_2 (Lal 2004). A change of total SOC by 10% would be equivalent to anthropogenic emissions from the past 30 years (Kirschbaum 2000). Therefore, compost application and its effects could have far-reaching benefits.

Materials and Methods

Sites

I examined soils from three different land uses. The first was a coffee plot treated with inorganic fertilizer (192 ft. x 64 ft.), the second a coffee plot treated with compost (192 ft. x 130 ft.), and the third plot a secondary (2◦) forest that was never converted to agriculture. All three plots were located at *Life Monteverde*. Both coffee plants were planted around seven years ago. The inorganic plot receives three rounds of synthetic fertilization each year, equaling a total of 922 kg of fertilizer per hectare per year. This fertilizer contains both a formula heavy in nitrogen and a complete formula. The compost treated plot is only 0.2 Ha, so under a typical fertilizing regime, it would only receive 184 kg of fertilizer per year. However, in 2017, it received 6810 kg of organic fertilizer, about 6 kg of organic fertilizer per plant (Daniel C. Vargas, personal communication).

Life Monteverde is located in the Cañitas area of Monteverde, Costa Rica. Monteverde is famous for its Cloud Forests and is considered a Premontane Wet Forest Life Zone at 1250 - 1800 meters. One study found the soil texture to be loamy sand in a secondary forest and composting farm, and loamy sand/sandy loam in an inorganic farm, all in the Cañitas area (Mendoza 2016). All samples taken appeared similar in color and composition. The 2° Forest had trees reaching canopy height (15-20 m), as well as a moderately dense understory with a leaf

litter layer completely covering the forest floor. Both compost and inorganic plots slanted down in an eastward direction, compost having a difference in elevation of only 3 m (1283 m & 1280 m). Inorganic had an elevational difference of 7 m (1286 m & 1279 m), and the secondary forest had an elevational difference of 10 m (1266m & 1256 m) sloping down in a southward direction (Table 1).

Table 1. The direction of the slope of each site with top of slope meaning the western side and bottom the eastern side in both the compost and inorganic sites. In the secondary forest (2° Forest), the top of slope represented the elevation of the northern side, while bottom of slope represented the southern side. All 3 sites were located at Life Monteverde, a farm located in a tropical premontane wet forest in Monteverde, Costa Rica.

Table 1. Site Slope Direction and Elevational Change			
Site	Sloping direction	Top of slope (m)	Bottom of slope
Compost	E	1283	1280
Inorganic	E	1286	1279
2° Forest	S	1266	1256

Sample Collection

Soil samples were taken from the surface to 50 cm depth in 10 cm increments for all plot types. This was done three times at each of the three plots, for a total of 15 samples from each plot category. These samples were collected with a soil core. The sample locations were adjusted to the plot size. The inorganic and compost plots were arranged into 3 x 3 quadrants. One sample point was placed in the middle of the top corner, one in the exact middle of the plot, and one in the middle of the bottom corner (Figure 1). Both of the plots sloped down in an eastward position. For the 2° Forest plot, 3 segments of forest were sampled based on the division of trails. The actual point sampled was 10m in from the trail on the 3 different segments, sloping down in a southward direction (Figure 2). The samples were tested for BD, as well as the presence of SOC (per hectare and percentage).

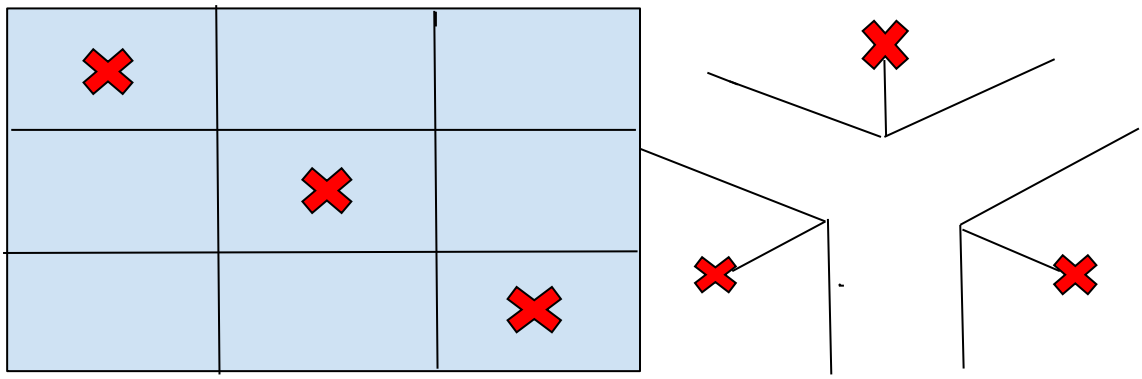


Figure 1 & Figure 2. Respectively, the dispersal of points in the coffee plots where samples were taken at 5 different depths, and the dispersal of points at 3 different sides of the trail (10 m from the trail) in the secondary

regenerated forest. All 3 sites were located at Life Monteverde, a farm in the lower premontane wet forest in the Cañitas area of Monteverde, Costa Rica.

Sample Processing

The samples were brought back to the lab at the Monteverde Biological Station. The samples collected were dried in a food dehydrator overnight at 54°C. The next day the samples were ground with a mortar and pestle to crush any large clumps of dirt before being sifted through a 2 mm soil sieve to remove gravel and root fragments to keep consistent with the methods used in Mendoza (2016). The dry weight of the soil was then determined before being ground again and sifted through a 0.15 mm sieve to prepare for the total soil organic carbon chemical analysis using the Colorimeter Method below (Baker 1976).

Organic Carbon Test (Colorimeter Method)

0.5g of soil was put into an Erlenmeyer flask. 2 mL of distilled water was pipetted into the flask, followed by 10 mL of 5% dichromatic potassium. 5 mL of 98.08% H2SO4 was then added. This solution was heated to 150°C in a water bath for 30 minutes. After the solution cooled to room temperature, 50 mL of Barium Chloride 0.4% was added. A blank solution was also made using the same chemical combinations without the addition of soil, and without any heating of the solution. These were left to sit overnight.

The next day, an aliquot of the supernatant was transferred to a colorimeter cuvette. These cuvettes were put into a spectrophotometer (200 Series, Model UV-200-RS, V-200-RS) to measure absorbance at 600 nm. These absorbance values were adjusted based on a sucrose-based standard curve for carbon concentration vs. absorbance (Figure 3; provided by Mendoza 2016 using the identical procedure).

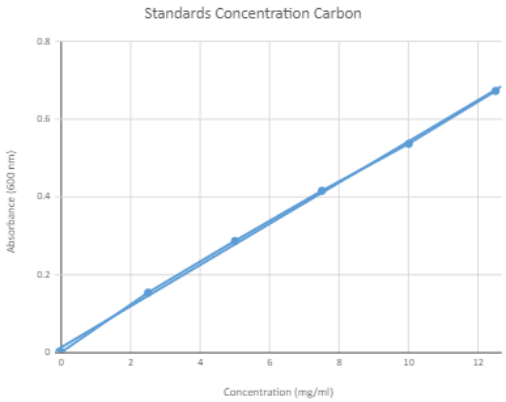


Figure 3. Standard curve for carbon concentration using a sucrose serial dilution. This was later used to determine percent organic carbon in soil samples with different land use. Percent organic carbon was determined using the equation: y= 0.053x + 0.0124 (Mendoza 2016).

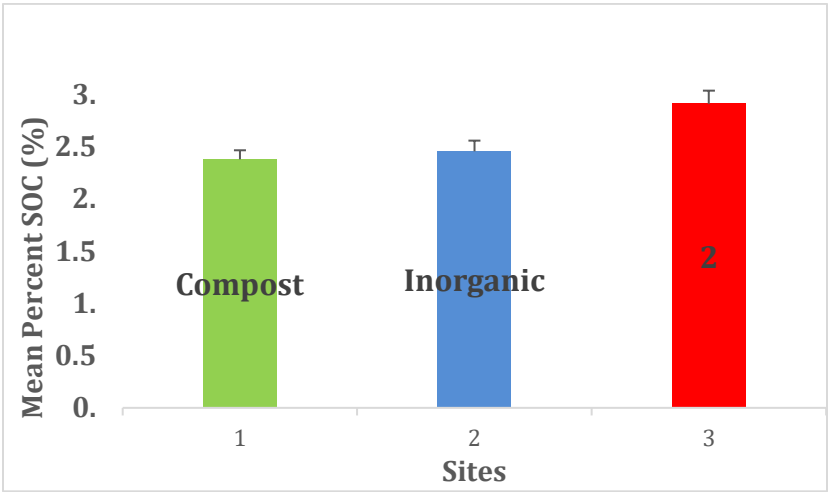
Bulk Density and Soil Organic Carbon per Hectare

BD was calculated using soil sample dry weight divided by its volume. In this case, where only 10 cm was measured out of the ground at a time, the volume measured was 31.42 cm^3. Using both the organic carbon percentage found per sample and the BD, soil organic carbon per hectare was calculated using the equation below (from Donovan 2012):

Carbon per Hectare (tons) = Th x D x C, where Th is sample thickness (10 cm), D is bulk density and C is % carbon

Results

The results showed significant statistical differences between percent SOC and site, according to the results of the two-way ANOVA (F= 17.55007, d.f. =2, p-value= 8.97616 e-6). The mean SOC percentage of the compost-treated plot was 2.38% +/- 0.09, while that for inorganic fertilizer was 2.45% +/- 0.10, and 2° Forest plots had a mean of 2.91% +/- 0.13 (Figure 4). According to the Fisher LSD test, there are significant differences ($p < 0.05$) between compost vs 2° Forest and inorganic vs 2° Forest at both 10 cm and 20 cm. Our results also found significant differences (at $p < 0.05$) for the differences between percent SOC and depth according to the results of the two-way ANOVA (F=15.56974, d.f. =4, p-value=5.32746 e-7). The mean SOC percentage of 10 cm = 3.00 % +/- 0.16, 20 cm =2.79% +/- 0.13, 30 cm =2.64% +/- 0.12, 40 cm= 2.34% +/- 0.09, and 50 cm = 2.13% +/- 0.09) (Figure 5). According to the Fisher LSD test, there are significant differences ($p < 0.05$) in 10 vs 50 cm in compost; 20 vs 50, 30 vs 50, 10 vs 50, and 10 vs 40 in inorganic; and 10 vs 30, 10 vs 40, 10 vs 50, 20 vs 40, and 40 vs 50 in the 2° Forest site. Our results showed no statistical difference for the combination of site and depth according to the two-way ANOVA (F=1.03739, d.f. = 8, p-value=0.43902) (Figure 6). To summarize, our results indicate that the % SOC decreases with depth, and is highest in the 2° Forest



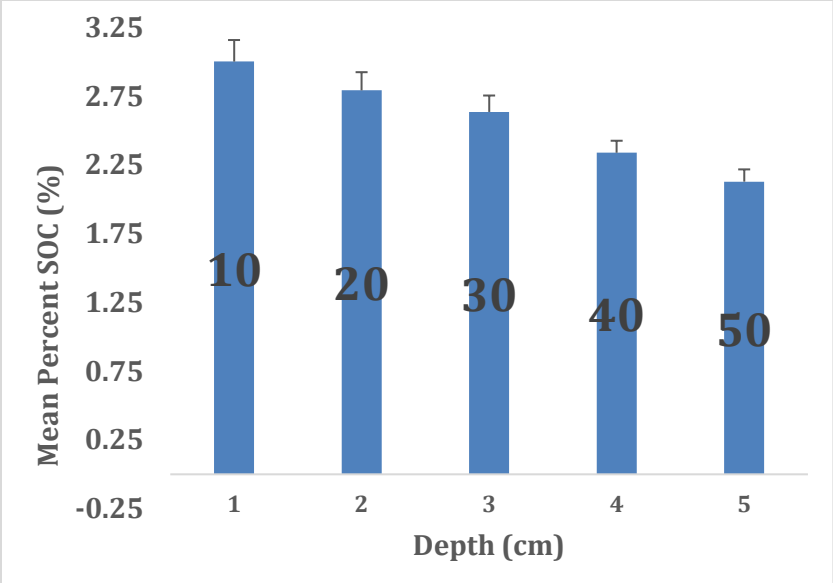


Figure 4 & 5. Mean percent SOC (%) with displayed standard error bars at all sites with a combination of the sampled depths, and at all depths with a combination of the three sites, at 3 samples per site: i) compost (green), inorganic (blue), and 2° Forest (red) ii) 10 cm, 20 cm, 30 cm, 40 cm, and 50 cm at Life Monteverde, a farm in a premontane wet forest. According to the Fisher LSD test, there are significant differences between compost vs 2° Forest and inorganic vs 2° Forest at both 10 cm and 20 cm in Figure 4. In Figure 5, according to the Fisher LSD test, there are significant differences in 10 vs 50 cm in compost; 20 vs 50, 30 vs 50, 10 vs 50, and 10 vs 40 in inorganic; and 10 vs 30, 10 vs 40, 10 vs 50, 20 vs 40, and 40 vs 50 in the 2° Forest site.

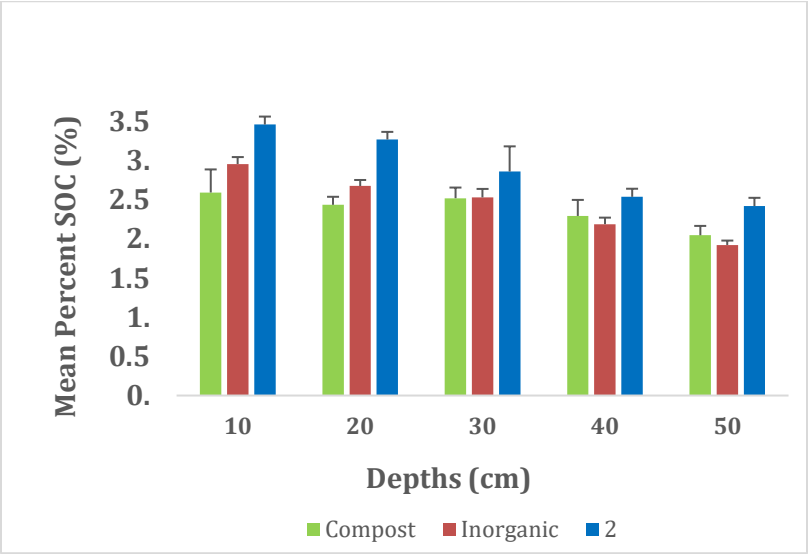


Figure 6. Mean percent SOC (%) at all sites with displayed standard error bars; compost (green), inorganic (blue), 2° Forest (red), and at all sampled depths of 10 cm, 20 cm, 30 cm, 40 cm, and 50 cm at Life Monteverde, a farm in a premontane wet forest, with 3 samples per site.

The results were significant between BD and site according to the results of the two-way ANOVA (F= 16.0514, d.f. = 2, p-value= 0.00002) with means (in g/cm^3) of compost = 0.74 +/- 0.03, inorganic = 0.82 +/ - 0.02, and 2° Forest = 0.66 =/- 0/03 (Figure 7). According to the Fisher

LSD test there were significant differences ($p < 0.05$) in inorganic vs 2° Forest at 10 cm; compost vs 2° Forest and inorganic vs 2° Forest at 20 cm; and compost vs inorganic and inorganic vs 2° Forest at 50 cm. There were significant differences between depth and BD according to the two-way ANOVA (F= 8.52927, d.f. = 4, p-value= 0.0001). The means (in g/cm^3) were 10 cm= 0.70 +/- 0.04, 20 cm = 0.67 +/- 0.05, 30 cm= 0.81 +/- 0.02,40 cm = 00.82 +/- 0.03, and 50 cm = 0.69 +/- 0.04 (Figure 8). According to the Fisher LSD test, there are significant differences ($p < 0.05$) in 10 vs 50, 20 vs 50, 30 vs 50, and 40 vs 50 in compost; and 10 vs 30, 10 vs 40, 20 vs 30, 20 vs 40, and 20 vs 50 in the 2° Forest sites. There were also significant differences between the combination of site and depth according to the two-way ANOVA (F= 2.68511, d.f. = 8, p-value= 0.02354) (Figure 9). To summarize, BD is lowest for the 2° Forest, and seems to increase up to 30 cm.

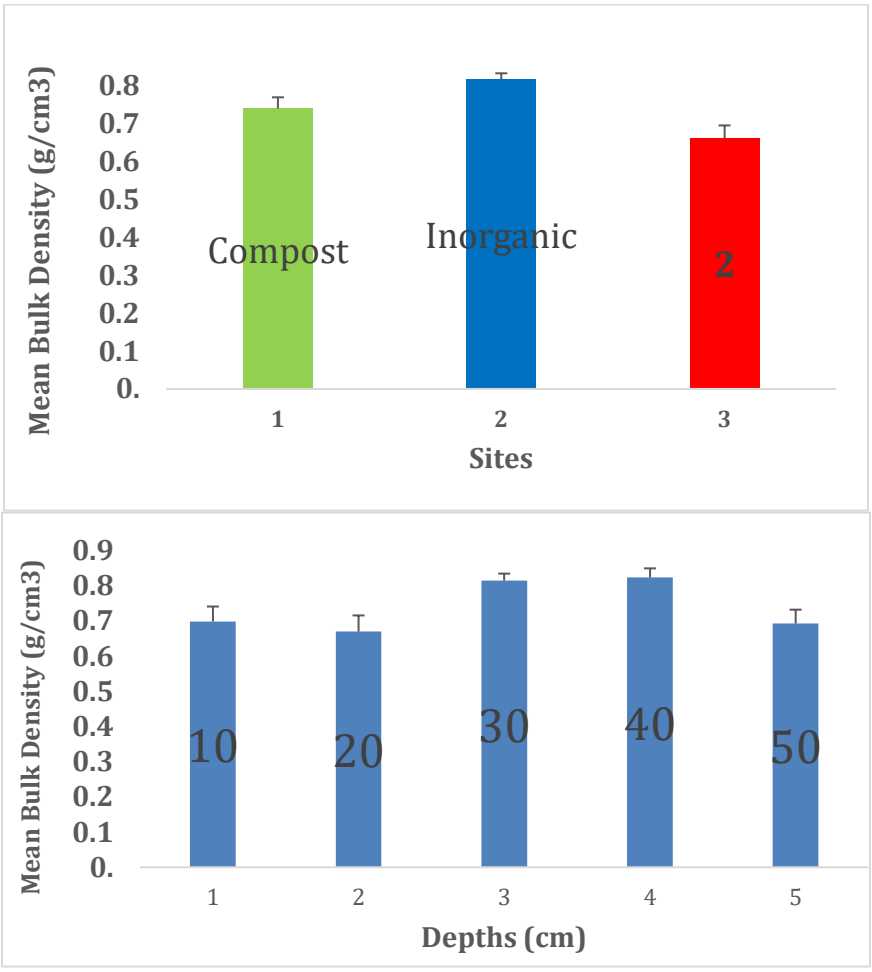


Figure 7 & 8. Mean bulk density (g/ cm^3) at all sites with displayed standard error bars with a combination of the sampled depths, and at all depths with a combination of the three sites, at 3 samples per site: i) compost (green), inorganic (blue), and 2° Forest (red) ii) 10 cm, 20 cm, 30 cm, 40 cm, and 50 cm at Life Monteverde, a farm in a premontane wet forest. According to the Fisher LSD test, in Figure 7, there are significant differences ($p < 0.05$) in inorganic vs 2° Forest at 10 cm; compost vs 2° Forest and inorganic vs 2° Forest at 20 cm; and compost vs inorganic and inorganic vs 2° Forest in 50 cm. In Figure 8, there are significant differences in 10 vs 50, 20 vs 50, 30 vs 50, and 40 vs 50 in compost; and 10 vs 30, 10 vs 40, 20 vs 30, 20 vs 40, and 20 vs 50 in the 2° Forest sites.

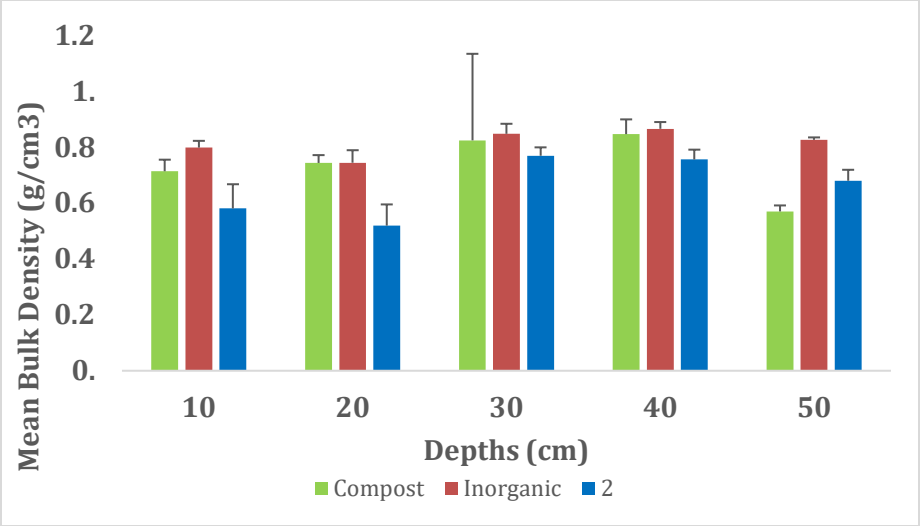
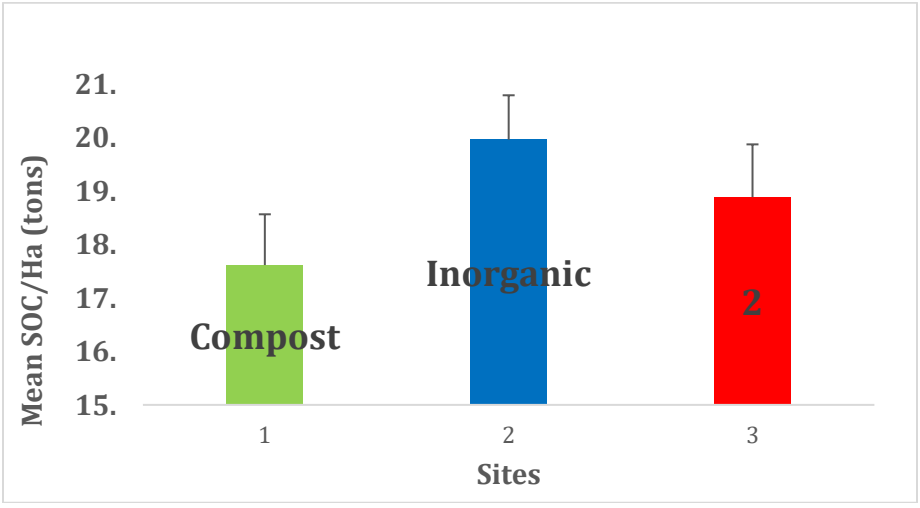


Figure 9. Mean bulk density (g/cm^3) at all sites with displayed standard error bars; compost (green), inorganic (blue), 2° Forest (red), and at all sampled depths of 10 cm, 20 cm, 30 cm, 40 cm, and 50 cm at Life Monteverde, a farm in a premontane wet forest, with 3 samples per site.

The results were not significant between C per hectare and site according to the two-way ANOVA (F= 2.73339, d.f. =2, p-value = 0.08119). The means (in tons) were compost= 17.62 +/- 0.95, inorganic= 19.97 +/- 0.83, 2° Forest= 18.89 +/- 0.99 (Figure 10). According to the fisher LSD test, in Figure 10, there are significant differences ($p < 0.05$) in compost vs inorganic at 10 cm. The results were significant between C per hectare and depth according to the two-way ANOVA (F=8.33173, d.f. = 4, p-value= 0.00012). The means (in tons) were 10= 20.64 +/- 1.25, 20= 18.27 +/- 0.89, 30= 21.46 +/- 1.12, 40= 19.10 +/- 0.53, 50= 14.65 +/- 0.83 (Figure 11). According to the Fisher LSD test, there are significant differences ($p < 0.05$) in 20 vs 30, 30 vs 50 in the 2° Forest site; 10 vs 50, 30 vs 50 in the inorganic site; and 10 vs 50, 20 vs 50, 30 vs 50, and 40 vs 50 in the compost site. The results were not significant between C per hectare and site with depth according to the two-way ANOVA (F= 0.97562, d.f. = 8, p-value= 0.47348) (Figure 12). The significant difference between depths regardless of sites mimics the same trends as BD, and emphasizes the relationship of all three variables with depth.



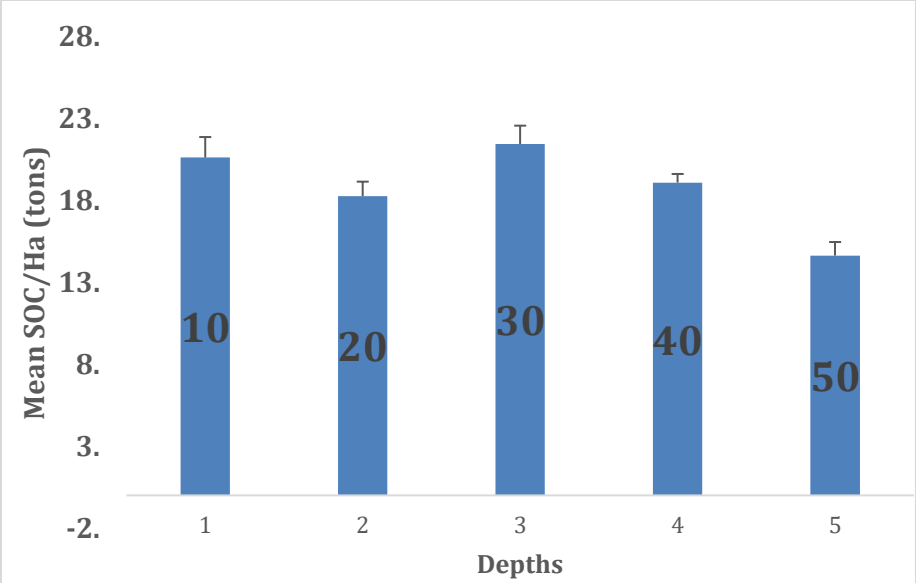


Figure 10 & 11. Mean C/Ha (tons) at all sites with displayed standard error bars with a combination of the sampled depths, and at all depths with a combination of the three sites, at 3 samples per site: i) compost (green), inorganic (blue), and 2° Forest (red) ii) 10 cm, 20 cm, 30 cm, 40 cm, and 50 cm at Life Monteverde, a farm in a premontane wet forest. According to the fisher LSD test, in Figure 10, there are significant differences in compost vs inorganic at 10 cm. In Figure 11, there are significant differences in 20 vs 30, 30 vs 50 in the 2° Forest site; 10 vs 50, 30 vs 50 in the inorganic site; and 10 vs 50, 20 vs 50, 30 vs 50, and 40 vs 50 in the compost site.

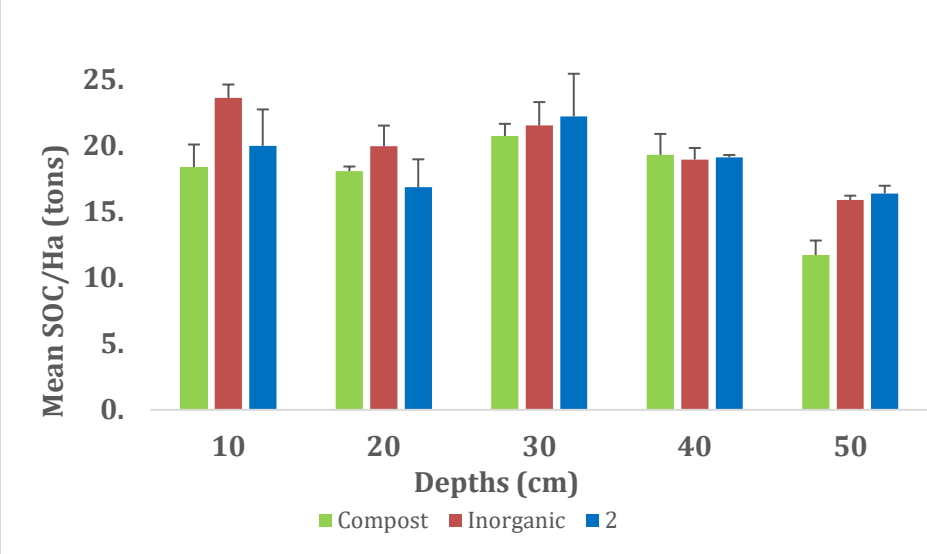


Figure 12. Mean C/Ha (tons) at all sites with displayed standard error bars; compost (green), inorganic (blue), 2° Forest (red), and at all sampled depths of 10 cm, 20 cm, 30 cm, 40 cm, and 50 cm at Life Monteverde, a farm in a premontane wet forest, with 3 samples per site.

Discussion

The significant differences between the mean percent SOC and site showed decreasing carbon stocks as measured from the 2° Forest to the compost sites, suggesting that these decreasing stocks co-occurred with decreasing plant richness (De Beenhouwer *et al.* 2016), and thus increasing agriculture or land transformation. This also displays the loss of C from the soil as a result of agriculture and points to tropical soils being a net source of C under agriculture (Lal 2004).

The similarity between percent SOC in the compost site and inorganic site can be explained by the aboveground biomass of the coffee plants donating additional humification from falling plant material (Mendoza 2016), explaining the slight disparity between the two. Both coffee plots were sampled between two rows of coffee plants. It is possible that the application of compost to the tree trunk accumulated mostly in that area, and thus did not have the same effects in between rows as it may have had right at the plant base (Dr. Justin Welch, personal communication). C leaching losses have also been proven to be important in the overall C balance of agricultural systems (Kindler *et al.* 2011).

The significant differences between BD and site exemplify what might be expected considering that agricultural soils have a 13% higher BD than forests, on average (Murty *et al.* 2002). This is caused by direct compaction of cattle and lack of de-compaction from soil decomposers. As BD values continue to increase, this may allow for greater C storage through increased compaction or greater aggregation. However, this fails to acknowledge the possibility of different mineral makeup and composition between the three sites, especially since biomass and rocks were removed from the samples before BD was calculated. Aggregation properties differ between each different mineral and composition type (soil, sand, etc.) as aggregate size is affected (Six *et al.* 2000). Nor is this accounting for the differences in past management strategies, such as the level of grazing which would have increased the compaction in the soil. Considering the important role of C leaching in croplands (Kindler *et al.* 2011), an increased BD may not have been beneficial in the two coffee plots if C was leached away too quickly.

There were no significant differences between SOC/Ha and site for all depths combined, despite the significant differences observed in both the percent SOC and BD between sites, at all sampled depths. However, it is possible that the application of compost had not been over a long enough period of time, as it has been shown that it takes up to 20 years for SOC in the top 30 cm to increase under high compost application (Ogle *et al.* 2004), and the coffee plots have only been around for seven years (Daniel C. Vargas, personal communication). It is also important to keep in mind that SOC/ Ha values were calculated using BD and % SOC. The 2◦ forest had a high % SOC and a low BD. The two coffee plots had the exact opposite, giving results for SOC/Ha that were not significant between the three sites.

The significant differences between the mean percent SOC in site regardless of depth and depth regardless of site aligned with previous studies that showed a decrease in SOC storage to a depth of 60 cm as a result of long-term cultivation, and the loss of C input from deeply rooted vegetation (Mikhailova *et al.* 2000). There is an increase in the amount of SOC loss at depth with the inorganic and compost sites used for coffee farming, as opposed to the forest that is unharvested (Davidson & Ackerman 1993, Stockmann *et al.* 2011). The decrease in SOC storage displayed here can also be explained by the relatively short time frame that the coffee plants had been established (Ogle *et al.* 2004).

On average, between the three sites, the BD increased at 30 and 40 cm, suggesting greater compaction in this mid-layer, and decreased decomposition with depth, until factoring in the drop in BD at 50 cm (Fontaine *et al.* 2007). As previously mentioned above, these results are not

considering the mineral composition. Clays and sands may protect similar amounts of C (Silver *et al.* 2000), but clay content is positively correlated with soil organic matter (Motavelli *et al.* 1994, 1995, Ritter *et al.* 1995). Since no specific tests were done in this study to identify layer specific soil texture, it is possible that differing clay amounts at different depths could have played a role in our results. Nor were past management strategies of the three sites taken into consideration. For example, it is possible that at 30 and 40 cm, there are remnants of past grazing, that 50 cm (at the time being more equivalent to 30 cm in depth) would have been shielded from, and thus increased compaction and aggregation in 30 and 40 cm. It would make sense that this would not be displayed in 10 or 20 cm in the coffee plots as that layer would be subject to cultivation and disturbance.

The significant difference between SOC/ Ha at the different depths with the highest mean store at 30 cm is in agreement with increased BD values indicating that based on the 20 year timeline, the SOC has leached down to the 30 cm depth, but has not yet had enough time to establish a SOC pool at 40 and 50 cm (Fontaine *et al.* 2007, Ogle *et al.* 2004) if soil composition and management type are disregarded.

This experiment set a basis for testing increasing SOC with depth, across different sites. Going forward, future experiments can be done with the assumption of differentiation between cropland and forest. However, compost does not currently appear to have any effects on SOC storage. Future experiments should take into account time accumulated, C leaching rates, decomposition rates, as well as past management strategies and soil composition. A tipping point should be researched in terms of the presence of excess carbon that can be captured in the form of soil particulate organic matter (POM). This could be reached by providing excess compost or composting more frequently. Clarification of the processes that move SOC deeper into the soil will establish more evenly distributed carbon credits, and ultimately provide a more sustainable way of farming in the face of climate change.

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Epiphyte root biomass in canopy soil along a cloud forest elevational gradient

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ABSTRACT

The epiphytic community is a key ecological group within tropical cloud forests and changes in composition and abundance at different elevations in response to different stressors. Studies have shown that epiphytes peak in density and richness at mid-elevations. Epiphytes send significant roots into canopy soil deposits in order to extract nutrients, and this study is aimed to build off previous epiphyte and canopy soil studies conducted in the cloud forest to analyse epiphyte root biomass (ERB) along an elevational gradient for the first time. While nutrient levels in soils do not change with elevation, ERB was found to. By sampling three canopy soil deposits in 11 different emergent trees on the Pacific slope of the lower montane wet and rain forests and measuring ERB, it was found that ERB significantly increases from 1540 m and peaks at 1640 m before decreasing towards the summit at 1785 m. This trend mirrors those found in past studies in epiphyte diversity along an elevational gradient. Along this steep elevational and life zone gradient, increasing moisture leads to increased ERB and increasing exposure to trade winds towards the summit are responsible in the decrease in ERB.

RESUMEN

Las comunidades epífitas son un grupo ecológico clave dentro de los bosques nubosos tropicales y cambios en la composición y abundancia a elevaciones diferentes en respuesta a diferentes factores estresantes. Los estudios han mostrado que las epífitas llegan a su máxima densidad y riqueza a elevaciones medias. Las epífitas mandan una cantidad significativa de raíces en los suelos del dosel para extraer nutrientes, y este estudio busca construir de estudios previos sobre epífitas y suelos del dosel conducidos en el bosque nuboso para analizar la biomasa de raíces de epífitas (ERB) a lo largo de un gradiente altitudinal por primera vez. Mientras que los niveles de los nutrientes en el suelo no cambian con la elevación, no se encontró lo mimo para ERB. Al muestrear los depósitos del suelo en el dosel en 11 árboles diferentes en la vertiente Pacífica del bosque humedo y bosque lluvioso montano bajo y midiendo el ERB, se encontró que el ERB aumenta significativamente de los 1540 m y llega a su máximo a 1640 m antes de disminuir cerca del pico más alto a 1785 m. Estas tendencias se asemejan a las encontradas en estudios previos en la diversidad de epífitas a lo largo de un gradiente altitudinal. A lo largo de este gradiente altitudinal y de zonas de vida, el aumento de la humedad lleva a un incremento de ERB y un aumento en la exposición a los vientos llegando a la cima que son responsables de la disminución de ERB.

INTRODUCTION

Epiphytes are fragile. Without access to the forest floor, they are stressed for water, and changes in moisture can have large effects in the community structure. Epiphytes play several important roles in ecosystem function in tropical forests (Gotsch *et al*. 2016). They intercept water and nutrients from the atmosphere and from intercepted host tree sources and may contribute significant inputs of these resources to the forest floor (Nadkarni 1992). They serve as habitat and food for birds and mammals, with over 200 species of birds documented as using epiphytic material in the Neotropics (Gotsch *et al*. 2016). In tropical cloud forests, dead organic matter including crown humus and intercepted litterfall comprises over 60% of the total epiphytic material (Nadkarni 1994). Some decomposing epiphytic and leaf material never make it to the

ground and forms layers and packs of humus and litterfall called canopy soil (Haristoy *et al*. 2014). These soils function to retain atmospheric nutrients, as habitat for canopy invertebrates, and substrate for wildlife and bird foraging (Gotsch *et al*. 2016, Nadkarni 1994). Canopy soil not only provides habitat for animals but is used by epiphytes to extract nutrients and water (Nadkarni *et al*. 2004). The epiphytic community's sensitivity to moisture suggests that epiphytic success varies with precipitation, and therefore elevation (Gotsch *et al*. 2016).

Canopy soils provide nutrients and water for epiphytes, and in certain cases, even the tree themselves (Cardelús and Mack 2010). By accessing soil reserves found in the canopy, epiphytes boost fitness and give themselves a competitive edge. Nearly all organic matter in a cloud forest canopy, especially root material, is found on top of branches and at branch junctions (Vance & Nadkarni 1992). In fact, about 80% of epiphytic root biomass (hereafter ERB) is found at branch junctions (Vance & Nadkarni 1992). By accessing these soil reserves found in the canopy, epiphytes boost fitness and give themselves a competitive edge.

Cardelús and Mack (2010) found that canopy soil-rooted epiphytes had higher N concentrations than atmospheric epiphytes, suggesting that they drew significant levels of nutrients from the canopy soils they exploit. However, they found no pattern between epiphytic leaf chemistry and elevation, which suggests that canopy soil-rooted epiphytes do not rely more heavily on the soil deposits at different elevations for nutrient extraction (Cardelús & Mack 2010). Vaillant (2001), Chan (2002) and Cardelús and Mack (2010) studied nutrient gradients in epiphytes along an elevational gradient, Vance and Nadkarni (1992) studied root density in canopy soil in comparison to forest floor soil deposits in a Monteverde cloud forest, but not along an elevational gradient.

Although no trend has been found between ground soil root biomass and elevation in the tropics (Girardin et al. 2012), it is important to study the effect of elevation on ERB because previous studies have shown that epiphyte diversity, epiphytic fern abundance, and epiphytic biomass all change at different elevations (Werner *et al*. 2012, Krömer *et al*. 2005, Kluge & Kessler 2010, Freiberg & Freiberg 2000). Freiberg and Freiberg (2000) found differences in canopy hummus biomass between lowland and montane forest in South America, which suggests that ERB could differ at different elevations as well.

Monteverde is a great place to study the effect of elevation on ERB because it is a short mountain with compressed life zones (Bolaños & Watson 1993). Secondly, extensive canopy soil research has been conducted here. (Vance & Nadkarni 1992, Nadkarni 1994, Nadkarni *et al*. 2004). In Monteverde, Costa Rica, the cloud forest on the Pacific side of the continental divide spans two Holdridge life zones: lower montane wet forest and lower montane rain forest. The lower montane wet forest spans from 1450 m to 1600 m in elevation, and the lower montane rain forest spans from 1550 m to 1850 m, to the summit and continental divide. While epiphytes and canopy soils are important to tropical montane cloud forests, (Gotsch *et al*. 2016) based on previous epiphyte biomass studies the differences in conditions such as moisture at different elevations could impact the root mass that epiphytes send into canopy soil deposits (Krömer *et al*. 2005, Kluge & Kessler 2010, Freiberg & Freiberg 2000).

This study aims to quantify epiphyte biomass change on canopy along an elevational gradient. According to Cardelús and Mack's 2010 study out of La Selva Biological Station and Chan's (2002) nutrient levels do not significantly different in canopy soils at different elevations. As such, any significant differences in ERB at different elevations is likely related to nutrient levels. Rather it is likely due to differences in moisture and wind that effect epiphytes ability to colonize certain elevations and their success there. I collected soil samples from the canopies of

trees at different elevations and measured root mass to test the potential relationship between these two variables.

METHODS

SITE INFORMATION

This study was conducted in the cloud forest outside of Monteverde, Costa Rica on the Pacific slope between 1540 m and 1785 m in elevation between 29 October 2018 through 5 November 2018. The lower montane rain forest which spans from 1550 m to 1850 m averages 3600-8000mm of rainfall annually while the lower montane wet forest below it receives half that; 1850-4000 mm (Bolaños & Watson 1993). The canopy height ranged from 25-35 m at 1540 m (Clark 1994) to 5-8 m at the summit (Lawton 1980), and all trees sampled were large, somewhat emergent trees. Samples were mostly conducted on emergent trees found in closed canopy of a primary forest.

SAMPLE COLLECTION

A large tree that visibly contained a large amount of soil and appeared climbable was selected roughly every 15-20 meters in elevation to get a large and consistent elevation range. Three samples were collected from each tree from the low and mid inner canopy of the tree. The samples were always from regions close to the junctions of branches or in particularly deep soil packs on the top of branches in the inner crown where total epiphytic biomass per branch surface is highest (Freiburg and Freiburg 2000, following Vance and Nadkarni's (1992) methods for canopy root sampling. Adapting from their method of branch junction sampling method in which surface litter was removed and cores (10 cm × 5 cm × 30 cm) were collected, a smaller block of soil of least 6 cm × 6 cm × 6 cm was cut using a pocket knife and stored (Vance & Nadkarni 1992).

SAMPLE PROCESSING

Once collected, each soil sample was standardized into 6 cm cubes (if larger), with the top being the top of the soil pack. Any above-soil organic material (stems, leaves, bryophytes etc.) was cleanly cut off. The cube was then set in a 2 mm mesh sieve, where the roots were thoroughly washed to remove any soil or non-root material. The saved roots were then placed on a fine mesh and set in a drying tank containing five heat light bulbs and dried for 24 hours, following guidelines set by Vance and Nadkarni (1992) before weighting them for each sample separately. Unfortunately, two samples had to be discarded (from 1540 m and 1615 m) due to insufficient depth.

STATISTICAL ANALYSIS

ERB increases with elevation until around 1600 meters and then it declines (Figures 1 and 2). A piecewise regression was used to statistically find the breakpoint in elevation at which ERB begins to decline. The analysis was conduced using the R package "segmented" after taking the log of biomass to linearize the relationship between this variable and elevation, because the

relationship in original scale is not linear (Figures 1 and 2). The analysis was conduced with average data per elevation (i.e. per tree) and with all data per elevation for comparison. After determining the breakpoint, two quadratic models were fit to the data before and after the breakpoint, using the "lm" base function in R. These models were also fit with average data and also with all samples per tree. All analyses were conducted in R version 3.4.3.

RESULTS

The relationship between elevation and biomass changes slope around 1640 meters. Specifically, the breakpoints were 1640.053 m (±16.529 m, standard deviation) when all data are considered and 1639.821 m (±17.625 m) using the average root sample biomass per tree. The breakpoint model is significant for all model terms when all the data per tree are used ($p<0.001$ for both slope and intercept terms) and tends towards significance when the average data are used ($p=0.07$ for intercept, $p=0.049$ for slope).

The quadratic regressions before and after the breakpoint of 1640 meters, fit using all observations per tree were highly significant ($p = 0.0005869$, LMW1 and $p = 0.0002557$, LMR2, Figure 1) and had very high R^2 values (LMW1 is $R^2 = 0.6817$, and LMR1 is $R^2 = 0.6681$). The quadratic regression with average data per tree was also significant (Non-linear regression test, $p = 0.005461$, LMW2 and $p = 0.01673$, LMR2, Figure 2) and more importantly found exceptionally high R^2 values (LMW2 is $R^2 = 0.969$ and LMR2 is $R^2 = 0.9345$)

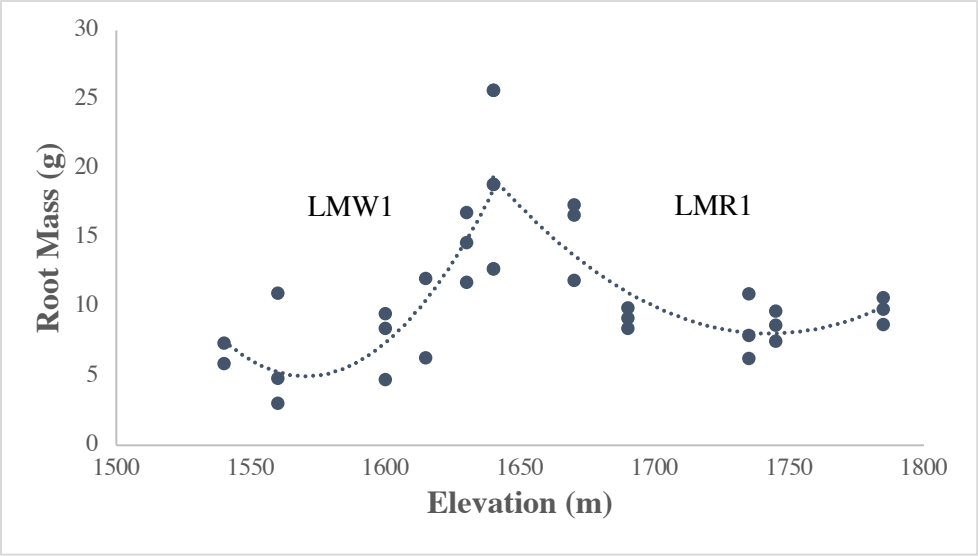

Figure 1: Epiphytic root samples (6 cm cube of canopy soil cut from branch junctions of cloud forest trees, washed through a 2 mm sieve, and dried for 24 hours) mass in grams in trees at increasing elevations in the lower montane wet forest and the lower montane rain forest to the summit on the Pacific slope around Monteverde, Costa Rica.
LMW: Mass = $0.0028(\text{Elevation})^2 - 8.6853(\text{Elevation}) + 6824.8$
LMR: Mass = $0.0011\ (\text{Elevation})^2 - 3.7307(\text{Elevation}) + 3258.8$

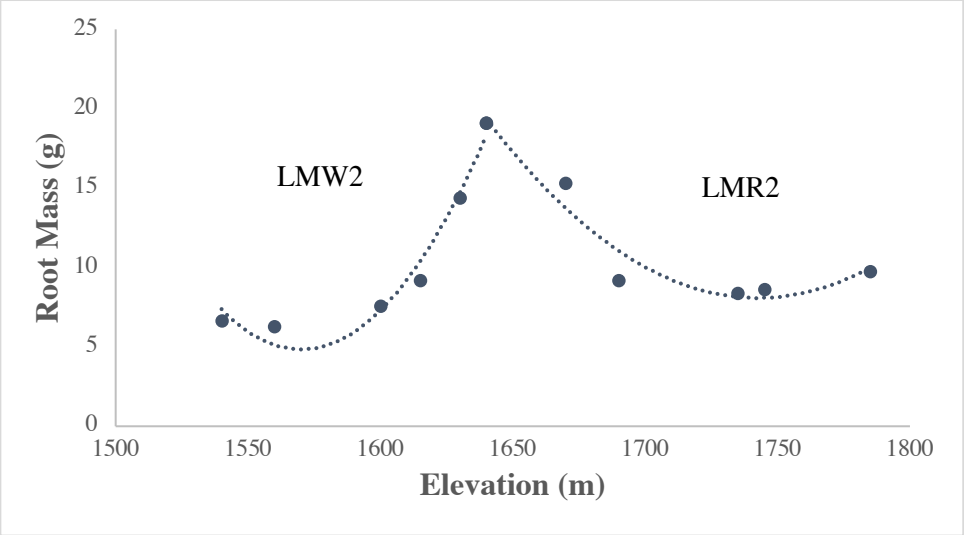

Figure 2: Change in average epiphytic root dry mass (in grams) per 6 cm cube of canopy soil cut from branch junctions of cloud forest trees along an elevational gradient on the Pacific slope in Monteverde, Costa Rica. The gradient spans the lower montane wet forest and the lower montane rain forest Holdridge life zones. Life zones change around 1600 meters. LMW: Mass = $0.0028(\text{Elevation})^2 - 8.6761(\text{elevation}) + 6816.3$ LMR: Mass = $0.00112(\text{Elevation})^2 - 3.7307(\text{Elevation}) + 3258.8$

ADDITIONAL OBSERVATIONS

Epiphytic bryophytes began to overtake vascular epiphytes as the main epiphytes present at the higher sites. Soil deposits were deepest at mid-elevation sites. Towards the summit, vascular epiphytes tended to grow more on the sides of branches and the trunk rather than on top.

DISCUSSION

The hump shaped trend present in ERB in relation to elevation found is not a unique trend in epiphyte studies. In a study conducted by Krömer *et. al (*2005) epiphyte species richness along an elevational gradient in Ecuador found that vascular epiphyte species richness forms a similar trend and peaks at mid-elevation sites and decreases the rest of the way to the summit. Kluger and Kessler (2010) studied fern species richness and density along an elevational gradient in Costa Rica and found that both fern species richness and epiphytic fern density peak at mid-elevation sites at well. So, the logical answer to why ERB along an elevational gradient mirrors epiphyte richness and density at different elevation is simply that more epiphytes are present to send roots into canopy soil deposits at those mid-elevation sites, at this case roughly 1600 m. This matches up well with visual observations during sample collection. Interestingly, the transition between LMW and LWR forest life zones also occurs at that elevation (Bolaños & Watson 1993).

There are three climate-based reasons why this distinct trend is present. The first two have to do with moisture. As it has been previously noted, epiphytes are quite sensitive to moisture and favor wetter areas (Gotsch *et al*. 2016) because they lack substantial root systems.

In forests like the one studied, moisture is present in two main forms, humidity and precipitation. Addressing humidity first, low elevations tend to have the lowest humidity, which explains the low epiphyte species richness there (Krömer *et al*. 2005) and the lower ERB found in this study. Moving up in elevation, the sites with the highest ERB at mid-elevation have been found to have a maximum of air humidity (Krömer *et al*. 2005). Although precipitation continues to increase towards the summit in Monteverde, in the study conducted by Cardelús and Mack (2010) at La Selva, an admittedly different moisture gradient, found that water use efficiency was lowest at their 1600 m site. Water use efficiency tends to be lowest when there is an excess in water available, suggesting highest levels of moisture at that elevation (Cardelús & Mack 2010). Overall, humidity is one of the major driving factors for epiphytic richness (Kluge & Kessler 2010).

In Monteverde, precipitation increases as you move up towards the summit (Clark 1994). The increase in precipitation from LMW to LMR forest is nearly double. The increase of rainfall from the lower to mid-elevations corresponds to an increase of epiphyte diversity (Krömer *et al*. 2005) and correlates to an increase in ERB. However, precipitation does not decrease from mid-elevations up to the summit like ERB does, rather it increases substantially. As such, although there might be a decrease in total moisture between 1600 m and the summit, it is not significant enough to explain the decrease in epiphyte species richness, vascular epiphyte density, and ERB (Krömer *et al*. 2005).

To explain the decrease in ERB nearer to the summit we must look to wind. In Monteverde, the majority of the wind comes in the form of trade winds from the Caribbean side that blow over the summit. As such, on the Pacific slope around Monteverde, wind increases with elevation (Clark 1994). Furthermore, forest conditions in ridge patches and ravine patches in the LMR forest do not differ significantly in sunlight, nutrients, or precipitation. The only significant difference in abiotic conditions is wind speed, which is twice as fast along the ridges (Lawton 1982). The increasing wind closer to the summit has two main effects: first it "trims" trees and vegetation, leading to stunted growth in canopy trees and secondly it strips soil (and epiphytes) from the canopy (Krömer *et al*. 2005, Nadkarni & Matelson 1991). The stunted canopy trees lead to less opportunities for epiphytes to grow (Krömer *et al*. 2005), While the decreased canopy soil, which is expected (Nadkarni & Matelson 1991) and was observed during sample collection, leads to less reason and opportunity for epiphytes to send our ERB. Another impact of high winds is that the surviving epiphytes in windy regions grow on the side of branches to avoid being knocked off (Madison 1977). As our samples were taken from the tops of branches and branch junctions, it is highly possible that at the higher elevation samples, we were simply not picking up on some of the ERB on that tree.

The final explanation for the decrease in ERB is that at higher elevations, vascular epiphytes decline in both richness and frequency and epiphytic bryophytes take up the available space (Krömer *et al*. 2005). This was borne out through observation as well, as practically the entire trees at the high elevation sites were covered in bryophytes, which do not have roots to send in to canopy soil.

Whether it is the abundance of soil and therefore epiphytic roots that leads to a bump in epiphyte diversity at mid-elevations or high epiphyte diversity that leads to high ERB at those sites, the two are very much interconnected. To summarize, there are three main abiotic causes in ERB variation along an elevational gradient. Humidity, precipitation, and wind each shape the trend observed and similar trends in epiphyte density and richness (Krömer *et al*. 2005,

Kluge & Kessler 2010). In short, these novel results show that ERB is determined by moisture, wind and access to canopy soil but also is determined by epiphyte diversity.

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Abundance of Arbuscular Mycorrhizal Fungi in Organic Farmland, Conventional Farmland and Secondary Forest

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ABSTRACT

Understanding the effects of different agricultural practices on arbuscular mycorrhizal fungi (AMF) communities is important for sustainable agricultural production, since AMF play an essential role in maintaining and promoting the health of crop-plant communities. Previous studies have demonstrated that organic farming methods promote greater diversity of AMF in crops like cereal and maize, and have begun to investigate the relationship between compost and AMF in coffee crops, but research regarding AMF abundance under different agricultural treatments, in a tropical ecosystem, is generally lacking. This study investigated the abundance of AMF in a secondary forest, an organic coffee plot, and a conventional coffee plot in a tropical cloud forest area in Costa Rica. Soil samples containing roots collected from each site were analyzed microscopically, and mean AMF root percent cover was determined for each site. AMF abundance was significantly greater in both the secondary forest and the organic coffee plot than in the conventional coffee plot. AMF abundance did not differ significantly between the secondary forest (25^{th} percentile: 22.650, median: 46.10, 75^{th} percentile: 72.675) and the organic coffee plot (25^{th} percentile: 34.400, median: 39.85, 75^{th} percentile: 49.250). These results show that different agricultural practices impact AMF communities, and highlight that organic farming both supports a greater abundance of AMF and is more resemblant of the abundance of AMF communities found in natural ecosystems. In particular, this study demonstrates the ability of organic farming to sustain more AMF relative to conventional farming, and the potential importance of this increased abundance for sustainable agriculture.

RESUMEN

Entender los efectos de las diferentes prácticas agrícolas en las comunidades de micorrizas arbusculares (AMF por sus siglas en ingles) es importante para una producción agrícola sostenible, ya que las AMF juegan un rol esencial en la promoción y mantenimiento de la salud de las comunidades de cultivos. Estudios previos han demostrado que los métodos de agricultura orgánica promueven una mayor diversidad de AMF en cultivos de cereal y maíz, y han empezado a investigar la relación entre el compostaje y las AMF en cultivos de café, pero estudios sobre la abundancia de AMF bajo diferentes tratamientos agrícolas, en un ecosistema tropical, son generalmente escazos. Este estudio investiga la abundancia de AMF en un bosque secundario, parcela de café orgánico, y parcela de café convencional en un bosque nuboso

tropical en Costa Rica. Muestras de suelo que contienen raíces fueron colectadas de cada sitio y se analizaron microscopicamente, y el promedio de cobertura de AMF en las raíces se determinó para cada sitio. La abundancia de AMF fue significativamente mayor tanto en el bosque secundario como en la parcela de café orgánico que en la parcela de café convencional. La abundancia no difiere entre el bosque secundario (percentil 25th: 22.650, mediana: 46.10, percentile 75th: 72.675) y la parcela de café orgánico (percentil 25th: 34.400, mediana: 39.85, percentile 75th: 49.250). Estos resultados muestran que las diferentes prácticas agrícolas impactan las comunidades de AMF, y sobresalta que la agricultura orgánica tanto soporta una mayor abundancia de AMF y se asemeja más a las comunidades de AMF encontradas en los ecosistemas naturales. En particular, este estudio demuestra que la habilidad de la agricultura orgánica para sostener más AMF comparada con la agricultura convencional, y la importancia potencial de esta abundancia aumentada de la agricultura sostenible.

INTRODUCTION

By 2050, the human population will be larger by 2 to 4 billion people (Cohen 2003). Global food demand is quickly increasing as a result (Tilman, et al. 2011), and under modern agricultural practices, consumption of agricultural products is responsible for the simplification and destabilization of ecosystems (Ehrlich and Holdren 1971). We now have a pressing need for the sustainable intensification of agriculture to provide for our growing global population, in a manner which mitigates the negative impacts of agricultural production whilst generating ecosystem services - such as the preservation of air, water and soil - via agroecosystems (du Jardin 2015). Arbuscular mycorrhizal fungi (AMF), a form of endomycorrhizal fungi belonging to the phylum Glomeromycota (Bonfante, et al. 2010), may play an increasingly important role in the way we approach sustainable farming.

AMF are obligate biotrophs, which means they must colonize the roots of a host plant and absorb carbohydrates from within plant cells to survive (Bonfante, et al. 2010). AMF have symbiotic associations with over 80 percent of terrestrial plant species (Harrier and Watson 2004). Upon colonization, AMF form arbuscules, or repeating branches of intracellular hyphae (the filamentous structure of the fungi) in the inner root cortex (the outermost layer of the root), which act as sites of nutrient exchange (Bonfante, et al. 2010). In this mutualistic interaction, AMF uptake carbohydrates from the plant, and the host benefits from the hyphal network formed by AMF, which facilitates the efficient horizontal movement of nutrients and communication between individuals of the same plant community (Bonfante, et al. 2010). AMF also have "high-affinity inorganic phosphate transporters," so they significantly improve soil nutrient uptake in the host, especially phosphorus (Bonfante, et al. 2010), in addition to translocating nutrients beyond the 'depletion zones' of the plant rhizosphere (Gosling, et al. 2006, Schneider, et al. 2015). AMF also increase resiliency to both biotic and abiotic stressors, improve hosts' water balance, and affect plant development through the alteration of phytohormones (Augé 2001, Gosling, et al. 2006, Gianinazzi, et al. 2010, Harrier and Watson 2004), which may contribute to root system remodeling (Gutjahr and Paszkowski, 2013). Of particular interest for agricultural purposes, AMF are a biostimulant, defined as any substance or microorganism applied to plants with the aim of enhancing nutrient acquisition efficiency, abiotic stress tolerance, and/or crop quality traits (du Jardin 2015). In addition, AMF prevent erosion via the formation of a macroporous structure that allows for the flow of air and water, whilst conferring soil stability (Oehl, et al. 2003).

An understanding of AMF is clearly important as their symbiosis with plants provides ecosystem services that promote crop productivity and quality in sustainable agricultural systems (Gianinazzi, et al. 2010). Consequently, an understanding of how different agricultural practices affect this mycorrhizal symbiosis is necessary for the creation of optimal management strategies for sustainable agroecosystems (Turrini, et al. 2017, Harrier and Watson 2004, Manoharan, et al. 2017). This understanding is especially important since less attention has been paid to the role of beneficial soil microorganisms (including AMF) in agriculture, since the 'first green revolution' (Gianinazzi, et al. 2010). Recent studies, conducted in varying geographic locations and agricultural systems, have analyzed different indicators of AMF health in relation to agricultural management methods. In east China, the addition of organic compost to a subtropical wheat-rice rotation agroecosystem significantly enhanced the hyphal and spore biomass of AMF (Qin, et al. 2015). In southern Sweden, organically farmed cereal fields sustained greater AMF diversity than their conventionally farmed counterparts (Manoharan, et al. 2017). In this study, high AMF diversity was positively related to barley phosphorus uptake and grain biomass production (Manoharan, et al. 2017). Several studies conducted in the tropics have illustrated that *Coffee arabica* individuals benefit from the inoculation of AMF and treatment with organic compost (Osorio, et al. 2002, Rivera, et al. 2007), but investigation of the qualities of AMF under organic versus conventional management in the tropics is lacking, where AMF may play an even more crucial role in nutrient transfer and plant productivity, since highly weathered tropical soils are low in both total and available phosphorus (Friesen, et al. 1997). Given the agricultural importance of AMF, this study seeks to assess, using percent root cover quantification – a common method of mycorrhizal colonization quantification (Mahdhi, et al. 2017) – how the abundance of AMF differs between a secondary forest, a monoculture plot of conventionally managed coffee plants, and a monoculture plot of organically managed coffee plants in a tropical cloud forest area.

MATERIALS AND METHODS

Study Site

Both coffee plots and the secondary forest were located at *Life Monteverde*, a 17-hectare farm in Monteverde, Costa Rica, in the premontane wet forest. The organic and conventional plots were adjacent to each other, separated by a trail and two live fences. Both plots were planted 7 years ago, both were coffee monocultures, and both were sun plantations of approximately 0.2 hectares. Both plots were managed identically excepting fertilizer use. The conventional plot (Fig. 1), receives three rounds of fertilizer and is treated with about 184 kg of fertilizer per year, consisting of both a nitrogenated formula and a complete formula. The organic plot is treated with 6,810 kg of organic compost (or about 6 kg of compost per coffee plant), created at the farm using livestock feces and organic waste. The patch of forest was completely removed from both plots, on the periphery of the farm.

Figure 1. A photograph from atop a hill, overlooking the conventional coffee plot in Life Monteverde. Coffee plants were observationally the same height, about 175 cm, and thus of similar age. The plot is surrounded by live fences (a single row of trees, on each side of the plot, that separate this plot from other plots). More plots and forest fragments make up the rest of the area. The organic coffee plot, on the other side of one of the live fences (but not pictured) looks essentially identical. Soil samples were collected from both coffee plots.

Sample Collection

Samples were collected from the organic plot on 29 October 2018, and from the conventional plot and forest on 7 November 2018. Using a 50 cm long soil core sampler, 10 topsoil samples were collected at each site (for a total of 30 samples), up to a depth of 10 cm. Cores were extracted 10 cm from the base of plant individuals, and each individual was approximately the same size (about 20 cm in diameter at knee height). Upon extraction, soil cores were placed into individual ziploc bags and sealed. Samples were collected at least one meter apart from each other within each site. Moreover, samples were not collected from adjacent individuals in the plots nor in the forest. In the forest, five samples were taken on each side of a man-made trail which divided the forest fragment into two sides. These samples were collected at least a couple meters deep in the forest, away from the trail. Within each plot, two soil samples were taken at each of five locations (the edge of each plot in each cardinal direction comprising four of the locations, and the approximate center of the plot comprising the fifth), to better encompass potential variation within a plot.

All coffee plants were of the same species, but plant individuals in the forest were of different, unidentified species. Samples from the organic plot were stored in the refrigerator, in their original ziploc bags, for approximately 3 days before being cleaned and processed, whereas samples from the conventional plot and forest were cleaned and processed on the same day they were collected. Refrigeration of roots, in their original soil samples, does not adversely impact AMF biomass (Dr. Karen Masters, personal communication).

AMF Staining

Out of the 10 samples from each site, 5 were randomly chosen for processing and analysis. Soil from each chosen sample was passed through a 2mm soil sieve (Fig. 2), and then each extracted root was rinsed with tap water and gently scrubbed by hand to remove soil particles. Roots were handled very carefully to avoid disturbing the outer root layer, where AMF arbuscules reside (Bonfante, et al. 2010). Four roots were chosen from each of the 5 samples, for a total of 20 roots per site, resulting in 60 total roots from the 3 sites. Roots were preferentially selected if they were at least 10 mm in length, were thin, and were pale or lacked pigment (to make microscopic analysis easier). Then, roots were cleared using a modified version of the Phillips and Hayman (1970) method (altered by Koske and Gemma 1989: Bagyaraj and Sturmer, 2008). Clean roots from a specific site were bathed in a glass beaker of approximately 100 ml of 3% KOH solution, at 90 °C for 90 minutes. Then roots were removed from the bath, using forceps, and placed in clean plastic petri dishes. Next, alkaline H_2O_2 was pipetted over the roots, until all roots were completely covered in solution (Fig. 3). A lid was placed on the petri dish(es) and the roots were then soaked for 30 minutes at room temperature, to complete the clearing process. Then, the roots were removed and placed in clean petri dishes, again using forceps for transfer, where they were completely covered by a solution of 1% HCl (administered using the same technique) and were left to soak, at room temperature, for one hour while covered. Finally, roots were transferred to clean petri dishes, where they were submerged in a room temperature solution of acidic glycerol with approximately 5 ml of 0.05% trypan blue dye. Trypan dye was stored in a cool, dark place in between each use. After covering the petri dishes, roots were left to soak overnight for 18 hours, to complete the AMF staining process. After 18 hours, roots were again moved to clean petri dishes prior to quantification of AMF coverage.

Figure 2. Roots from a soil sample, after sieving, but prior to gentle scrubbing to remove remaining soil particles. Roots that were preferentially selected for were generally similar in pigment and morphology to those pictured - pale or translucent, and thin.

Figure 3. Several roots soaking in alkaline H_2O_2 during chemical processing, after samples were cleaned and placed in petri dishes.

Quantification of AMF abundance

Each root was placed on a microscope slide after processing, and observed using a compound microscope under 10x magnification. After scanning the entire root, the region with the highest observed quantity of AMF, which appeared blue and looked like vesicles and/or hyphal branches (Fig. 4), was chosen to be photographed using a microscope digital camera adaptor. Once photos were taken for all 60 roots, the photos were exported to Microsoft PowerPoint, where each was sized to 28.63 cm wide and 19.05 cm tall. A transparent 8x8 grid (22.67 cm wide and 8.23 cm tall, with individual cells of 2.84 cm width and 1.14 cm height), created using the table function of PowerPoint, was placed horizontally on each photo. The rectangular grid was aligned with the longest side parallel to the longest side of the photo, within the root area (no part of the grid extended into any blank space in the background of the photo). The grid was placed over the portion of the photographed root that appeared to be most populated by AMF. Then, the number of grid squares (out of 64) in which there were any AMF were counted as 'present.' The number of 'present' squares were totaled for a root, and were then divided by 64 (total number of grid squares) to calculate the percent cover of AMF. This quantification process was repeated for all 60 roots.

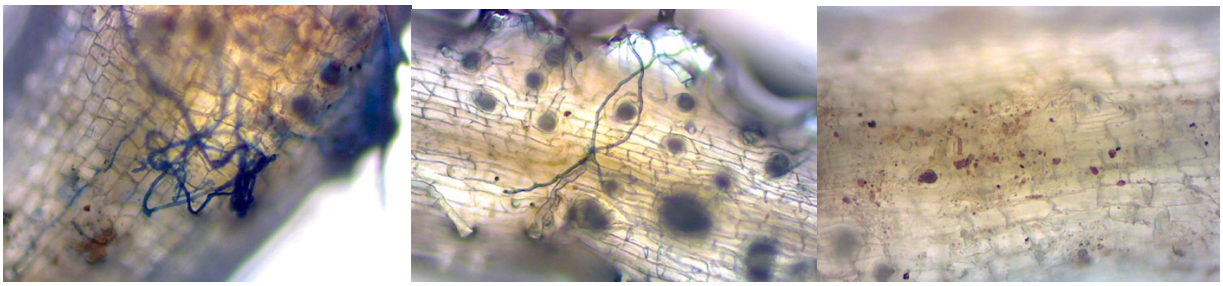

Figure 4. Photographs of arbuscular mycorrhizal fungi (AMF) in plant roots, taken at 10x magnification under a compound microscope. AMF may exist as hyphae, arbuscules, and/or vesicles. From left to right: a root from an organic coffee plot, a root from a conventional coffee plot, and a root from a nearby secondary forest. These photographs are not necessarily representative of the mean AMF percent cover for each of these 3 sites, but provide clear examples of how AMF appear under the microscope after processing, and show the types of photos used during percent quantification.

RESULTS

Percent cover data were not normally distributed, so a non-parametric analysis was used to examine the differences in median AMF percent cover of roots between secondary forest, organic coffee plot, and conventional coffee plot. Sites differed in root AMF percent cover (Kruskal-Wallis 18.375, df = 2, p = 0.0001023). Median AMF percent cover per root was lower in the conventional plot than in the organic plot and the forest. The latter two were not different from each other (Fig. 5).

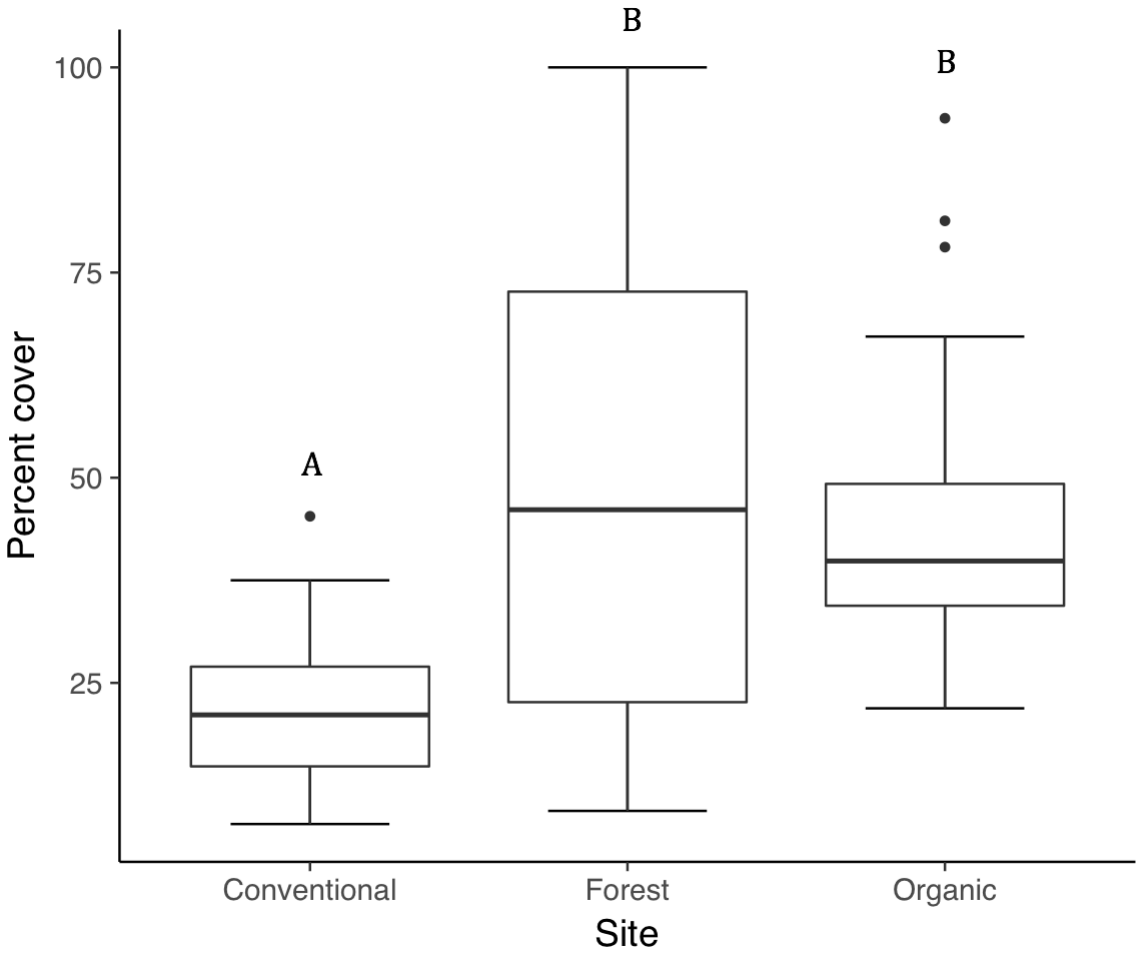

Figure 5. Arbuscular mycorrhizal fungi (AMF) differ in percent cover on roots of coffee plants from a conventional coffee plot and an organic coffee plot, and tree individuals from a nearby secondary forest in premontane wet forest in Monteverde, Costa Rica. AMF percent covers were calculated using digital photographs of roots at 10x magnification under a compound microscope. Boxes show the 25th and 75th percentile of the data, the median is represented by the solid line inside the box. Whiskers represent 1.5 times the interquartile range. Different letters on top of boxes indicate significant differences in post-hoc pairwise comparisons between means according to Mann-Whitney U tests ($p<0.05$ adjusted for multiple comparisons using the Holm's method) following the detection of significant differences between treatments using a Kruskal-Wallis test.

Additional Observations

The coffee plants in the conventional plot and the organic plot appeared equally healthy, upon observation.

DISCUSSION

While AMF were present in all three sites, the conventionally managed plot had a significantly lower density of AMF colonization than the other two sites. Secondary forest and the organic plot, conversely, supported similar abundances of AMF. Results from this study lend insight into how different agricultural management practices, specifically of a valuable tropical crop, affect

AMF abundance. These results also demonstrate how such practices alter AMF abundance from its relatively undisturbed state in a secondary forest, in the Costa Rican premontane wet forest.

The lack of difference in AMF abundance between the organic coffee plot and secondary forest may signify that the organic crop is more resemblant of natural AMF communities. This finding aligns with a previous study which showed that tropical coffee production systems share the same diversity of AMF species as a tropical montane cloud forest patch in Mexico, as measured by AMF spores in topsoil (Arias, et al. 2012). Higher AMF abundance may also indicate that the use of compost in tropical agriculture promotes healthier soils, since microbial parameters (AMF included) are effective and consistent indicators of management induced changes to soil quality (Bending, et al. 2004).

The reduced abundance of AMF in the conventional plot highlights the negative impact of conventional agricultural practices on the prevalence of beneficial AMF communities in tropical cropland, and could have critical implications for crop health (Gosling, et al. 2006). The symbiosis between crop plants and AMF generally increases plant performance in soils of low nutrient status, as AMF provide plants with nutrients, particularly those nutrients which are less mobile, like phosphorus (Thingstrup, et al. 1998). Thus, abundant and diverse AMF communities may be required for the maintenance of acceptable yields of many crop species when grown in low-P soils (Barea and Jeffries, 1995; Bethlenfalvy and Linderman, 1992; Miller et al., 1995). Such a positive response by coffee plants to AMF inoculation has been documented under tropical soil conditions (Rivera, et al. 2007). Osorio *et al*. (2002) found that *Coffee arabica* seedling growth was improved by organic treatment and AMF inoculation, when compared to coffee plants in unamended soil. Furthermore, mycorrhizal coffee plants have exhibited better growth than non-mycorrhizal plants due to higher accumulations of N, Ca and Mg (Vaast and Zasoski, 1992). Since both plots appeared to be equally healthy (similar in height and density, etc.) in this study, the reduced abundance of AMF in the conventional plot might be compensated for by the current application of agrochemicals.

The higher density of AMF colonization in the organic plot, when compared to the conventional, is likely due to the exclusion of fertilizers, fungicides, and many biocides - substances which typically reduce AMF inoculation and colonization in crops (Gosling, et al. 2006, Thingstrup, et al. 1998). Additionally, the use of compost itself in the organic plot, and not solely the absence of agrochemicals, may be responsible for this result. AMF inoculation and compost application both independently increased the yield and nutrient uptake of wheat in a laboratory experiment in Pakistan, but the highest improvement was observed with the inoculation of AMF with compost (Jan, et al. 2014). This may also be the case in coffee crops, given that compost-treated coffee supports higher AMF abundance, in a tropical cloud forest area. Further studies should be conducted to assess the specific relationship between compost and AMF communities, and to provide insight into how the combination of AMF and compost may best be exploited to improve yield.

Unfortunately, organic agriculture in developing countries, like Costa Rica, produces -43% of the yield of conventional agriculture in these same areas, on average (Seufert 2012). While this trend may be due to a multitude of abiotic and biotic factors, promoting AMF abundance may be one method of encouraging higher plant productivity (Friesen, et al. 1997) and higher biomass output (Manoharan, et al. 2017). Additionally, since AMF are biofertilizers, meaning they increase nutrient use efficiency (Rouphael 2015), the increased abundance of AMF in organic farming may become a substitute for the reduced fertilizer and biocide inputs that organic farms often still use (Gosling, et al. 2006). Therefore, because organic coffee farming in

a tropical cloud forest allows for significantly greater propagation of AMF than conventional coffee farming, organic agricultural practices may be crucial for further reducing reliance on agrochemicals and generating ecosystem services, in the tropics, whilst potentially increasing yield (Andrade, et al. 2009), through the sustenance of AMF.

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Cloud Forest moss leaf morphology and mist capture

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ABSTRACT

Mosses absorb water primarily through their leaves via foliar water uptake. This input of moisture in cloud forests provides opportunities for many moss species to thrive. This study examines moss diversity in Pacific slope Tropical Premontane Wet, Lower Montane Wet, and Lower Montane Rain forest in Monteverde, Costa Rica, from 1550 to 1780 m. From this community, nine common moss species differing in morphology were selected to measure differences in their water retention, rate of water capture and rate of water loss. 36 morphospecies were found largely with traits that correlated with low surface area to volume ratios (80% without awn, 69% without leaf curvature, 70% with smooth leaves). Moss water retention and rate of water capture increased with density of leaves (max increase of 3,660 ± 239% of dry weight). Conversely, morphospecies with slowest water loss had least dense leaves. Branching species and those with awns also performed better in water capture but worse in water retention. A high surface area to volume ratio likely provided more sites for water to condensate from a gaseous phase. Mosses with greater water retention abilities, however, were able to store more water due to fewer pathways for heat transfer.

RESUMEN

Los Musgos absorben agua principalmente con sus hojas a través de la captación foliar de agua. Esta introducción de humedad en los bosques nubosos crea oportunidades para que muchas especies de musgos puedan prosperar. Este estudio examina la diversidad de musgos en la vertiente Pacífica en el Bosque Tropical Mojado Premontano, Mojado Montano Bajo, y Lluvioso Montano Bajo en Monteverde, Costa Rica desde los 1550 a los 1780 metros. Para esta comunidad, nueve especies de musgos fueron diferenciados por características físicos para determinar diferencias en la retención de agua, tasa de captura de agua, y tasa de perdida de agua. 36 morfoespecies fueron encontrados en general con características correlacionadas con bajas relaciones de superficie a volumen (80% sin aristas, 69% sin curva de la hoja, 70% con hojas lisas). La retención de agua por los musgos y la tasa de captura de agua aumenta con la densidad de las hojas (incremento máximo de 3,660 ± 239% del peso seco). Inversamente, las morfoespecies con una menor pérdida de agua tienen hojas menos densas. Las especies con ramificaciones y aquellas con aristas también tienen una mejor captura de agua pero peor retención de agua. Una mayor proporción superficie volumen provee más sitios para la condensación del agua de la fase gaseosa. Los musgos con mayores abilidades de retención de agua, sin embargo, son capaces de almacenar más agua debido a las pocas vías para la transferencia de calor.

INTRODUCTION

WITH THE INCREASING INFLUENCE OF CLIMATE CHANGE, ecosystems are under threat. Cloud forests, ecosystems characterized by mist from adiabatic cloud formation (Goldsmith et al. 2012), are only present in 1.4% of tropical forest area and are increasingly impacted by climate change through lifting cloud cover (Pounds et al. 1999, Goldsmith et al. 2012, Karmalkar et al. 2008). Low-level cloud cover provides an additional source of water without increasing soil wetness and encourages species, such as many mosses, that engage in foliar water uptake (Kerr and Beardsell 1975; Boucheret al. 1995; Burgess and Dawson 2004; Breshears et al.2008; Ewing et al. 2009, Goldsmith et al. 2012). Factors such as water availability have significant impact on leaf morphology (Xu et al. 2009) and thus cloud forests

provide a unique environment to study how leaf morphology impacts the success of mosses in their water capture and retention. Determining what morphological features are abundant and successful in cloud forests of Monteverde, Costa Rica can impact new technologies for fog harvesting, using moss leaf morphology as a guide for artificial structures, and help predict moss community structure and richness as climate continues to change.

Foliar water uptake, the ability to absorb water directly through leaves, is characteristic of mosses (Brown 1982; Brown and Bates 1990). It is critical in the misty days of dry season in cloud forests that are otherwise receive no water (Rundel 1982, Goldsmith et al. 2012). This method of hydration immediately increases water content. (Boucher et al. 1995, Breshears et al. 2008, Gouvra and Grammatikopoulos 2003, Grammatikopoulos and Manetas 1994, Yates and Hutley 1995). With elevation, capacity for foliar water uptake in moss improves (Goldsmith et al. 2012). Several studies of moss diversity and capacity for holding water in the Monteverde Cloud Forest Reserve find similar trends (Nichols 2013, Hagen-Botbol 2016, Rose 2016). Beyond elevation, morphological differences likely play a key role in water holding capacity (Riani 2017).

Riani's (2017) hypothesis agrees with findings of a study on water capture mechanisms of the desert moss *S. caninervis* (Pan et al. 2016). Here, 4 key structures on the awn (leaf hair point growing from the tip of the leaf structure) are crucial to *S. caninervis*'s survival in the desert (Pan et al. 2016). This includes longitudinal nano-grooves, longitudinal micro-grooves, small barbs, and tightly packed clusters of awns (Pan et al. 2016). All these structures facilitate nucleation - in this context, the self-organization of water into a liquid phase from a supersaturated gas phase (Pan et al. 2016). It is likely that other mosses share similar characteristics, using morphological structures to assist nucleation or avoid water loss via evaporation in the Monteverde dry season.

Cloud forest mist quantity and frequency are changing with increasing temperature due to anthropogenic pressures (Goldsmith et al. 2012, Karmalkar et al. 2008). Climate simulations find that highlands are predicted to experience more consecutive mist-free days during the dry season (Pounds et al. 1999, Karmalkar et al. 2008). The Pacific slope will experience more warming than the Atlantic, with both slopes experiencing more consecutive dry season days as a result (Karmalkar et al. 2008, Goldsmith et al. 2012). Moisture supply will decrease, and cloud formation will occur at higher altitudes (Pounds et al. 1999, Karmalkar et al. 2008). All these findings indicate a future in Monteverde with higher temperatures, less, and more variable precipitation, putting organisms that rely on precipitation and cloud cover at risk.

Exploring adaptations for foliar water uptake in other cloud affected ecosystems (cloud forest versus coastal desert) may provide insight to how moss community richness changes with limiting factors in moisture availability. To cope with dry season, it is possible mosses have adapted morphologies to make water capture and retention more effective. Because moisture is abundant in this environment, it is likely that water capture is not as crucial, and instead water retention is favored for withstanding drier periods. Should Monteverde mosses demonstrate similar morphological aspects as in desert environments, we would expect high surface area to volume ratios as a common mechanism to generate more nucleation sites (Pan et al. 2016, Goldsmith et al. 2012). This study investigates differences in water capture, retention, and holding (capacity) among different moss morphologies for common moss species found on the Pacific slope in Tropical Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests of Monteverde, Costa Rica to begin to determine what physical traits are important in water uptake and retention. By determining which traits provide the greatest advantage in fitness

we might be able to predict how community structure and richness will be altered with climate change.

MATERIALS AND METHODS

FIELD STUDY

The field study was conducted from 17 October 2018 to 6 November 2018, at end of wet season, on Pacific slope trails directly behind the Estación Biológica in Monteverde, Costa Rica. The study site encompassed three Holdrige lifezones, premontane wet, lower montane wet, and lower montane rain forests, from elevations 1550-1780 (Holdrige 1967). Annual rainfall ranges between 1.85 – 4 m, with mist input averaging around .625 m / yr. The mean annual temperature is 12 - 17°C. Canopy height in this primary forest is between 20 – 30 m. The study focused on understory mosses that occur in this community to assess moss diversity in the area. Samples of mosses were taken near and along the continental divide among several altitudinal gradients.

FIGURE 1. Sample sites in Tropical Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests on trails behind the Estación Biológica in Monteverde, Costa Rica from which common mosses of different morphologies were collected to assess their water uptake and retention. Images and samples taken during end of wet season in late October 2018.

Moss samples were separated by morphospecies and analyzed for a suite of traits that could impact water uptake and retention: leaf shape, presence of a midrib, cell shape, leaf texture, branching, leaf curvature, and presence of awns (a thin protrusion at the end of the leaf). Unique morphospecies were stored, marked, and photographed three times at varying magnifications (0.8x, 3.5x, 10x) to capture morphological differences. Once all differences were categorized morphospecies were assembled into a diversity key of the community, with relative abundances of each key trait.

The nine morphospecies of moss chosen for lab trials were determined by their relative abundance and morphological characteristics that allowed comparison between species to isolate key traits. Distance between leaves (categorized by eye into three relative groups – low density, medium density, and high density) were recorded for these morphospecies. Fifteen samples of each morphospecies were collected. Moss mats were cleaned of dirt and debris in tubs of water and again through a rinse in running water. Volume of mosses were standardized to the best

ability possible by creating 2 cm diameter spheres measured with a cutout of a 2 cm PVC pipe, and again with calipers.

FIGURE 2. Morphospecies of mosses collected for lab trials from study sites of the Pacific slope of Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests Forest of Monteverde, Costa Rica. From left to right, row one, morphospecies 34 (A), 17 (B), 29 (C), 28 (D), 3 (E). From left to right, row two, morphospecies 20 (F), 36 (G), 25 (H), and 9 (I). Used as abundant mosses between 1520 – 1800 m in forest behind Estación Biológica.

LAB TRIALS

Samples were inundated in water at ambient temperature for 10 hours, with water barely covering the tops of each sample. Samples were hung to drip-dry during a 40-minute time period on mesh and weighed for water holding capacity after dripping ceased for each individual.

FIGURE 3. (Left) 10-hour water bath setup for inundation of moss samples. (Right) Drip-dry setup for moss samples collected at various altitudes of Pacific slope Tropical Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests in Monteverde, Costa Rica, in order to calculate water holding capacity.

To measure water retention, samples were dried in a Garden Master food dehydrator under low heat (35°C) and a circulating fan. Samples were dried for 10 hours. In this 10-hour period, sample weight was recorded at intervals of 10 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, and

10 hours to calculate the rate of dehydration. The final 10-hour weight was used to calculate percent water as a ratio of wet mass to dry mass for each sample. After 10 hours, all samples had reached a satisfactory asymptote in weight to be considered fully dried.

Rates of water capture were recorded by placing samples in a glass aquarium attached to a Reptifogger terrarium humidifier. Samples were elevated from the bottom of the aquaria to prevent contacting water that pooled. The ultrasonic cool mist humidifier distributed mist evenly from the center at a rate of 1.04 ml/minute. Samples were weighed at intervals of 10 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 10 hours, and 24 hours. These weights were not deemed comparable to the saturated weights due to the lack of drip drying – mosses were overly saturated after 24 hours.

FIGURE 4. (Left) Food dehydrator used in the process of determining desiccation rates of moss samples. Dehydrator was set to a heat of 35°C during sampling. (Right) Glass aquarium and misting apparatus used in the process of determining water capture rates of moss samples. Aquarium was covered and lightly sealed with humidifier entering through the center. Humidifier was set to output 1.04 ml/min for the duration of sampling. Wire mesh elevated samples off the bottom to reduce the effects of pooling water. Samples were placed equidistant from the tube to avoid effects of proximity.

RESULTS

FIELD STUDY

There were 36 moss morphospecies found in the Monteverde Cloud Forest behind the Biological Station. These species displayed a variety of traits with some being more common than others (Figure 5). Filiform and round leaf shapes were the most common leaf types (36% of morphospecies had filiform leaves, 36% were round, 28% were narrow), though several round species also had awns (5 morphospecies). Many "round" morphospecies were not entirely round, but rather closer to "round" than "narrow" (Morphospecies 12, 24, 34, 35). The majority of mosses also had branching structures (58% branching, 42% none).

Most leaves had a "smooth" texture, meaning no presence of additional structures. Toothed leaves (those with ragged edges or jutting structures) and node leaves (segmented with protruding "joints") were also present, with a single morphospecies exhibiting a rough leaf texture (70% smooth, 19% toothed, 8% nodes, 3% rough). Additionally, few species displayed an "awn", though when present, some species had more than one (83% no awn, 5% one awn, 6% two awns, 6% > two awns).

TABLE 1. Key of distinguishing traits for moss morphospecies within the Pacific slope Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests surrounding Estación Biológica in Monteverde, Costa Rica (See Appendix 1 for species breakdown). Node distance (how densely packed leaves were) was determined by relative observation between species and general categorizations from there

Branching	Leaf Density	Leaf Shape	Awns	Midribs	Leaf Texture	Cell Shape	Leaf Curvature
Single Stem	"1"	Round	None	None	Smooth	Elongate	Plane
Branching	"2"	Narrow	One	One	Toothed	Isodiametric	Upward
	"3"	Filiform	Two	Two	Nodes	Long Hexagonal	Downward
					Rough	Spindle	Reflexed

The majority of mosses had no midribs (appearing like a central vein), and only one species had two midribs (61% none, 36% one, 3% two).

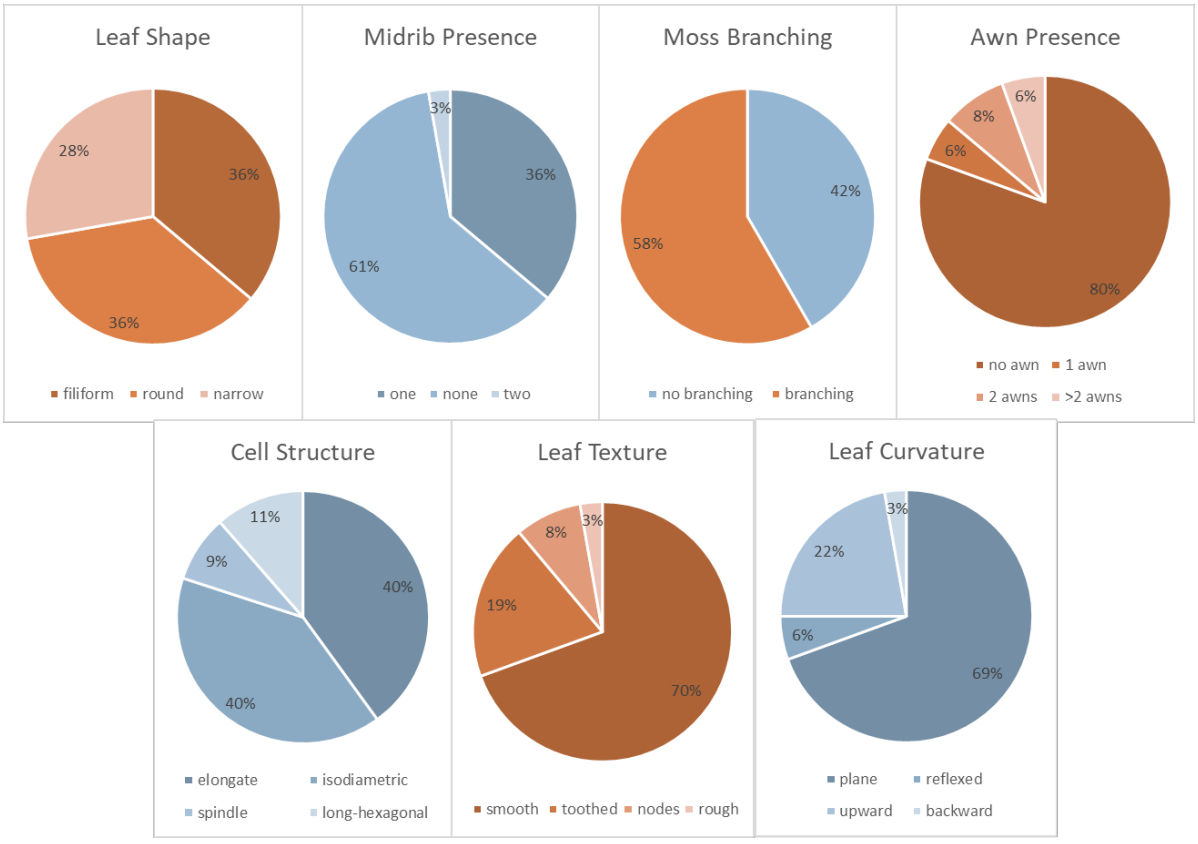

FIGURE 5. Percent occurrence of each observed distinguishing trait for moss samples within the Pacific slope Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests surrounding Estación Biológica in Monteverde, Costa Rica (See Appendix 1 for species breakdown).

TABLE 2. Observed distinguishing traits for nine chosen moss morphospecies within the Pacific slope Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests surrounding Estación Biológica in Monteverde, Costa Rica (See Appendix 1 for species breakdown). Node distance (how densely packed leaves were) was determined by relative observation between species and general categorizations from there.

	Leaf Shape	Midrib	Cell Shape	Texture	Branching	Awn	Leaf Curve	Leaf Density
A	round	0	elongate	smooth	alt	1	upward	3
B	round	0	long hexagonal	nodes	alt	>2	plane	2
C	filiform	1	long hexagonal	smooth	none	0	plane	3
D	narrow	0	elongate	smooth	alt	0	plane	3
E	filiform	0	long hexagonal	smooth	none	0	upward	3
F	narrow	1	isodiametric	smooth	none	0	plane	3
G	filiform	0	elongate	smooth	none	0	plane	2
H	filiform	1	isodiametric	toothed	none	0	plane	1
I	round	0	isodiametric	nodes	alt	>2	plane	3

LAB STUDY

Water holding capacities differed with moss species (ANOVA; $F = 70.78$, $df = 8$, $p < 0.0001$, $n = 135$; Figure 6) with morphospecies I having the highest water holding capacity at 3,660 ± 239 % of its dry mass. Post-Hoc Scheffe tests determined significant differences amongst species ($p<0.05$). Morphospecies I was shown to significantly differ from all other morphospecies (Scheffe; $p<0.0001$). Morphospecies G had the lowest water holding capacity of 526 ± 23 % of its dry mass. Morphospecies G did not significantly differ from Morphospecies B, H, or C (Scheffe; $p>0.05$).

Morphospecies I and B were significantly different (Scheffe; $p < 0.0001$; B: 743.5 ± 30.28 %) with the primary difference being distance between leaves. Round leaves such as A and I had the highest water holding capacity (Figure 6; Mean A: 1,702.63 ± 123.69%), both with a higher density of leaves. I was significantly different from all other morphospecies and A was significantly different from C (Mean; 1,015.38 ± 79.01%) and below (Scheffe; $p = 0.01719$). Furthermore, A and I both had awns while all other species besides B did not.

Narrow leafed species had the second highest water holding capacity with D and F coming in 3rd and 5th, respectively (Figure 6; Mean F: 1,228.28 ± 46.99 %, Mean D: 1,584.85 ± 121.99 %). D was significantly different from species H (Mean: 924.596 ± 87.65 %) and below (Scheffe; $p = 0.0276$) while F was only significantly different (besides I) from G (Scheffe; 0.0131).

Besides morphospecies B, branching mosses appeared to also have higher water holding capacity (morphospecies I, A, and D). Morphospecies A and E (Mean: 1542.40 ± 20.92 %) both has leaves with upward curves, perhaps assisting with their success. Other traits appeared to have little significance in water holding capacity.

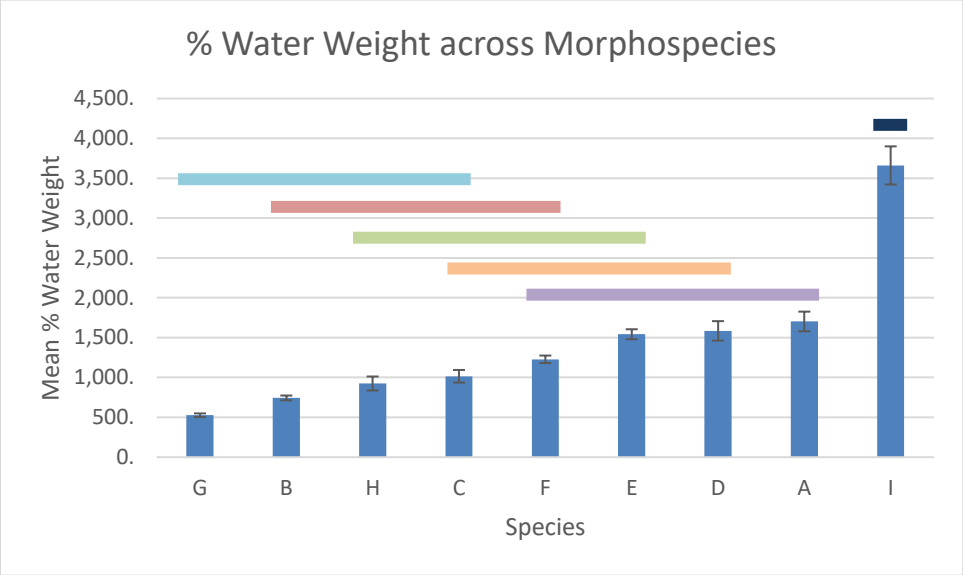

FIGURE 6. Percent water mass (wet wt./dry wt.) of mosses after 10 hours of inundation in water baths. Mosses collected from Pacific slope Tropical Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests of Monteverde, Costa Rica behind Estación Biológica. Samples collected in surrounding area from 1520 m to 1800. Alphabetical identifications indicate different morphospecies. Groups with different horizontal bars are significantly different (ANOVA; $F = 70.78$, $df = 8$, $p < 0.0001$, $n = 135$, Scheffe; $p < 0.05$)

An analysis of covariance (ANCOVA) revealed that water retention capacities were significantly different (Figure 7) between study species (ANCOVA; log(time)*species $F = 60.43$, $df = 8$, $p < 0.0001$). Rates of water capture also differed between species (ANCOVA; log(time)*species $F = 41.88$, $df = 8$, $p < 0.0001$), indicating that morphological differences lead to different rates of water capture (Figure 8) and water retention (Figure 7) properties. Looking at the relative traits and significant differences (Figure 9, Figure 10), it can be observed that leaf density is consistently important in determining water capture and retention, with the least dense morphospecies, B,G, and H, being significantly different and inferior at water capture (Figure 10, B/C Chisq; $p = 0.0060$), as well as significantly different and superior at water retention (Figure 10, G/F Chisq; $p < 0.0001$). The reverse is true for higher leaf density. Figure 10 is the culmination of Chisq Post-Hoc ANCOVA - ranking morphospecies in their effectiveness of water capture and retention. Those at the top of the list are superior at the respective functions. This allows connections to be drawn between physical characteristics and their impact on water capture and retention. It appears that branching species and those with awns are consistently better at water capture, being key distinguishing features between high leaf density significance groups IV and III (Figure 10). It also appears that no midrib performed better than one midrib in the same case. The reverse appears to be true in water retention, with general trends of species with awns and branching performing worse than those without (Figure 10)

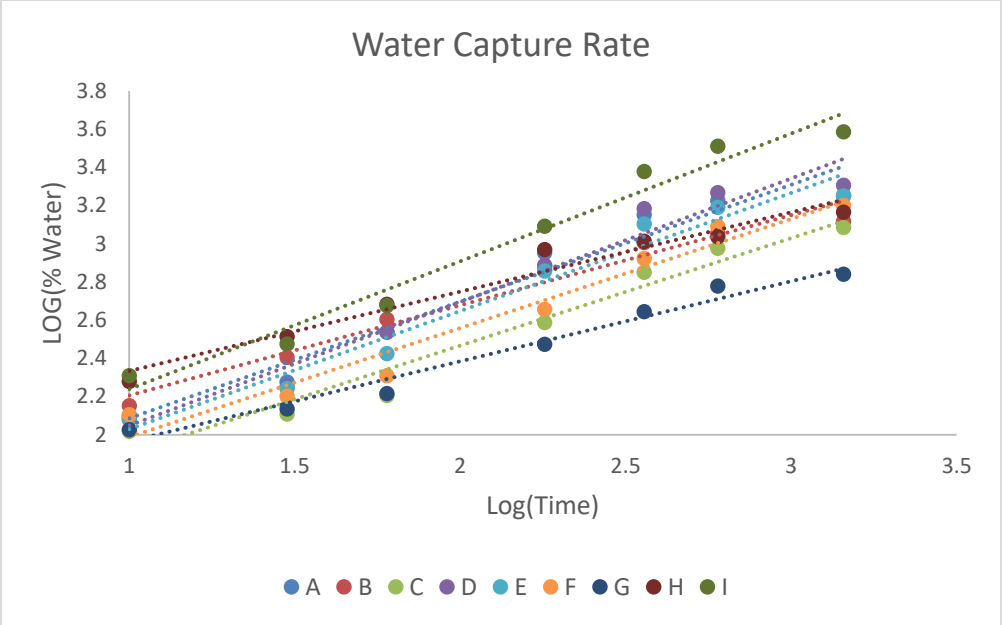

FIGURE 7. Log transformation of the percent of original water weight of moss samples over a 10 hour period in GardenMaster food dehydrator. Moss samples collected from Pacific slope Tropical Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests of Monteverde, Costa Rica behind Estación Biológica. Samples collected in surrounding area from 1520 m to 1800. Rates of dehydration (interaction between log(Time) and morphospecies) were significantly different between study species (ANCOVA; log(Time)*species $F = 60.43$, $df = 8$, $p < 0.0001$). Alphabetical identifications and color of points and lines indicate different morphospecies (See Appendix 1 for species identification).

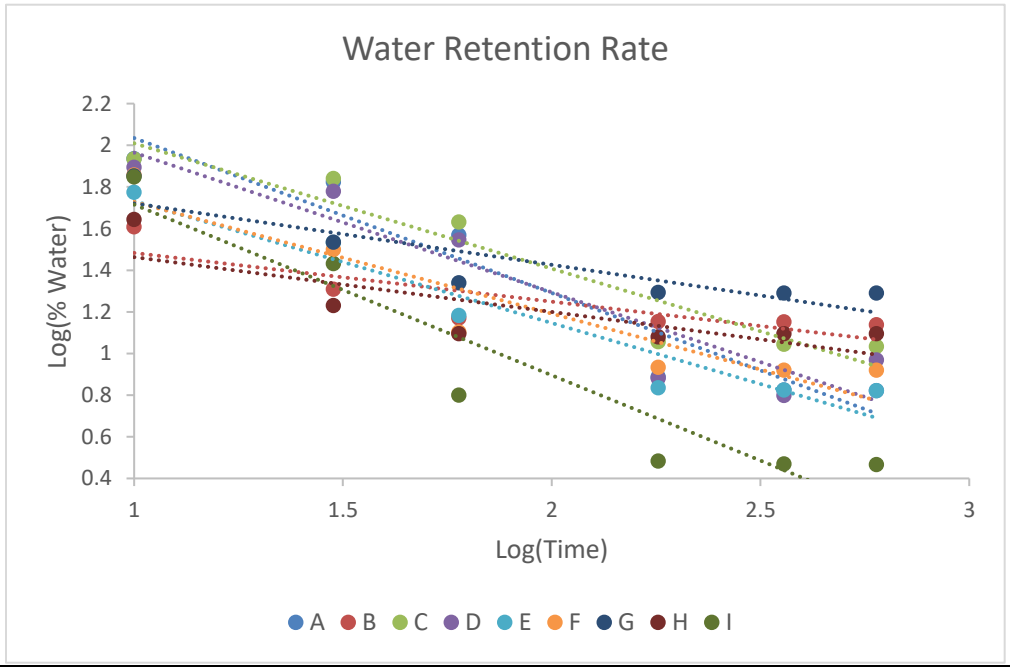

FIGURE 8. Log transformation of the percent water weight of moss samples over a 24-hour period in aquarium misting apparatus. Moss samples collected from Pacific slope Tropical Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests of Monteverde, Costa Rica behind Estación Biológica. Samples collected in surrounding area from 1520 m to 1800. Rates of dehydration (interaction between log(Time) and morphospecies) were significantly different between study species (ANCOVA; log(Time)*species $F = 41.88$, $df = 8$, $p < 0.0001$).

Alphabetical identifications and color of points and lines indicate different morphospecies (See Appendix 1 for species identification).

TABLE 3. Figure-Table of p-values for Chisq post-hoc ANCOVA. Moss samples collected from Pacific slope Tropical Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests, Costa Rica behind Estación Biológica. Samples collected in surrounding area from 1520 m to 1800. Blue values indicate relationships between species in water capture and orange values for relationships in desiccation. Bolded values are considered significant (Chisq; $p < 0.05$).

ANCOVA Chisq		WATER CAPTURE								
		A	B	C	D	E	F	G	H	I
DESICCATION	A		**<0.0001**	**0.0360**	0.2529	1.0000	0.2520	**<0.0001**	**<0.0001**	0.0659
	B	**<0.0001**		**0.0060**	**<0.0001**	**<0.0001**	**0.0001**	0.0701	0.1395	**<0.0001**
	C	**0.0037**	**<0.0001**		**<0.0001**	**0.0113**	1.0000	**<0.0001**	**<0.0001**	**<0.0001**
	D	1.0000	**<0.0001**	0.1268		0.4092	**0.0006**	**<0.0001**	**<0.0001**	1.0000
	E	**0.0001**	**<0.0001**	1.0000	**0.0121**		0.1433	**<0.0001**	**<0.0001**	0.1395
	F	**<0.0001**	**<0.0001**	0.2697	**<0.0001**	1.0000		**<0.0001**	**<0.0001**	**<0.0001**
	G	**<0.0001**	0.6480	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**		1.0000	**<0.0001**
	H	**<0.0001**	1.0000	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	1.0000		**<0.0001**
	I	1.0000	**<0.0001**	**<0.0001**	0.1268	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	

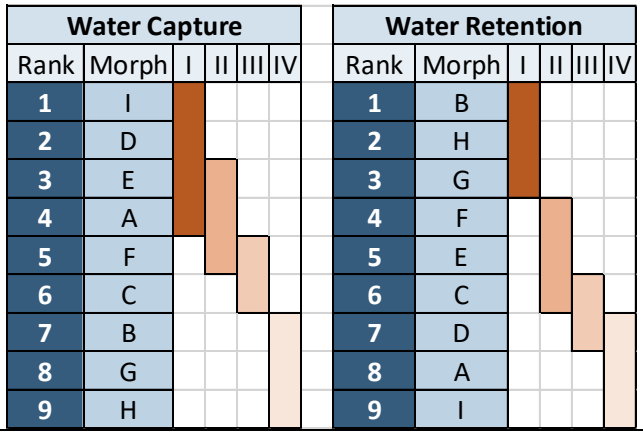

FIGURE 10. Figure-Table of significance for Chisq post-hoc ANCOVA. Moss samples collected from Pacific slope Tropical Premontane Wet, Lower Montane Wet, and Lower Montane Rain forests of Monteverde, Costa Rica behind Estación Biológica. Samples collected in surrounding area from 1520 m to 1800. Morphospecies of the same color are not significantly different (Chisq; $p < 0.05$). For water capture, morphospecies are ranked from best at capture to worst. For water retention, morphospecies are ranked from best at retention to worst.

DISCUSSION

36 morphospecies were found, many with unique combinations of recorded physical traits. Some of these species held traits that potentially maximized water capture. Most species were round or filiform in shape. The abundance of filiform species correlates with the findings of a study which determined that awns were a crucial mechanism for fog capture of the desert moss *S. caninervis* (Pan et al. 2016). Functionally, filiform species could be considered similar to awns, with the key difference being the stiffness of the thin structure protruding from the leaf. The majority of mosses also had branches which increase surface area and may enable mosses to intercept greater

quantities of mist. However, beyond these two factors, most species minimized surface area. The abundance of round species (as they observationally take up more room) may result in a smaller surface area. Awns, toothed, and node structures on leaves were uncommon, as were leaves with some manner of curvature.

Data from lab trials supports that a higher surface area to volume ratio maximizes water capture while a lower surface area to volume ratio maximizes water retention. Water retention and water capture showed strong trends with leaf density, with the most densely packed stems providing both the best species for water capture as well as the worst species for water retention. This makes physical sense via the effect of a high surface area to volume ratio, wherein water capture is easier, attaching to more surfaces, but unable to be stored within the volume of a moss and resist a dry climate. Awns, also known as leaf hair points, help to increase water content by speeding up dew formation and snagging water droplets in microgrooves (Pan et al. 2016, Tao and Zhang 2011). Those with awns in the Monteverde community performed consistently better in water capture, agreeing with findings for desert *Syntrichia caninervis* (Pan et al. 2016). Species such as morphospecies I may have been successful in water capture and water holding capacity in part due to multiple, tightly packed awns such as those in *S. caninervis* (Pan et al. 2016). Branching may have been a crucial aspect in improving water capture, providing more surface area for water droplets to adhere to.

The reverse is true in water retention where awns and branching structures are worse at holding water. Again, with concepts of surface area to volume ratios, this is a logical trend. A significant trend appears wherein species suited for water capture are poorly adapted for water retention. This is likely due to the reasons established above; while more protruding structures provide more sites for nucleation to occur, it also provides more pathways for heat transfer. Only one morphospecies, E, was relatively adept at both water capture and retention. This can possibly be attributed to its high leaf density and filiform leaf shape that would theoretically aid in capturing water, while maintaining capacity for water retention through observationally thick leaves and a single stem.

These trends are further supported when comparing two similar species. Morphospecies I and B maintained consistent qualities aside from distance between leaf nodes, where morphospecies I had more dense packaging of leaves. For likely this reason, morphospecies I was better at water capture and total holding capacity, but worse at water retention. offers more support for the idea that increasing surface area to volume ratio aids water capture.

Continued anthropogenic pressures will have increasingly large impacts on cloud forest ecosystems over time. Both Atlantic and Pacific slopes will experience increased water stress and more consecutive dry days, though pressures on moisture will have greater effect on the pacific side (Karmalkar et al. 2008). Because Monteverde's cloud base will continue rising, providing less moisture outside of the soil system, moss diversity and richness are threatened. In this scenario, morphospecies with fewer traits suited for water retention may suffer - in other words, those adept at water capture. It is possible that these species will gravitate to higher elevations to cope with reduced moisture and higher temperatures, as has been observed with tropical tree species in Costa Rica (Feeley et al. 2013). If species are unable to cope in this manner, or as changes in cloud cover become more extreme, the Monteverde community may become more morphologically homogeneous over time, with traits corresponding to low surface area to volume ratios.

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Thank you to the Monteverde Biological Station for this unique opportunity to study in a beautiful landscape. Thank you to my parents for providing me the means to participate in this program. Thank you to Orlando for his constant contribution to the success of this program and thank you to Gisella, Johel, Alan, Moncho, and Karla for giving us the knowledge required to take on a task such as this. Thank you to everyone in this program for making it a time to remember.

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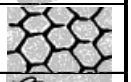
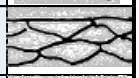
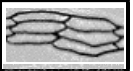
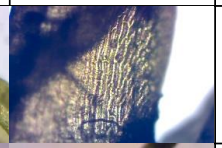
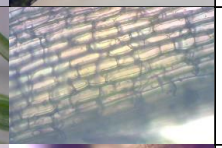
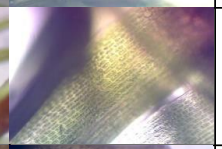
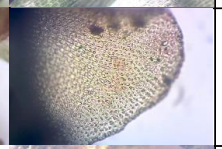
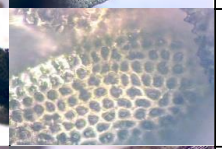
APPENDIX 1.

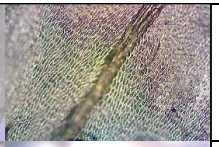
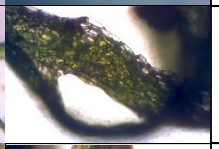
TABLE 4. Glossary of terms for physical traits and corresponding images.

Term	Description	Image
Awn	leaf hair point growing from the tip of the leaf structure - stiff, bristle-like appendage	
Branching	Central stem has branching structures with leaves along both branches and stem	
Downward	Leaf curves down towards the underside	
Elongate	Long, narrow "rectangular" cells	
Filiform	Leaf shape that is almost threadlike	
Isodiametric	Hexagonal shaped cells	
Leaf Density	How tightly leaves are packed on a stem	
Long Hexagonal	Hexagonal shaped cells stretched width-wise	
Midrib	A strengthened vein along the middle of a leaf	
Narrow	Leaf shape that comes to a narrow point	
Nodes	Large bumps appearing to segment leaves - appearing like joints	
Plane	Flat leaf curvature	
Reflexed	Leaf curvature creating a valley within the center of the leaf	
Rough	Bumpy structures on leaf	
Round	Leaf shape that is similar size in width and length	
Single Stem	No branching, only one "central stem" present	
Smooth	No additional structures on leaf - smooth in texture	
Spindle	Almond shaped cells that are fairly uniform	
Toothed	Additional barb like structures on the edges of leaves	
Upward	Leaf curves up towards the top	

APPENDIX 2.

TABLE 5. Identified morphospecies of moss from study sites in a Pacific slope Tropical Lower Montane Wet-Rain Forest of Monteverde, Costa Rica. All specimens collected at end of wet season in areas surround La Estación Biológica in Monteverde, Costa Rica.

Morphospecies	0.8 x	3.5 x	10 x	Leaf Shape	Midrib	Cell	Leaf Texture	Branching	Awns	Rolling	(Nichols, 2013)
Morpho 1 *Herbertus spp.*				narrow	0	elongate	smooth	none	2	reflexed	Morpho 10
Morpho 2	N/A			narrow	0	elongate	smooth	yes	0	upward	
Morpho 3 **(E)** *Leycobryum martianum*				filiform	0	long-hexagonal	smooth	none	0	upward	Morpho 7
Morpho 4				filiform	0	long-hexagonal	smooth	none	0	upward	
Morpho 5				round	0	isodiametric	smooth	none	0	backward	
Morpho 6 *Thuidium spp.*				round	0	isodiametric	rough	yes	1	upward	Morpho 9
Morpho 7				narrow	1	elongate	smooth	yes	0	plane	

Morpho 8				narrow	1	spindle	smooth	yes	0	plane	
Morpho 9 **(H)** *Trichocolea spp.*				round	0	isodiametric	nodes	yes	4	plane	Morpho 19
Morpho 10				filiform	0	isodiametric	smooth	yes	0	plane	
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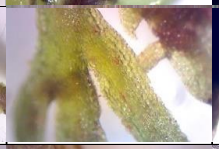
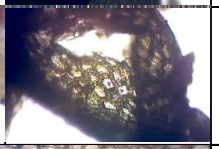
Morpho 12				round	1	isodiametric	smooth	yes	0	plane	
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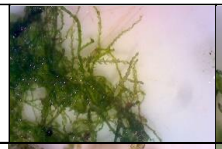
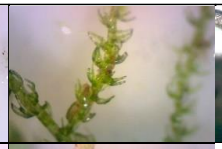
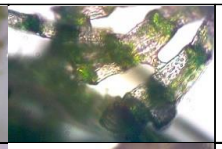
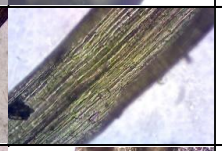
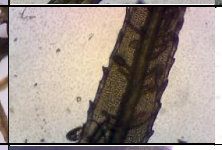
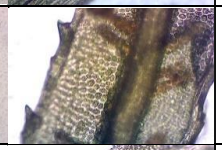
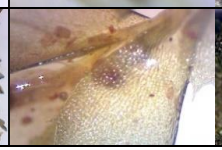
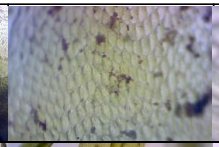
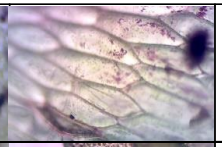
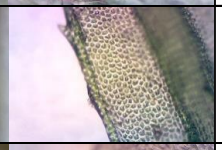
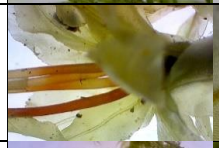
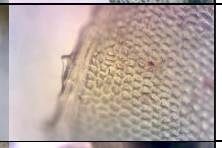
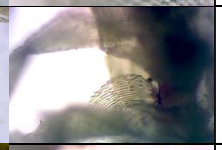
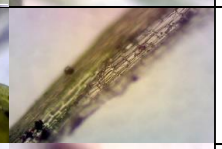
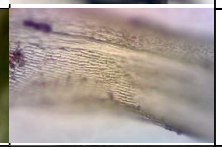
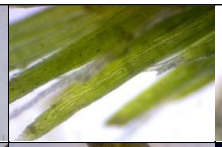
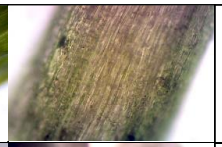
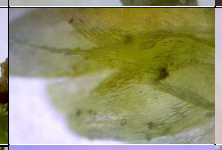
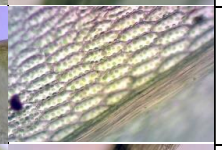
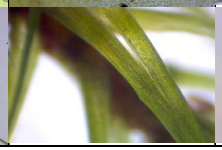
Morpho 14				narrow	2	elongate	smooth	yes	0	plane	
Morpho 15				narrow	0	isodiametric	toothed	yes	2	plane	

Morpho 16				round	0	isodiametric	nodes	yes	2	plane	
Morpho 17 **(B)** *Pilotrichella spp.*				round	0	long-hexagonal	nodes	yes	4	plane	Morpho 3
Morpho 18				filiform	1	elongate	toothed	none	0	plane	
Morpho 19				filiform	1	isodiametric	toothed	none	0	upward	
Morpho 20 **(F)**				narrow	1	isodiametric	smooth	none	0	plane	
Morpho 21				filiform	0	long-hexagonal	smooth	none	0	plane	
Morpho 22				narrow	1	elongate	smooth	yes	0	plane	
Morpho 23				round	0	isodiametric	toothed	yes	0	plane	

Morpho 24				round	0	spindle	smooth	yes	0	plane	
Morpho 25 **(H)**				filiform	1	isodiametric	toothed	none	0	plane	
Morpho 26				round	1	isodiametric	toothed	none	0	plane	
Morpho 27				narrow	0	elongate	smooth	yes	0	plane	
Morpho 28 **(D)**				narrow	0	elongate	smooth	yes	0	plane	
Morpho 29 **(C)**				filiform	1	long-hexagonal	smooth	none	0	plane	
Morpho 30				filiform	0	elongate	smooth	yes	0	reflexed	
Morpho 31				filiform	1	elongate	smooth	none	0	plane	

Morpho 32				filiform	0	elongate	smooth	none	0	plane	
Morpho 33				round	0	isodiametric	smooth	yes	0	upward	
Morpho 34 **(A)** *Hypnum spp.*				round	0	elongate	smooth	yes	0	upward	Morpho 13
Morpho 35				round	1	spindle	toothed	none	0	plane	
Morpho 36 **(G)**				filiform	0	elongate	smooth	none	0	plane	

Moss and Leaf Succulence as Buffers to Climate Change for Cloud Forest Epiphytes

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ABSTRACT

Climate change is decreasing dry season mist frequency in Tropical Montane Cloud Forests (TMCFs). This mist is essential to the abundance and diversity of epiphytes that grow there. Epiphytes may mitigate the effects of climate change via leaf succulence or growing on moss. To test this, two epiphytic *Peperomia* sp. (thick and thin) were placed with and without moss in four different misting conditions over the course of 20 days. Mist conditions represented historical (every other day), projected (every fifth day), and extremes (mist everyday and no mist). It was found that presence of moss did not significantly impact mean leaf length, width, and thickness of both thick- and thin-leaved *Peperomia* spp., regardless of mist condition. Leaf succulence however did decline drastically in the dry mist condition (average leaf thickness at time zero = 0.815, average at time four = 0.323) while leaf width and leaf length did not change, demonstrating the succulence of the leaf working to maintain the overall health of the plant. This indicates that as the climate of TMCFs become drier due to climate change, moss will not be a great buffer for the increasing amount of dry days, but leaf succulence in more succulent species may act as a slight buffer.

RESUME

El cambio climático está disminuyendo la frecuencia de la niebla durante la época seca en los bosques nubosos montanos tropicales (TMCF por sus siglas en ingles). Esta niebla es esencial para la abundancia y diversidad de epífitas que crecen ahí. Las epífitas pueden mitigar los efectos del cambio climático a través de la suculencia de las hojas o creciendo en musgo. Para probar esto, dos especies de *Peperomia* (gruesa y delgada) se colocaron con y sin musgo en cuatro condiciones diferentes de niebla en el transcurso de 20 días. Estas representan las condiciones históricas (día de por medio), proyectada (cada cinco días), y los extremos (niebla cada 5 días y no niebla). Se encontró que la presencia de musgo no impacta significativamente el largo, ancho y grueso de tanto las hojas gruesas como las delgadas de *Peperomia* spp, sin importar la condición de niebla. La suculencia de las hojas, sin embargo, disminuye drásticamente en la condición seca (promedio de hojas gruesas al tiempo cero = 0.815, promedio al tiempo cuatro = 0.323) mientras que el ancho y largo de las hojas no cambio, demostrando que la suculencia de las hojas funciona para mantener la salud general de las plantas. Esto indica que al volverse más caliente el clima de TMCF debido al cambio climático, el musgo no va a ser un gran amortiguador para el aumento de días secos, pero la suculencia de las hojas en especies más suculentas puede actuar como amortiguador.

INTRODUCTION

Tropical Montane Cloud Forests (TMCFs) occur where mountains are frequently enveloped by tradewind-derived orographic clouds and mist in combination with convective rainfall (Still et al. 1999). Dry season mist is essential for many Cloud Forest epiphytes, as this is their only source of water and nutrients (Still et al. 1999). With deforestation and climate change, trade wind-orographic clouds are lifting the cloud base, decreasing mist frequency in the dry season (Pounds et al. 1999; Freedman et al. 2001; Sperling et. al. 2004). In response to drier conditions, some species may seek refuge in higher altitudes, some may suffer in their current habitats, others may not be able to withstand the climate and go extinct, at least locally (Pounds *et al* 1999). Epiphytic species in the tropics compose about 25% of the vascular plants and contribute significantly to local cloud forest biodiversity (Ibisch et al. 1996; Kress 1986). Epiphytes are often mist dependent, due to absorption and retaining of atmospheric water and nutrients directly, this dependency is especially important in areas with a pronounced dry season (Nadkarni and Solano 2002; Eggli & Nyffeler 2009).This becomes a problem as the predicted cloud cover is expected to move up as climate warms (Still et al. 1999). These Cloud Forest epiphytes are expected to be negatively affected by predicted climate changes due to their need for mist (Nadkarni and Solano 2002).

In order to survive these drier conditions, epiphytes are going to need extra sources of water. Morphological adaptations in epiphytes that reduce water stress include leaf and stem succulence (Herrera 2000). Succulence, which allows water storage for further use when its availability decreases and when its demand increases, is one of those plant features typically viewed as adaptive to arid environments (Eggli & Nyffeler 2009). This suggests that succulence can act, as this needed water source for many epiphytes as drier days become more common.

Epiphytes lacking key morphological adaptations to water stress may rely more heavily on water-holding substrates to buffer dry conditions, like moss. Bryophytes, or moss, can store water (Hedderson & Longton 1996). Gregor (2018) found that depending on the morphology of the moss, water storage could be more than 200% of its dry mass, indicating its large water holding capacity. With moss' ability to hold significant amounts of water and collect mist within their shoots from precipitation, they could be beneficial to epiphytes that grow on them. Prats (2015) found that more epiphytes grow where there is more moss growth, and that mosses and epiphytes have similar moisture and growth requirements. This suggests that as the climate warms, and the cloudbank in tropical montane cloud forests continues to rise, creating more days between mist in the dry season, moss may be a possible extra water source for plants. This may be a crucial relationship in keeping many epiphytic species moist enough to survive.

Peperomia spp (Piperaceae) are commonly found as epiphytes in Neotropical Cloud Forest. There are several species that can be found in Monteverde that vary in plant succulence. Due to *Peperomia*'s poorly developed root systems (Herrera *et al.* 2000) they frequently experience water deficit and are therefore sensitive to drought periods. This is because they are epiphytes without the ability to store large amounts of water in tanks or pseudobulbs, and therefore respond quickly to drought (Gutierrez 2012). Leaf succulence in some *Peperomia* spp can offset water sensitivity because there is more hydrenchyma, a water storing tissue, present within the plants leaves (Schmidt 1987). Conversely, thin leaved *Peperomia spp.* may be more dependent on dry season mist due to fewer hydrenchyma.

Nadkarni and Solano (2002) found that when different species of epiphytes were transplanted to lower elevations to simulate future drier climate conditions they found significant reduced longevity of *Peperomia* at the lower elevation relative to the mid-elevation site, suggesting that *Peperomia* will be a species disproportionally affected by these drier conditions.

Here, I examine if leaf succulence and association with moss ameliorate immediate impacts of reduced mist frequency for *Peperomia* spp. *Peperomia* spp with more and less leaf succulence, with and without moss are exposed to different mist frequencies. This will show how many epiphytes are responding to climate change as mist frequency in Tropical Cloud Forests declines.

MATERIALS AND METHODS

Study Site

Collection of epiphytic *Peperomia* spp was conducted in forest above La Estacíon Biológica Monteverde, Puntarenas, Costa Rica. This forest is classified as a Lower Montane Wet Forest. It is located at 1550 meters in elevation on the Pacific side, and is considered secondary forest with a canopy height of around 30 meters (Bolaños and Watson 1993). Average precipitation in Monteverde is 2.5 meters of rainfall per year and 0.675 meters of mist per year, the latter being a unique and extremely important characteristic of Cloud Forests (Nadkarni and Wheelwright 2000).

Study Species

Epiphytic *Peperomia* spp. were the focus of this experiment to test if different succulence between species and the presence of moss would mitigate the effects of climate change. 80 thick *Peperomia tetraphylla* and 80 thin *Peperomia angustata* were collected from the forest around Estacion Biologica Monteverde. The succulent *P. tetraphylla* had leaves that were thicker and more turgid. Thin leaved *P. angustata* had leaves that were thinner, more limp and pliable (Fig.1) *P. tetraphylla* had smaller leaves that averaged around 5.5mm, *P. angustata* had larger leaves averaging around 12 mm.

Figure 1. More succulent *Peperomia tetraphylla* (left) and thin-leafed *Peperomia angustata*. (right) were placed in differing mist conditions with and without moss to see how leaf morphology changed over time.

Collection

The identification of *Peperomia* sp. was done using reference photos. The two distinct *Peperomia* sp. were determined observationally, looking at the leaf succulence. Two species of *Peperomia* were collected, *P. tetraphylla* with thick succulent leaves, and *P. angustata* with thin leaves. Both species were collected attached and not attached to moss. The roots were kept intact and the whole *Peperomia* individual was taken, including with moss if present. All plants were collected on living horizontal tree branches or from fallen branches and trunks. 160 plants were collected in total: 80 of each species. The collection took place over two days in October 2018.

Changes in Leaf Morphology with Mist Frequency

This study was set up based off of Gutierrez's 2012 study. Each tank housed 20 thick *P. tetraphylla*, and 20 less succulent *P. angustata*. 20 plants per species were then split into 10 without moss and 10 with moss surrounding the roots. Each plant that was collected with moss had all of its roots within said patch of moss. This was repeated for four aquaria that were 10inch by 19.5inch, which housed the four different climate conditions.

AQUARIA CONDITIONS – The four mist conditions were mist everyday (continuous control), mist every other day (historical) mist for five days followed by no mist for five days (pulsed) and no mist (dry control) (Guitierrez 2012; Prats 2015). Misting treatments consisted of six-hour intervals that released half a liter of water per treatment using three Perfect Aire LLC Ultra Sonic Humidifiers. Each tank was equipped with wire mesh that sat a few inches above the bottom of the tank, where the plants and moss resided. This was to keep the plants from sitting in any accumulated water. Each tank had a cardboard top with a hole in the middle, to house the misting tube and to keep mist from escaping. Accumulated water was disposed of, by removing the wire racks of plants, pouring the water out, then returning the wire rack to the drained aquaria.

MEASUREMENTS – Individual plant were numbered 1-10. Two leaves from each were marked. Leaves that were similar in size to one another were chosen; youngest leaves found at the top of the plant were avoided, as they were usually not fully-grown. Each leaf was measured for length, width, and thickness every five days for 20 days, with the first day being time zero. Measurements were made with a digital caliper to the nearest 0.01mm. Inflorescences were only found on thin *P. angustata*., up to two were measured per plant, if present. If a marked leaf fell off at any point within the 20 days a new leaf of similar size was marked and measurements continued on that new leaf. If a plant died, no new measurements were taken. Data were analyzed using a linear mixed model (LMM) test; the stat compared all variables but linked individual plant measures to one another.

RESULTS

Leaf Lengths at the Start of Trials

The LLM showed *P. tetraphylla* and *P. angustata* differed significantly in their leaf length (LMM Species Chi-square = 280.39, $p < 0.0001$; see Table 1). Overall, *P. tetraphylla* had a mean leaf length = 12.39 +/- 0.057, while *P. angustata* leaves had a mean length = 21.81 +/-0.179. When separating plants between those with moss and those without, *P. tetraphylla* with moss had slightly but significantly shorter leaves, with a mean = 11.89+/- 0.067, while those without moss had a mean = 12.89 +/- 0.084. However, *P. angustata* with moss had a mean leaf length = 22.38 +/- 0.26 and leaves from plants without moss had a

mean length = 21.25 +/- 0.245. Overall, leaf lengths for plants chosen for the without moss group tended to be longer for *P. tetraphylla*, and shorter for *P. angustata*. Initial differences in leaf length for moss and no moss groups were seen for both species (Species*Moss Chi-square = 4.33, p = 0.04; Table 1).

Further, when plants were separated into different conditions of mist frequency, their leaf lengths tended to be longer for plants placed in the dry and historical mist conditions for *P. tetraphylla* and pulsed and continuous for *P. angustata* (Condition Chi-square = 3.61, p = 0.31). *P. tetraphylla* chosen for continuous mist had a mean leaf length = 12.21 +/- 0.117, while those placed in historical had a mean leaf length = 12.77+/- 0.109 those in pulsed = 12.09+/- 0.112 and those in dry conditions with no mist had a mean leaf length = 12.52 +/- 0.111. Likewise, *P. angustata* chosen for continuous mist had a mean leaf length = 22.34 +/- 0.252, while those placed in historical had a mean leaf length = 21.08 +/- 0.336, those in pulsed = 23.27 +/- 0.412 and those in dry conditions with no mist had a mean leaf length = 20.48 +/- 0.388. Leaf length differed for mist conditions significantly for both *Peperomia* species (Species*condition Chi-square = 7.43, p = 0.05, Table 1).

Changes in Leaf Length with Time and Mist Condition

Leaf lengths maintained their initial differences over time (LMM Time Chi-square = 10.19, p = 0.001; Table 1). Initial differences in leaf length were not maintained over time for both *Peperomia* with and without moss (Figure 2; LMM moss*time Chi-square = 3.29, p = 0.069) thin *P. angustata* with and without moss did differ significantly from their initial measurements (LMM species*moss*time Chi-square = 7.00 p = 0.008). Mist frequency also had an impact on whether leaf length changed over time (LMM Condition*Time Chi-square = 45.84, p = P< 0.01), regardless of presence or absence of moss (LMM Species*Condition*Moss Chi-square = 5.44, p = 0.14) however, thin leaved *P. angustata* did significantly change (LMM Species*Condition*Moss*Time Chi-square = 8.66, p = 0.03).

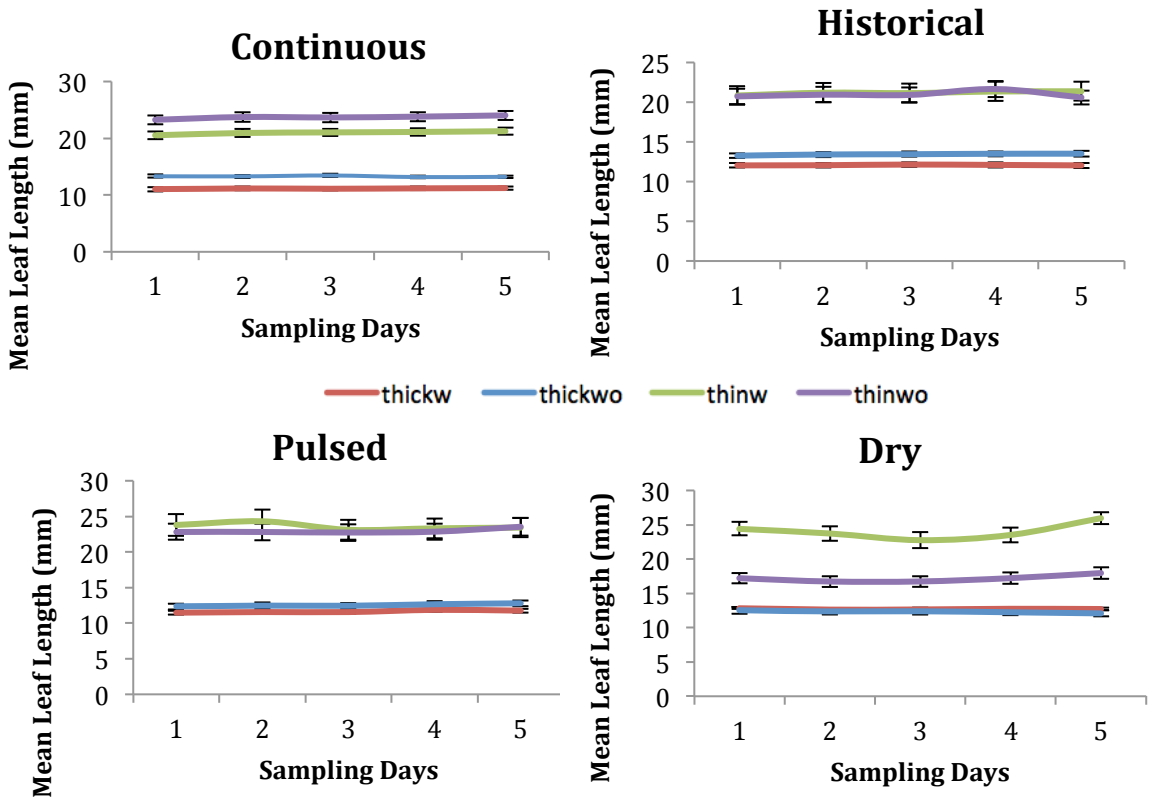

Figure 2. Mean leaf lengths for both thin *P. angustata* and thick *P. tetraphylla* without moss as well as thin *P. angustata* and thick *P. tetraphylla* with moss within four different misting conditions. Leaf length (mm) was measured for a total of 20 leaves per treatment per species, with and without moss for each sampling day. Error bars represent standard error. Treatments resulted in very little fluctuations of leaf length for both succulent and thin species, with and without the presence of moss within the different conditions. There are significant relationships found between all variables, but they reflect initial differences in mean leaf length (mm) that were maintained throughout the trials.

Leaf Widths at the Start of Trials

The LLM showed *P. tetraphylla* and *P. angustata* differed significantly in their leaf width (LMM Species Chi-square = 411.58, $p < 0.0001$; see Table 2). Overall, *P. tetraphylla* had a mean leaf width = 5.79 +/- 0.02, while *P. angustata* leaves had a mean width = 11.41 +/- 0.097. When separating plants between those with moss and those without, *P. tetraphylla* with moss had slightly but significantly narrower leaves, with a mean = 5.66 +/- 0.30, while those without moss had a mean = 5.92 +/- 0.03. However, *P. angustata* with moss had a mean leaf width = 11.73 +/- 0.14 and leaves from plants without moss had a mean width = 11.097 +/- 0.14. Overall, leaf widths for plants chosen for the without moss group tended to be narrower for *P. tetraphylla*, and wider for *P. angustata*. Initial differences in leaf length for moss and no moss groups were seen for both species (Species*Moss Chi-square = 2.62, $p = 0.10$; Table 2).

Further, when plants were separated into different conditions of mist frequency, their leaf widths tended to be wider for plants placed in the pulsed and historical mist conditions for *P. tetraphylla* and pulsed and continuous for *P. angustata* (Condition Chi-square = 18.19, $p < 0.001$). *P. tetraphylla* chosen for continuous mist had a mean leaf width = 5.74 +/- 0.036, while those placed in historical had a mean leaf width = 5.98+/- 0.038 those in pulsed = 5.83

+/- 0.053 and those in dry conditions with no mist had a mean leaf width = 5.62 +/- 0.046. Likewise, *P. angustata* chosen for continuous mist had a mean leaf width = 11.73+/- 0.133, while those placed in historical had a mean leaf width = 10.95 +/- 0.183, those in pulsed = 12.89 +/- 0.232 and those in dry conditions with no mist had a mean leaf width = 9.98 +/- 0.152. Leaf width differed for mist conditions significantly for both *Peperomia* species (Species*condition Chi-square = 15.53, p = 0.001, Table 2).

Changes in Leaf Width with Time and Mist Condition

Leaf width maintained their initial differences over time (LMM Time Chi-square = 5.20, p = 0.02; Table 2). Initial differences in leaf width were not maintained over time for both *Peperomia* species with and without moss (Figure 3; LMM moss*time Chi-square = 0.0001, p = 0.99) however this was true for both *Peperomia* species (LMM species*moss*time Chi-square = 3.99, p = 0.04). Mist frequency also had an impact on whether leaf width changed over time (LMM Condition*Time Chi-square = 157.74, p = p < 0.0001), however this was regardless of presence or absence of moss (LMM Species*Condition*Moss Chi-square = 2.86, p = 0.41) and this was true regardless of species (LMM Species*Condition*Moss*Time Chi-square = 0.92, p = 0.82).

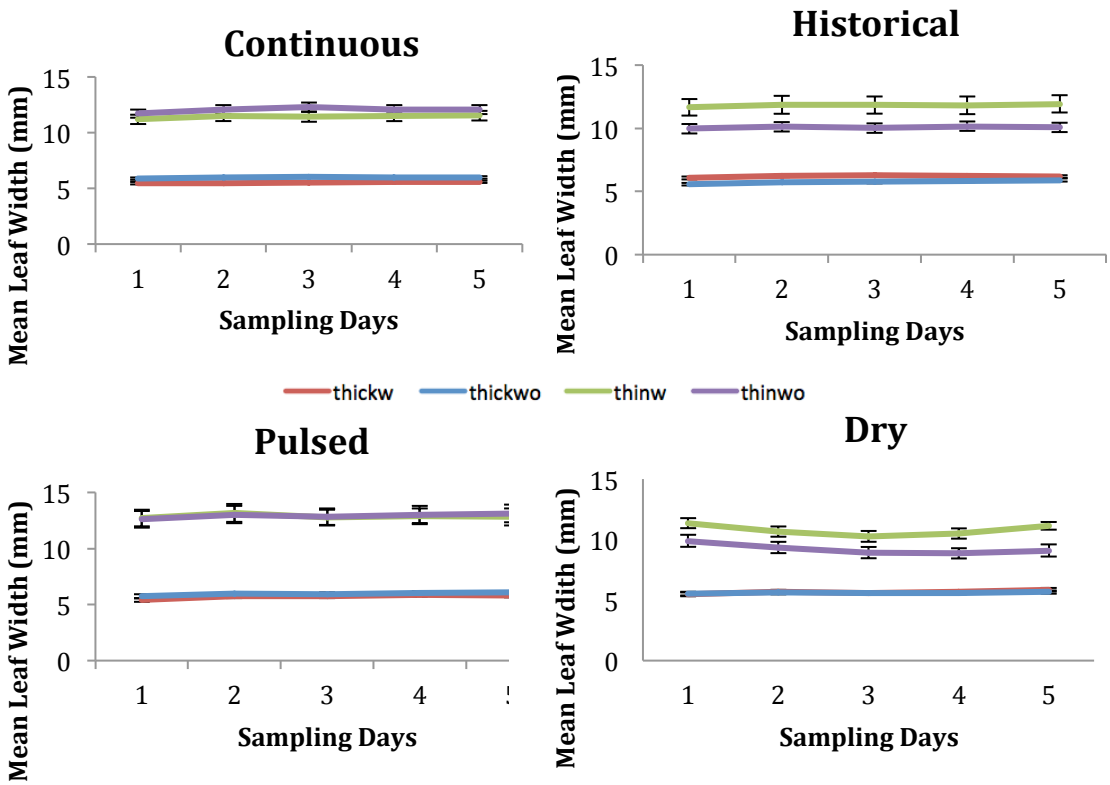

Figure 3. Mean leaf widths for both thin *P. angustata* and thick *P. tetraphylla* without moss as well as thin *P. angustata* and thick *P. tetraphylla* with moss within four different misting conditions. Leaf width (mm) was measured for a total of 20 leaves per treatment per species, with and without moss for each sampling day. Error bars represent standard error. Treatments resulted in slight fluctuations of leaf width for both succulent and thin species in the continuous treatment within the first five days of the treatment, but then leveled out. All other conditions resulted in very little fluctuations in width growth between the two species with and without the presence of moss within the different conditions. There are significant relationships found between all variables, but they reflect initial differences in mean leaf length (mm) that were maintained throughout the trials.

Leaf Thickness

Leaf Thickness at the Start of Trials

The LLM showed *P. tetraphylla* and *P. angustata* differed significantly in their leaf thickness (LMM Species Chi-square = 105.47, $p < 0.0001$; see Table 3). Overall, *P. tetraphylla* had a mean leaf thickness = 1.12 +/- 0.014, while *P. angustata* leaves had a mean thickness = 0.73 +/- 0.012. When separating plants between those with moss and those without, *P. tetraphylla* with moss had slightly but significantly thicker leaves, with a mean = 1.20 +/- 0.018, while those without moss had a mean = 1.04 +/- 0.021. Likewise, *P. angustata* with moss had a mean leaf thickness = 0.80 +/- 0.020 and leaves from plants without moss had a mean thickness = 0.66 +/- 00.12. Overall, leaf thickness for plants chosen for the without moss group tended to be thicker (LMM Moss Chi-square = 13.27, $p = 0.0003$). Initial differences in leaf thickness for moss and no moss groups were seen for both species (Species*Moss Chi-square = 0.25, $p = 0.618$; Table 3).

Further, when plants were separated into different conditions of mist frequency, leaf thickness tended to be thicker for historical and pulsed mist conditions for *P. tetraphylla* and continuous and pulsed for *P. angustata* (Condition Chi-square = 124.67, $p < 0.0001$). *P. tetraphylla* chosen for continuous mist had a mean leaf thickness = 1.14+/- 0.022, while those placed in historical had a mean leaf thickness = 1.37 +/- 0.021 those in pulsed = 1.28 +/- 0.022 and those in dry conditions with no mist had a mean leaf thickness = 0.71 +/- 0.024. Likewise, *P. angustata* chosen for continuous mist had a mean leaf thickness = 0.79 +/- 0.019, while those placed in historical had a mean leaf thickness = 0.74 +/- 0.014, those in pulsed = 0.97 +/- 0.029 and those in dry conditions with no mist had a mean leaf thickness = 0.39 +/- 0.014. Leaf thickness differed for mist conditions significantly for both *Peperomia* species (Species*condition Chi-square = 10.20, $p = 0.017$, Table 3).

Changes in Leaf Thickness with Time and Mist Condition

Leaf thickness maintained their initial differences over time (LMM Time Chi-square = 24.04, $p < 0.0001$; Table 3). Initial differences in leaf thickness were not maintained over time for both *Peperomia* species with and without moss (Figure 4; LMM moss*time Chi-square = 2.58, $p = 0.11$) and this was true for both *Peperomia* species (LMM species*moss*time Chi-square = 5.28, $p = 0.021$) Mist frequency had an impact on whether leaf thickness changed over time (LMM Condition*Time Chi-square = 2025.56, $p = p < 0.0001$), within the dry condition both species of *Peperomia* thickness declined more rapidly with the absence of moss, and were more stable in th epresence of moss within the first five days (LMM Species*Condition*Moss Chi-square = 3.83, $p = 0.28$) but not with regards to species (LMM Species*Condition*Moss*Time Chi-square = 22.60, $p < 0.0001$). Within the dry misting condition both plants species with and without the presence of moss began to decline greatly after five days, where both species increased slightly in both the continuous and historical misting conditions regardless of moss presence.

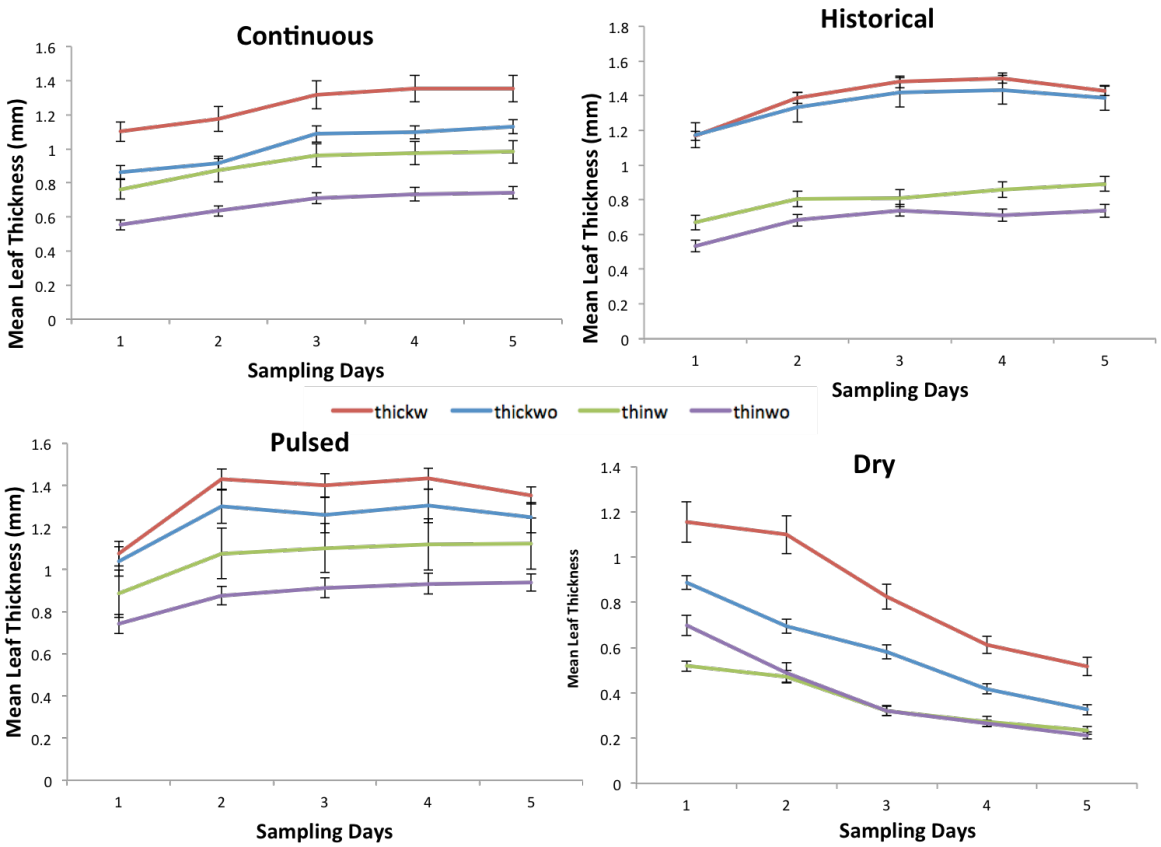

Figure 4. Mean leaf thickness for both thin *P. angustata* and thick *P. tetraphylla* without moss as well as thin *P. angustata* and thick *P. tetraphylla* with moss within four different misting conditions. Leaf thickness (mm) was measured for a total of 20 leaves per treatment per species, with and without moss for each sampling day. Error bars represent standard error. Treatments resulted in similar trends between continuous, historical misting condition. Within the pulsed misting treatment, thick *P. tetraphylla* with and without moss showed increase within days that had mist, and decline within days without any mist. Thin *P. angustata* with and without moss increase slightly throughout the twenty days, with the highest increase being during the first five days. In the dry condition, all species with and without moss decreased significantly throughout the twenty days. There are significant relationships found between all variables, but they reflect initial differences in mean leaf length (mm) that were maintained throughout the trials.

Table 1. Comparing all combinations of control plant identity variables between each other, in response to mean leaf length, using an LMM test. The four main variables being tested are: species (of thick and thin leaved *Peperomia* spp), condition (the four different misting treatments: continuous, historical, pulsed, and dry), presence of moss, and time (five day increments of sampling days with 20 days in total). The "*" indicates the response between the combination of the two variables indicated. Up to all four of the variables were combined. The "*" in the Pr(>Chisq) section indicates significance, between the variables, and (when present) significance between the combination of variables.

Responseto Length	Chisq	Df	Pr(>Chisq)
species	318.5698	1	< 2.20E-16*
condition	3.6092	3	0.3068704
moss	0.0304	1	0.8616875
time	10.1947	1	0.0014085*
species*condition	7.4304	3	0.0593741*
species*moss	4.3278	1	0.037494*
condition*moss	16.5671	3	0.0008674*
species*time	1.5967	1	0.2063733
condition*time	45.8367	3	<0.0001*
moss*time	3.2983	1	0.0693537*
species*condition*moss	5.4395	3	0.1422995
species*condition*time	14.5629	3	0.002231*
species*moss*time	7.0008	1	0.0081475*
condition*moss*time	5.7717	3	0.1232589
species*condition*moss*time	8.6579	3	0.034203*

Table 2. Comparing all combinations of control plant identity variables between each other, in response to mean leaf width, using a LMM test. The four main variables being tested are: species (of thick and thin leaved *Peperomia* spp), condition (the four different misting treatments: continuous, historical, pulsed, and dry), presence of moss, and time (five day increments of sampling days with 20 days in total). The "*" indicates the response between the combination of the two variables indicated. Up to all four of the variables were combined. The "*" in the Pr(>Chisq) section indicates significance, between the variables, and (when present) significance between the combination of variables.

Response to Width	Chisq	Df	Pr(>Chisq)
species	411.5766	1	<2.20E-16*
condition	18.195	3	0.0004009*
moss	0.398	1	0.5281355
time	5.2049	1	0.0225238*
species*condition	15.5304	3	0.0014152*
species*moss	2.6223	1	0.1053713
condition*moss	3.5455	3	0.3149069
species*time	39.0398	1	<0.0001*
condition*time	157.7436	3	<2.20E-16*
moss*time	0	1	0.9967578
species*condition*moss	2.8565	3	0.4142759
species*condition*time	171.4396	3	<2.20E-16*
species*moss*time	3.9976	1	0.045565*
condition*moss*time	1.5433	3	0.6723141
species*condition*moss*time	0.9234	3	0.8197819

Table 3. Comparing all combinations of control plant identity variables between each other, in response to mean leaf thickness, using a LMM test. The four main variables being tested are: species (of thick and thin leaved *Peperomia* spp), condition (the four different misting treatments: continuous, historical, pulsed, and dry), presence of moss, and time (five day increments of sampling days with 20 days in total). The "*" indicates the response between the combination of the two variables indicated. Up to all four of the variables were combined. The "*" in the Pr(>Chisq) section indicates significance, between the variables, and (when present) significance between the combination of variables.

Response to Thickness	Chisq	Df	Pr(>Chisq)
species	105.47	1	<2.20E-16*
condition	124.6703	3	<2.20E-16*
moss	13.2751	1	0.000269*
time	24.044	1	9.42E-07
species*condition	10.2049	3	0.016903*
species*moss	0.2487	1	0.617972
condition*moss	2.0803	3	0.555906
species*time	1.3405	1	0.246952
condition*time	2025.5578	3	<2.20E-16*
moss*time	2.5811	1	0.108146
species*condition*moss	3.8289	3	0.280543
species*condition*time	55.1487	3	<0.0001*
species*moss*time	5.2799	1	0.021573*
condition*moss*time	2.1954	3	0.532849
species*condition*moss*time	22.5987	3	<0.0001*

DISCUSSION

Change in *Peperomia* leaf length and width in both thick and thin leaved species was not affected significantly by the presence of moss. The length and width of both thick and thin species of *Peperomia* spp with moss did not significantly differ from the plants without moss after the 20 days in four different misting treatments. The mean leaf thickness of both thick and thin leaved plants, with and without moss, in the continuous and historical misting treatment all increased slightly throughout the 20 days. However, the thickness of both thick and thin leaved plants with and without moss were affected by the pulsed and dry misting conditions The dry condition, which received no water at all showed that presence of moss in both species seemed to only mitigate effects of drying for up to five days, as plant thickness greatly suffered after the first five days. It can also be seen that both plant species without moss begin to decline more drastically after five days, signifying that after five days, both plants with and without moss needed more water. This trend is easily seen in the pulsed misting condition as well, as all plants greatly increased in length within the first five days of treatment, which had mist everyday, and then during the next five days were there was no mist, plants begin to decline in thickness.

The thick-leaved *P. tetraphylla* declined in thickness while in the dry misting condition that received no water, where the length and width of the plants did not change in the same dry condition. This suggests that leaf succulence acted as the extra water source in order to keep the overall health of the plant high. The hydrenchyma tissue that makes this

succulence is helpful for water storing but shrinks and suffers water loss at a much higher rate in response to drought than other tissues (Schmidt 1987) causing the thickness for the thick-leaved species to decrease, even though it was maintaining the overall health of the plant with this extra water. Where as when the thin-leaved species thickness decreased the whole plant was clearly dying and drying out, indicating that it ran out of its extra water resource much before the more succulent species. It can be assumed from this observation that if more dry days were to be measured, the overall health of the more succulent species would be better due to have more of the extra water reserve compared to that of the less succulent species. Gutierrez (2012) found the same thickness decline within the same dry misting condition within her study of succulence in epiphytic *Peperomia* and resilience to climate change. Prats (2015) also found a similar trend when he tested the same misting conditions but on epiphytic orchid species, the leaf width responded significantly in the absence of mist, where leaves shriveled.

Although many of the tests between the four different variables (species, condition, moss, and time), and the combinations of these variables had significant data to support the differences between mean length, width, and thickness, they reflect initial difference in size rather than effects of treatment. This is because the slope trends are not biologically significant when taking into consideration the starting mean measurements of the different plants being different. This caused significance to be calculated based off the different starting measurements, which were not consistent throughout, leading to inapplicable data.

This data indicate that as TMCFs mist frequency declines with climate change, moss is not going to be a great buffer for epiphyte resistance to the increasing dry days. This is supported by Cecco (2015) study that found similar results stating that moss is not going to buffer the impacts of climate change on, *Lepanthopsis comet-halleyi,* the orchid species she was testing. However, leaf succulence in more succulent species will buffer the drier days. Gutierrez (2012) also found that more succulent species of *Peperomia* tested in the same four misting conditions as here, indeed had better overall health.

Moss, although known for its water storing abilities (Hedderson & Longton 1996), needed a resurgence of water at the same time that the plants did. According to Gregor (2018) different moss morphospecies have different amounts of water holding capacity ranging from 239 % of its dry mass to 23 %, indicating that depending on the moss species the epiphyte grows on, different quantities of water may be available. Also, it has been proposed that moss are a useful indicator of suitable microclimates for vascular epiphytes although they may not directly improve water supply (Scheffknecht *et al.* 2010), instead, they depend on the same level of moisture in microhabitat as epiphytes. Epiphytes as a growth form are water-stressed regardless of vasculature (Coley & Kursar 1996), so perhaps their apparent association with moss is a result of a common requirement for wet habitats (Cecco 2015), and the extra water that was expected to be available was not available due to the moss using it to keep itself alive.

As mist frequency decreases rapidly in Monteverde, due to its negative correlation with climate change (Pounds *et al.* 1999), epiphytic *Peperomia spp.* with more succulent leaves may have a bit more of a buffer compared to less succulent, but in general both species will be affected if dry consecutive days surpass five at a time. This indicates for the future that succulent species may become largely dominant, decreasing the overall diversity of epiphytes within the Cloud Forest. This implication would lead to extreme changes within ecosystem functions and overall biodiversity.

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Invertebrate Diversity Between Moss Species

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Abstract:

Mosses can provide shelter from predators, food, and more consistent temperatures and moisture to invertebrates. Different moss species may not be equal at providing these benefits. The diversity and composition of invertebrate living in three different morphospecies of moss within Monteverde cloud forest were tested to see what, if any effect different morphospecies of moss makes on the invertebrate community. There was no significant difference in the diversity (table 1), abundance ($p = 887$, $F = 0.12$), species richness ($p = 0.95$, $F = 0.051$), or composition of invertebrates between moss species (Morista = 70%). More samples might show a slight difference in the species richness living in species A (figure 5). The invertebrate are mostly generalist species that show no strong preference for moss type.

Resumen:

Los musgos pueden proveer a los invertebrados refugios contra depredadores, para alimentarse, y con una temperatura y humedad más constante. Diferentes especies de musgos pueden no proveer todos estos beneficios por igual. La diversidad y composición de invertebrados que viven en tres diferentes morfoespecies de musgo en el bosque nuboso de Monteverde fueron evaluados para ver si existe un efecto de alguna de las diferentes morfoespecie de musgo en la comunidad de invertebrados. No hubo diferencias significativas en la diversidad (tabla 1), abundancia ($p=0.887$, $F=0.12$), riqueza de especies ($p=0.95$, $F=0.051$), o en la composición de invertebrados entre morfoespecies de musgos (Morisita=70%). Una mayor cantidad de muestras mostraría una ligera diferencia en la riqueza de especies que viven en la morfoespecie A (Figure 5). Los invertebrados son especies mayoritariamente generalistas que no muestran una preferencia fuerte por los tipos de musgo.

Introduction

Mosses are an important habitat for many species of invertebrates. They can serve as food, shelter from predators, and breeding grounds (Gerson 1969), and because the structure of certain moss species can keep temperature and moisture levels constant (Merrifield and Ingham 1998). Certain invertebrates such as Collombola can feed directly on moss or on other substances that grow on moss, like nitrogen fixing cyanobacteria, fungi, or algae (Bozanic, 2008, Lindo and Gonzalaz, 2010). The different surface area that the moss creates provides a unique habitat and environment for invertebrates to live in, creating niches that invertebrates could specialize in. Moss also increases the chance of interception and retention of aerially delivered vegetation fragments (Nadkarni, 2000) that can add more structural diversity and more niches. For these possible reasons, the diversity and abundance of invertebrates is far greater on branches and trees that contain moss than those that do not (Angelini and Silliman, 2014).

Not all mosses are equal in providing these benefits. The structural differences within the moss structure and potentially the leaf components could alter their abilities to control moisture or provide nutrients. The air pockets formed by the branching structure of the moss, which help maintain temperature and moisture, can vary vastly. Some mosses can absorb over 10 times its dry weight of water, while other mosses have water content of only 5% (Merrifield 1998). Denser moss structures also contain higher water

retention, which can help Fungi grow, and provide a new food source for other organisms (Jonnson, et al., 2015). The different densities could also affect the hiding places for different invertebrates.

Moss with thicker leaves and higher retention has been shown to increase tardigrade density (Jonsson, et al., 2015). Mosses that are more open textured tend to support Arthropods at greater moss depths (Gerson 1969), but Isopods and Diplopoda prefer compact moss patches (Bozanik, et al., 2013). Some species of moss accumulate higher proportion of dead material than others, which can make the moss less nutritious (Yanoviak, Nadkami, and Walker, 2004).

Studies in more temperate areas have shown that different types of moss can have different diversities and compositions of invertebrate communities living within them (Jonnson, Trekels, Driesen, & Vanschoenwinkel), but not much research has been done in tropical cloud forests. I will test whether the components and diversity of invertebrate communities living within moss will vary based on different moss morphospecies.

Materials and Methods

Area:

This experiment took place took place in the Lower Montane Tropical Rain Forest from 1720m to 1770m along a trail that goes through a primary forest. Samples were taken from late October to early November during the late wet season.

Species:

Three different common morphospecies of mosses were collected that had different morphospecies. There was some mixture of moss species with in each sample, so samples containing over 75% of the chosen species were used. Species A has very thin and complex branching with tiny almost nonexistent leaflets. Species B has relatively large leaflets and very little branching and the mats tend to be less thick than the other species. Species C is highly branched like species A, but tends to have a thick almost branchlike structure.

Figure 1: Species C (top left), Species B (bottom), Species A (top right). Collected from live trees in tropical Montane rainforests of Monteverde, between 1720 and 1770m in elevation.

Experiment:

Thirtysix150 mm^3 samples were collected of three different moss morphospecies at 1 m height up different live trees. 12 samples of each moss species were collected. I used berlese funnel method to separate the invertebrates from the moss (Merrifield and Royce). The mesh placed over the samples was too coarse to keep out outside insects from flying into the sample, so flies from samples 1 – 8 were not counted in the results, and a finer mesh was used to keep out flies that did not originate from the moss. The invertebrates were separated from the ethanol using a sieve, and identified to the nearest order and sorted based on morphospecies. The Shannon-Weiner index was used to compare the overall diversity of invertebrates within each moss type. The Morista index was used to test if the macroinvertebrates with in each moss species were different from one another. The average abundance and species richness per sample were calculated.

Results:

Twenty-six species and 67 individuals were found in species A, 32 species and 62 individuals were found in species B, and 30 species and 59 individuals were found in species C. The most common order found was Collembola in all three species and there were also Diptera, Araneae, Acari, Coloptera, Leodoptera and Hemiptera at both adult and juvenile stages, and a few Hymenoptera, Isopoda and one Psocoptera (figure 2). There is no significant difference in abundance ($p = 887$, $F = 0.12$) (figure 3) or in species

richness (p = 0.95, F = 0.051) (figure 4), per sample between the three species. There is no significant overall difference between moss species in the Shannon index (table 1). The Morista Index shows a 70% overlap between species. The rarefication curve suggests that species A may show a lower relative species richness compared to the other two species if more samples are taken (figure 5).

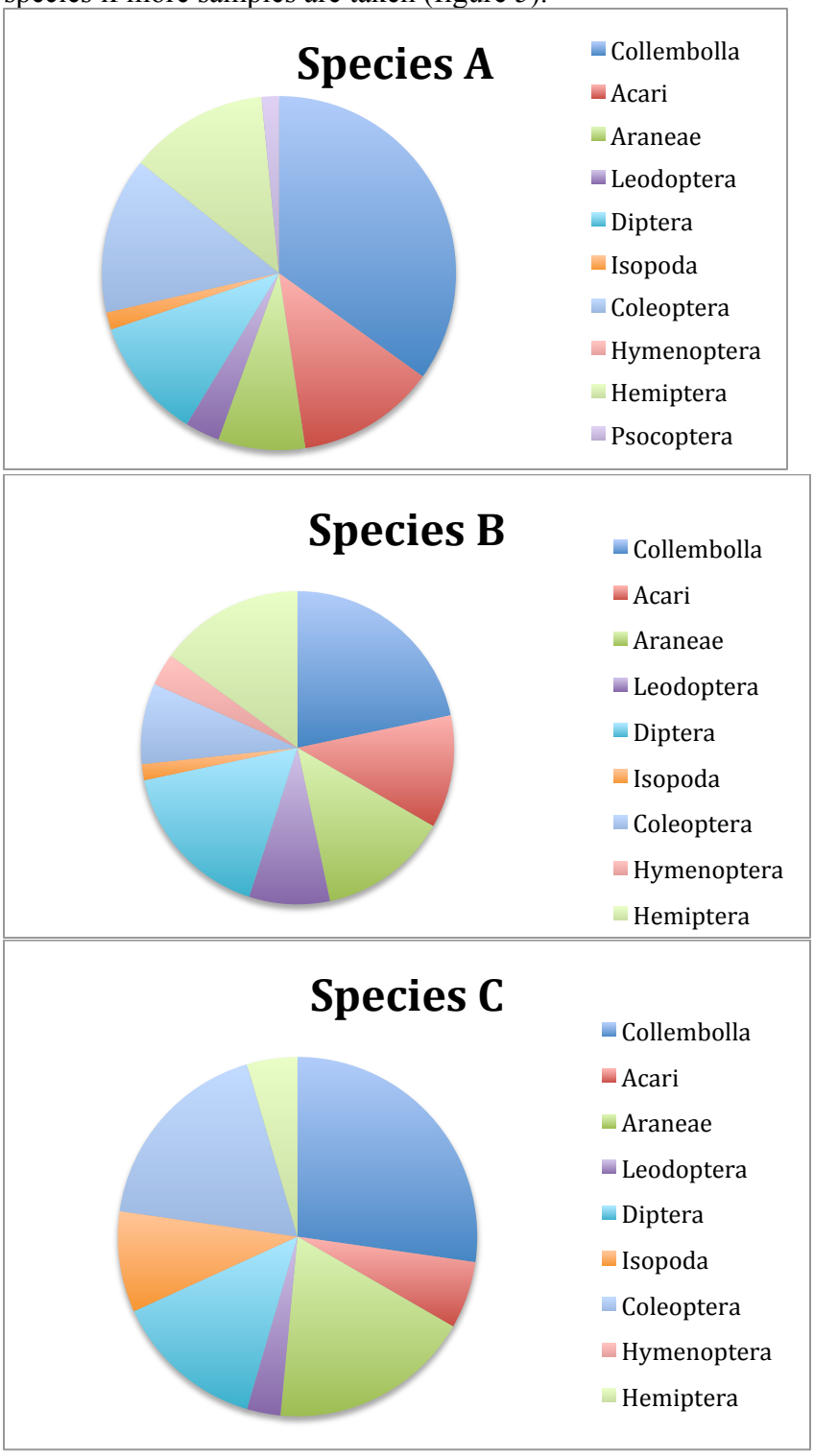

Figure 2: Relative abundance of each order of invertebrates found in moss species A (top), in moss species B (middle), and in moss species C (bottom)

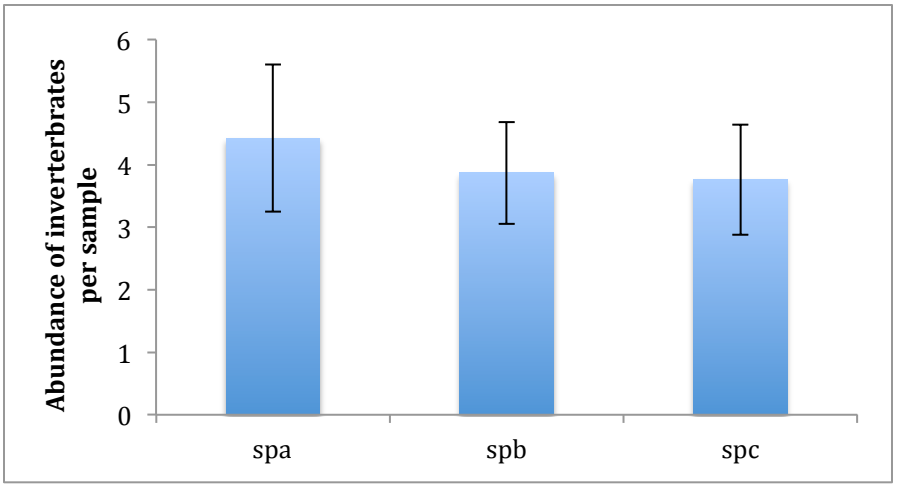

Figure 3: The average abundance of invertebrates found per moss sample for each morphospecies of moss spa (species A), spb, (species B), and spc (species C). Error bars are 1 standard error. The differences are not significant (p = 0.887).

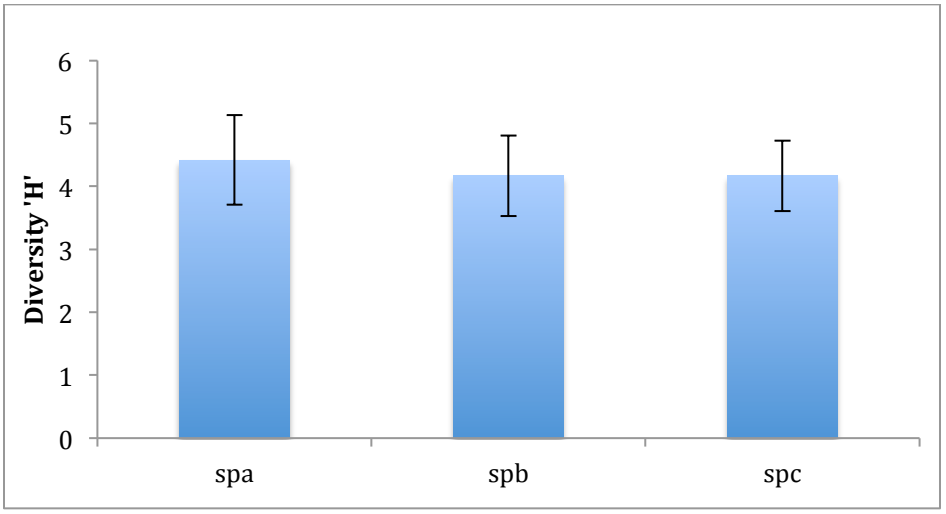

Figure 4: The diversity of invertebrates per sample using the Shannon index for each moss morphospecies. Error bars are one standard error. The differences are not significant (p = 0.95)

TABLE 1: A comparison of overall diversity of invertebrates between each moss morphospecies using the Shannon-Weiner index.

	Diversity t-test		
	t	df	p
spa vs spb	-1.4385	126.96	0.15275
spa vs spc	-0.96807	122.77	0.33491
spb vs spc	0.44326	120.85	0.65837

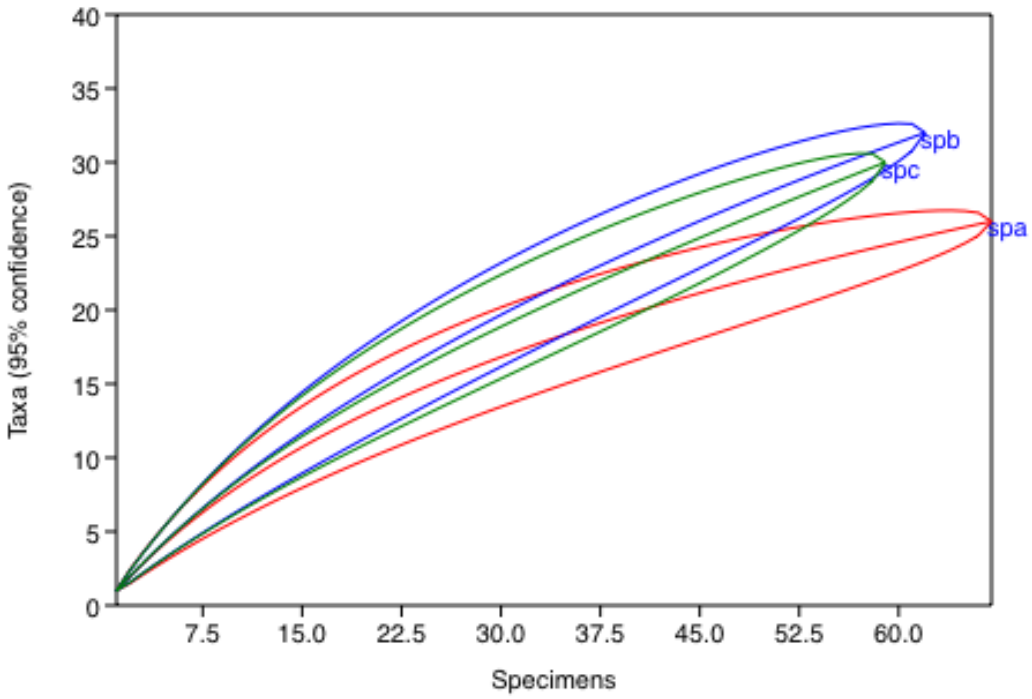

Figure 5: Rarefaction curve predicting the number of new species of invertebrates that will be found based on the number of samples of moss taken for each moss species.

Discussion:

The morphology of different moss species does not affect the invertebrate communities living there. This is different from what was found in other studies (Trekels, Driesen, & Vanschoenwinkel, 2017), and suggests that the invertebrates are mostly generalist species, and are randomly distributed showing no moss preference or niche partitioning. Although species A might prove to have different species richness with more samples, most invertebrate communities are highly diverse regardless of which species they are found in. Possible reasons for species A being slightly different could be because there is less leaf biomass within a given area than the other two species, which might mean there may be less to feed on, but more samples should be taken to verify this pattern. The other two moss species may be so similar to each other if they have similar moisture retention abilities despite their different morphology, or if the only factor that matters in Monteverde is biomass and not structure.

The species found in the moss were as expected based on other studies. Collembola often feeds on moss along with detritus and fungi, and Diptera and Accari were also frequently associated with moss (Miller, Wagner and Woods, 2008). Among nematodes plant associates were not strongly affected by moss morphology, so perhaps a similar pattern is absorbed for this case. Spiders feed on Collembola and studies have shown a positive correlation between the two populations (Miller, Wagner and Woods 2008). Though there is no sufficient difference to find a true trend, figure 2 shows the moss species having the most Collembola having the fewest spiders (Araneae). This is possibly because the spiders could have eaten some of the Collembola or there may be other factors of the moss that negatively or positively impact Collembola and spiders.

Possible reasons for this result were a lack of sample size. The mosses patches were seldom isolated and often had other many moss species surrounding them and some other moss and epiphylls were growing mixed within the moss samples. Having multiple species increase invertebrate diversity (Trekels, Driesen and Vanschoewinkel, 2017) for each of the mosses. There also may not have been as great of a difference in diversity between mosses due to moisture availability, since one of the effects of different morphology is moisture regulation, the time of year, and whether the moss is in the shade or in the sun can alter the effectiveness of the moisture regulation mosses provide (Božanić, 2008). There may be some variation in invertebrate species due to tree type or microhabitats regardless of presence of moss. Age of moss communities can also impact the complexity of the community structure, increasing the number of predators and detritivores (Jonnson, et al. 2015, Angelini and Silliman, 2014).

More samples should be taken to determine if there is a difference in invertebrate communities in species A. It would then be interesting to test the mosses water retention ability and nutrient content to determine which factor is likely to effect invertebrate communities the strongest and to test more morphospecies of moss. Regardless of the morphospecies, moss is home to an incredible diversity of invertebrates and should be protected to maintain biodiversity.

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Trail Gradient Determines Leaf Fragment Selection by Leaf Cutter Ants in the Wild

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ABSTRACT

Leaf cutter ants in the genus *Atta* are often used as model organisms in studies of central place foraging. Workers carry smaller than optimal leaf fragments for their body size from their foraging site to their nest via several foraging trails. Laboratory experiments have demonstrated that these forager ants tend to carry smaller leaf fragments when foraging on uphill trails, but no experiment has confirmed this trend in the field. Here I examine how trail gradient affects foragers choice in leaf fragment size in wild colonies in Monteverde, Costa Rica. Trails chosen were the most extreme versions available of both uphill and downhill trails for each colony, with ants traveling back to the central nest. Laden ants and their loads were weighed in the field. In general ants traveling downhill towards the nest were found to carry heavier leaf fragments (0.73 ± 0.01) in relation to their body size than and ants traveling uphill (0.60 ± 0.01). Ant mass did not differ between the two trails, indicating the lack of a colony-level response to compensate for difficulties imposed by uphill gradients. My results confirm the results from laboratory experiments and suggest that ants adjust load sizes based on extreme trail gradients. The results also confirm that trail gradients contribute to determine optimal load sizes in central place foraging in natural conditions.

Las hormigas cortadoras de hojas en el género *Atta* son usadas a menudo como organismos modelo en estudios de forrajeo central. Las obreras cargan fragmentos de hojas de tamaño óptimo con respecto al tamaño de su cuerpo desde su lugar de forrajeo a sus nidos a través de varios senderos de forrajeo. Experimentos de laboratorio han demostrado que estas forrajeadoras tienden a cargar fragmentos de hojas más pequeños cuando forrajean en senderos cuesta arriba pero ningún experimento ha confirmado esta tendencia en el campo. Aquí examinó como el gradiente del sendero afecta la selección de las obreras en el tamaño de los fragmentos de las hojas en colonias silvestres en Monteverde, Costa Rica. Los senderos escogidos fueron los más extremos disponibles tanto cuesta arriba como cuesta abajo para cada colonia, con hormigas viajando de vuelta hacia el nido central. Las hormigas cargadoras y su carga fueron pesadas en el campo. En general las hormigas viajando cuesta abajo hacia el nido cargaron fragmentos más pesados (0.73 ± 0.01) en relación a su tamaño corporal que las hormigas viajando cuesta arriba (0.60 ± 0.01). La masa de las hormigas no difiere entre los dos senderos, indicando una falta de respuesta a nivle de la colonia para compensar por las dificultades impuestas por el gradiente cuesta arriba. Mis resultados confirman los resultados obtenidos en experimentos de laboratorio y sugieren que las hormigas ajustan el tamaño de su carga basadas en los gradients extremos de los senderos. Los resultados también confirman que los gradientes de los senderos contribuyen a la determinación óptima del tamaño de carga en el forrajeo central en condiciones naturales.

INTRODUCTION

Central place foraging theory suggests that individual organisms should forage in a way that maximizes their efficiency when gathering resources from a particular site to take back to a central location (Orians 1979). Many organisms practice central place foraging such as birds

which gather materials for their nests or beavers which gather materials for their dams. Insects such as bees and ants also practice central place foraging (Lewis et al., 2008). The ability to adapt foraging behavior accordingly and forage effectively by reducing time and energy costs or increasing energy gain per unit time is evolutionarily relevant (Norton 2014). Many social insects, particularly ants, behave consistently with the predictions of the central place foraging theory (Holway and Case 2000).

Leaf-cutter ants (genus *Atta* and *Acromyrmex*) are ideal organisms for studies of central place foraging (Burd and Howard 2005). They build trails that last for months or even years and may be partially or completely cleared to facilitate movement and foraging efficiency (Rockwood and Hubbell 1987). A single trail can stretch along the forest understory up to 2 or 3 km (Howard 2001) and can be up to 30 cm wide (Lewis et al 1974). Forager ants carry loads in their mandibles from foraging locations up to 100m away from their nest back to their fungi chambers (Hölldobler and Wilson 1990). Ants vary greatly in size within a nest, and the selection of fragment size by individual workers varies in size in relation to the size of the ant (Burd 2000). Individual ants might be expected to select leaf fragments flexibly to maximize their loading ratio and efficiency of leaf fragment transport per unit time based on previous experience (Norton et al. 2014). However, workers are often observed to transport smaller fragments than expected for their size and maximization of optimal load (Rudolph and Loudon 1986). This is true of all sizes of ants within the colony. Only 2-6% of ants carry rate maximizing loads (Burd 2001).

Several hypotheses have arisen to explain this discrepancy between theory and observed results. One is that if ants harvest smaller fragments it will lead to less traffic at the foraging site and make transport more efficient (Burd 1996, 2000). *Atta Columbica*, another species of leaf cutting ants, have been found to make up for cutting smaller leaf fragments by increasing the number of foragers on a specific trail (Dussutour et al 2009). Another theory is that fragment size affects processing speed within nest and therefore ants take smaller pieces back to optimize efficiency at the nest (Burd and Howard 2005, Garret at al. 2016). Another is that colonies will optimize foraging as a whole group instead of as an individual, meaning that they may not carry as much as they can individually, but they optimize efficiency as a colony (Burd 1996, 2000, Burd and Howard 2005). For instance, colonies may recruit workers of different sizes to the different situations which would suggest a colony-level adaptation to terrain (Shutler and Mullie 1991, Dussutour et al 2009). The optimum efficiency on a colony level is obtained when colonies recruit larger ants to forage farther distances and carry larger fragments in relation to their body size (Shutler and Mullie 1991).

Calculation on optimal loads in leaf cutter ants have been conducted on horizontal trails but in reality, ant foraging trials include many uphill and downhill sections (Lewis, et al 2008). Previous studies with laboratory colonies have suggested that individual leaf cutter ants modify loads in the presence of height constraints or steep trail gradients (Lewis et al. 2008, Dussutour et al. 2009, Norton et al. 2014). These results suggest different energetic costs for laden workers

traveling uphill versus workers traveling downhill (Lewis et al 2008). Lewis, et al (2008) manipulated trail gradients in a lab setting using short distances of travel and a single relatively young and small captive colony and found that leaf transport rate was highest for *A. cephalotes* ants traveling 90º downhill and on horizontal gradients and low for ants traveling on 90º uphill trails. Ants were found to carry the highest load to body size ratio (hereafter load ratio) when the trail ahead was 90º downhill. They did not find differences in ant mass, so the hypothesis that colonies recruit different ant sizes was not supported (Lewis et al 2008). Norton et al. (2014) found similar results with a laboratory colony of *Acromyrmex octospinosus*, finding that ants selected heavier loads when returning to the nest vertically downwards than when returning horizontally or vertically upwards. These findings from both genera of leaf cutter ants suggest that this behavior may be general for all leaf cutter ants. To my knowledge similar experiments have only been conducted in the lab on captive colonies and never on wild colonies.

In this study, I tested the prediction generated under restricted laboratory conditions that wild *A. cephalotes* preferentially cut leaf sizes based on the gradient of the trail ahead. I specifically tested the result from Lewis, et al (2008) that loading ratio was highest on downhill and horizontal trails but declined when ants carried loads uphill. I also tested whether ant masses vary between trail types to evaluate the prediction that ant mass does not differ between downhill and uphill trails.

MATERIALS AND METHODS

Data Collection

Data were collected from three colonies of *A. cephalotes* found on hilly terrain in the premontane wet forests Monteverde Costa Rica. Each colony sampled was several kilometers away from each other. Data were collected between 9 a.m. and 1 p.m. on sunny days at an elevation of 1200-1300 m in the Premontane wet forest life zone (Haber 2000) during the wet season. Rainfall for this moist forest is 2000-4000 mm annually and mean annual temperature is 17-24 degrees Celsius. This life zone constitutes the upper elevational limit for *A. cephalotes* in Monteverde, as it is not tolerant of cloud forest conditions (Longino 2000). Data were collected during the month of November 2018 on mornings with no rain.

Ant weight and load size (leaf weight) were recorded from ants carrying leaves back to the nest. Trails were chosen based on the extreme of their gradient and the two most extreme trails, both uphill and downhill, were chosen for each colony. Specific gradients of each trail were measured using a clinometer. Ants were picked from their trail by their leaf using forceps and were then separated them from their leaf load. Ants were then wrapped gently in tinfoil to immobilize them during weighing. The mass of the foil used to wrap ants was subtracted from the mass of the ant to get actual ant weights. Each ant was returned to the trail directly after measuring. Each ant's leaf fragment was weighed after the ant. Weights were recorded to the nearest 0.001g in the field using a portable scale. Ants were sampled systematically. A mark was set at a point on the trail

and ants were chosen based on which one was passing that mark after the last ant had been weighed and released. If more than one ant was present at the mark at one time, the ant with the larger leaf was chosen. Thirty ants were measured per trail at each colony on each day. Data were taken for four days at two colonies and three times for the third colony for a total sample size of 656 ants.

Data Analysis

Loading ratios were calculated by adding ant mass and load mass and dividing this quantity by ant mass (Lewis et al. 2008). Linear mixed models (LMMs) were used to compare loading ratio and ant mass, individually, between trail type and between colonies while controlling for dependency of observations collected from the same colony and on different days. Hence, colony identity and collection date were included as random effects in the models. Models were also fit using colony identify as a fixed effect to test for variation between colonies in the measured variables. Variables were log transformed to meet the assumption of normality. P-values correspond to analyses of deviance conducted with the function "Anova" of the R package "car". Pairwise post-hoc comparisons of means were conducted using Tukey tests with the R package "emmeans". All analyses were conducted in R 3.4.3.

RESULTS

Gradients of each trail were measured and are shown in Table 1. Up trails were a found to be steeper gradients overall compared down trails.

Table 1. The gradient of each up and downhill trail by colony. Trails chosen were the most extreme options. Trail gradients are similar but not the same. Ants were sampled on these gradients in a tropical premontane wet forest in Costa Rica at approximately 1200 m elevation. Samples were taken from three different colonies for a total of 656 samples over one month.

	Colony 1	Colony 2	Colony 3
Up trail	55 degrees	50 degrees	60 degrees
Down trail	35 degrees	30 degrees	15 degrees

Load Ratio

In general, *A. cephalotes* adjusted their load ratio differently between uphill and downhill trails (LMM: Chisq = 68.966, df = 1, p <.0001). Ratio for uphill trails was 13% lower than downhill trails (Fig. 1). The mean value for down trail 0.73 ± 0.01 (standard error) and the mean loading ratio value for the uphill trail was 0.60 ± 0.01.

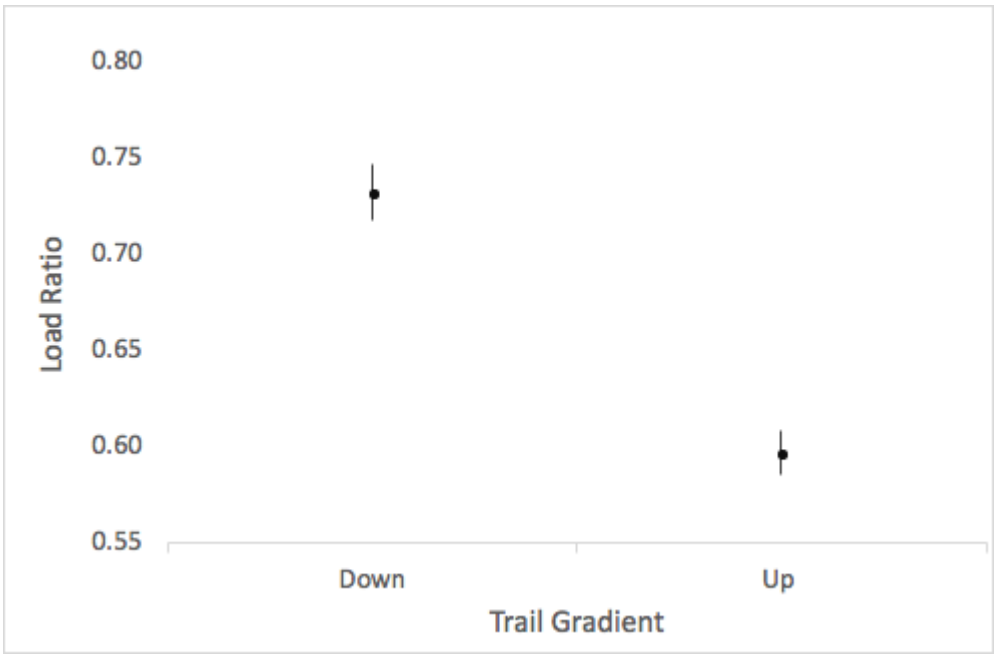

Fig. 1: Leaf cutter ants (*Atta cephalotes*) adjust their load ratio between uphill and downhill foraging trails. Ants were sampled in a tropical premontane wet forest in Costa Rica at approximately 1200 m elevation. Samples were taken from three different colonies for a total of 656 samples over one month. Trail gradient was measured using a clinometer and load ratio was calculated using the formula s ([ant mass + load mass]/ant mass). Error bars represent one standard error.

Load Ratio Separated by Colony

The LMM that included colony as a fixed effect revealed that the difference in load ratio between up and down trail was colony specific (colony*trail interaction: Chisq = 9.3232, df, = 1, p = 0.002). Specifically, load ratios were higher in downhill trails for only 2 of the 3 colonies (Fig. 2). Means and standard errors were: colony 1 down = 0.67 ± 0.01, up = 0.70 ± 0.02, colony 2 down = 0.93 ± 0.03, up = 0.55 ± 0.02, colony 3 down = 0.60 ± 0.02, up = 0.52 ± 0.02.

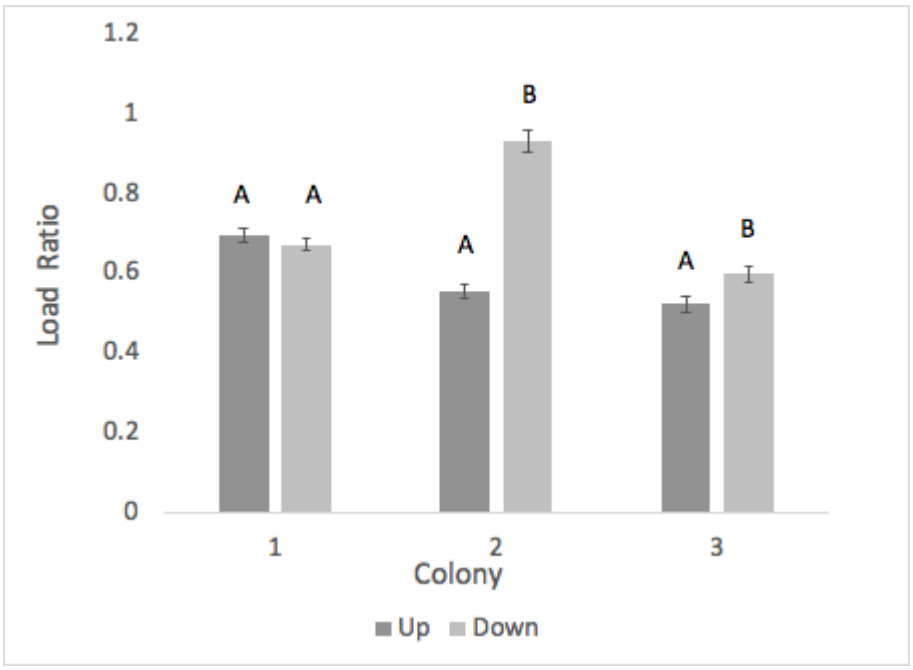

Fig. 2: Load ratio of forager ants between trails going uphill and downhill compared between three colonies sampled. Data were collected in a tropical premontane rainforest in Costa Rica at approximately 850 m elevation. Letters above bars represent significant difference between trail gradients, not a comparison between colonies. Colony one had no difference, colony two had a significant difference between trail gradients where loading ratio was much higher in the down trail, and colony three also had significant difference between trail gradients where loading ratio was higher in the down trail Different letters indicate significant differences in post-hoc pairwise comparisons between means according to Tukey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model.

Ant mass

A. cephalotes mass was not found to be significant between trail gradients (LMM: Chisq = 2.1999, df = 1, p = 0.138). The mean value for the down trail was -2.11 ± 0.02 (standard error) and the mean mass for the uphill trail was -2.08 ± 0.02.

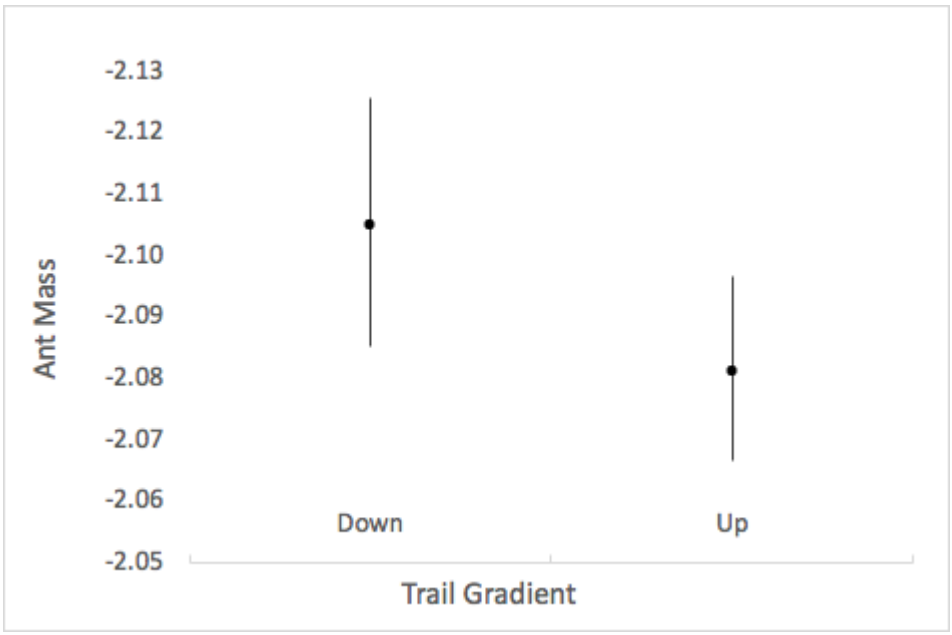

Fig. 3: Leaf cutter ants (*Atta cephalotes*) do not differ in mass between trail types. Ants were sampled in a tropical premontane wet forest in Costa Rica at approximately 1200 m elevation. Samples were taken from three different colonies for a total of 656 samples over one month. Ants were weighed with a portable scale weighing to the nearest 0.0001g. Error bars represent one standard error.

Ant Mass Separated by Colony

The LMM that included colony as a fixed effect revealed that the difference in ant mass between up and down trail was colony specific (colony*trail interaction: Chisq = 15.8122, df, = 1, $p < 0.0001$). Specifically, ant mass was higher in the downhill trail of colony 2 and lower in the downhill trail of colony 3 (Fig. 4). Means and standard errors were: colony 1 down = -1.98 ± 0.02, up = -1.99 ± 0.03, colony 2 down = -2.44 ± 0.03, up = -2.14 ± 0.02, colony 3 down = -1.89 ± 0.02, up = -2.13 ± 0.03.

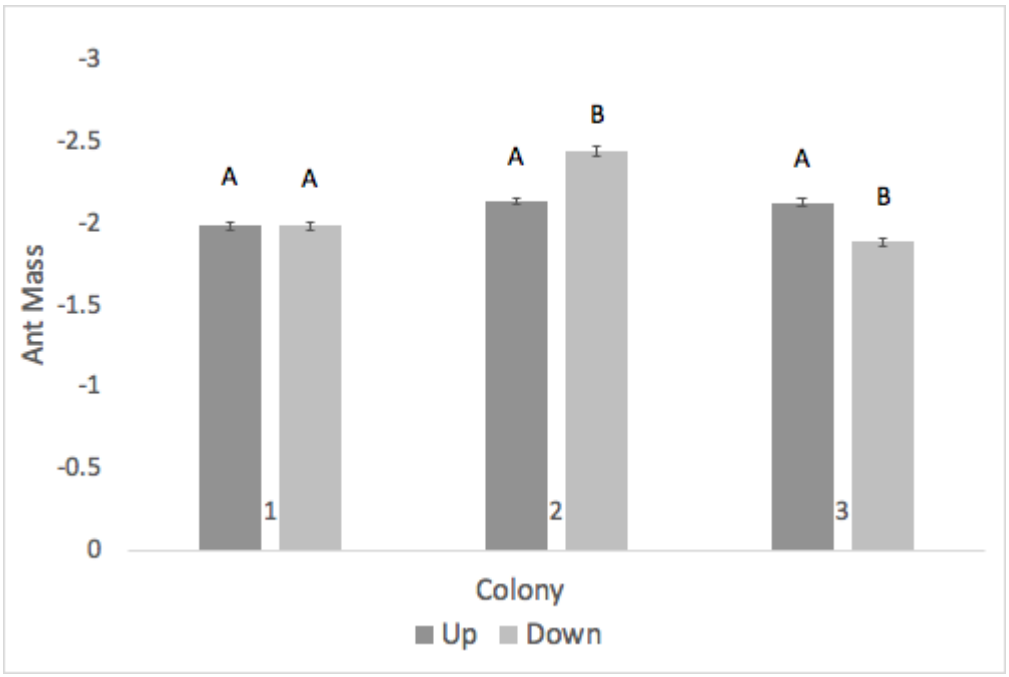

Fig. 4: Mass of forager ants between trails going uphill and downhill compared between three colonies sampled. Data were collected in a tropical premontane wet forest in Costa Rica at approximately 1200 m elevation. Samples were taken from three different colonies for a total of 656 samples over one month. Colony one had no difference, colony two had a significant difference between trail gradients where ant mass was much higher in the down trail, and colony three also had significant difference between trail gradients where ant mass was higher in the up trail. Different letters indicate significant differences in post-hoc pairwise comparisons between means according to Tukey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model.

DISCUSSION

Central place foraging may be maximized by individual foragers altering the mass of their loads based on the gradient of the trail ahead of them. Previous studies have researched load size selection by leaf cutter ants in terms of ant mass and trail gradient in the laboratory, but have failed to find consistent relationships that could implicate a single factor as the reason for load size selection between either (Roces 1990; Burd 1995). This study found that trail gradient had significant effects on *A. cephalotes* foraging behavior in the wild. Specifically, *A. cephalotes* workers alter their loading ratios according to the gradient of the trail ahead. Foragers traveling downhill tended to carry heavier loads than foragers carrying leaf fragments uphill. Lewis et al. (2008) found that ants in a captive laboratory colony carried heavier loads when traveling down a 90º gradient than ants traveling up a 90º gradient, but trail gradients and loading ratios have never been tested in the wild. I found similar results to Lewis et al. (2008) in wild colonies on less steep gradients. The steepest downhill gradient in the wild colonies sampled for this study was 35º and the steepest uphill gradient was 60º, showing that the results of Lewis et al (2008) are consistent in wild colonies on less steep gradients. When separated by colony, only colony 2 and 3 showed significant differences in loading ratio between trail types, and colony 1 loading

ratio was nearly the same between up and downhill trails.

The genus *Atta* are known to respond to gravitational cues (Vilela et al. 1987), and my results suggest that on steeper downhill trails ants may be able to exploit gravity to reduce their energetic costs of carrying heavier loads, making higher loading ratios feasible. The possibility that differences in trail congestion were responsible for the differences in load ratio between trail types was excluded because none of the trails sampled were very active except for the uphill trail of colony 1, which was the colony that did not show differences in load ratio or ant mass. Trail congestion as a result of a high volume of active ants leads to slower travel times and decreased foraging efficiency (Freeman and Chaves-Campos 2016). This study was conducted in the highlands of Costa Rica which is the upper limit for *A. cephalotes* residence which leads to less active trails overall (Freeman and Chaves-Campos 2016). Congestion could, however, be a reason for differences in load ratios between colonies given that the most congested trails were observed at colony 1 which did not have any difference in load ratio between trails. Additionally, no evidence was found to support the theory that colonies will optimize foraging as a whole group instead of as an individual by carrying smaller fragments because smaller fragments were consistently found to be carried uphill (Burd 1996, 2000, Burd and Howard 2005). There was also no evidence to support that there is any suggest a colony level adaptation to terrain by recruitment of larger foragers (Shutler and Mullie 1991, Dussutour et al 2009) because each colony showed different results for average ant mass by trail gradient. This makes it unlikely that the differences in load ratio between trail types are a consequence of differences in ant mass because only one out of the three colonies recruited larger ants for uphill trail gradients.

Burd and Howard (2005) show that smaller load selection in *A. columbica* can be explained if fragment processing within the nest is taken into account, because smaller fragments make for faster processing times. As in previous lab studies (Lewis et al. 2007, Norton et al. 2014) no attempt was made to measure fragment processing time inside the nest, as the main focus of this study was above ground foraging. It is possible however that both trail gradients and constraints such as fragment processing time could act together to limit optimal load sizes. The processing costs inside the nest identified by Burd and Howard (2005) play an important role in explaining the discrepancy between rate maximizing loads and actual loading ratios but so does trail gradient. It's probable that both of these factors work together to inform load size selection by foragers.

A. cephalotes trail gradients in nature are more likely to be downhill because foraging sites are above ground and the nest is underground. Because *Atta* species often forage in the canopy of trees (Wirth et al. 2003), downhill sections will be an element of many foraging trails. Trail gradient in past studies have focused on mean trail gradient but the variance in gradients on a typical *A. cephalotes* foraging trail may actually be more critical than the mean gradient (Lewis et al 2008). Every foraging trail has numerous short uphill and downhill sections and therefore load size selection may be determined by these extreme gradients, rather than by the overall mean gradient. In this study sample trails were chosen based on the most extreme gradients

available and found load ratio differences between trails supporting this theory.

This study is the first test similar to Lewis et al. (2008) and Norton et al. (2004) done on a field colony and the results are similar, concluding that captive colonies and wild colonies optimize foraging in the same way. Given that lab studies have been conducted on both genera of leaf cutter ants (*Atta* and *Acromyrmex*) these results suggest that leaf cutter ants in general may adjust load ratios according to terrain gradients in the field. These results have implications for central place foraging theory across many species. Environmental conditions affect all central place foragers and can have significant energetic consequences (Lewis et al. 2008). Trail gradient as an environmental factor, shown here, can be applied to many terrestrial central foraging species (Taylor et al. 1972). These environmental factors play a large role in the foraging behavior of central place foragers and must therefore be taken into consideration when making predictions about the habits of central place foragers.

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Ectoparasitic mites reduce chemical defense in the Neotropical millipede (*Nyssodesmus python*)

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ABSTRACT

Parasites negatively impact host fitness in a variety of ways. This study quantified the impact of external mites on the chemical defense system of the large forest-floor millipede *Nyssodesmus python* (Diplopoda:Polydesmida) collected from primary Cloud Forest habitat. A second experiment tested if external mites were attracted to *N. python's* chemical defense. Milligrams of excreted cyanide were quantified for *N. python* with mite loads ranging from 0 to 79 using a sodium picrate assay. There was a significant decrease in cyanide secreted as mite load increased. A doubling of mite load from 10 to 20 lowered cyanide excretion by 0.017 mg (18.6%). *N. python* sex, stage, and length were compared to cyanide excretion and mite load. Only stage and length impacted cyanide excretion, with adults and longer specimens excreting significantly more than juveniles and shorter specimens. Sexes excreted similar amounts. Mites did not have a significant preference between sexes, stages, or lengths of *N. python.* When mite location on *N. python* was compared to cyanide excretion, mites found anywhere besides the base of the legs of the millipede (Non-LL mites) have a greater impact on cyanide excretion than mites found at the base of the legs (LL mites; mite species examined in attraction test). Of the three morphospecies of undescribed mites found on *N. python*, one mite species was tested for an attraction to cyanide from crushed passion vine leaves (*Passiflora*) and counting mites nearby. The mites were not significantly attracted to the cyanide compared to a drop of water. These findings add to a greater understanding of factors influencing *N. python*'s chemical defenses. This also shows that one mite species likely does not feed on or is attracted to *N. python*'s hydrogen cyanide. They also show that young, shorter *N. python* individuals are more susceptible to predation because of their lower cyanide excretion. The additional factors such as high mite load may weaken young *N. python* and their overall fitness.

RESUMÉN

Los parásitos afectan negativamente el éxito de su hospedero de varias maneras. Este estudio cuantifica el impacto de ácaros externos en la defensa química del milpies terrestre Nyssodesmus python (Diplopoda: Polydesmida) colectados en bosque nuboso primario. Un segundo experimento provó si los ácaros externos fueron atraídos a las defensas químicas de N. python. Los miligramos de cianuro excretado fue cuantificado para los milpies con cargas de ácaros de 0 a 79 usando el ensayo de picrato de sodio. Hay una disminución significativa en el cianuro secretado al aumentar el número de ácaros. Al doblar el número de ácaros de 10 a 20 se disminuye la secreción de cianuro por 0.017 mg (18.6%). El sexo, estadío, y tamaño de N. python se compararon con la excreción de cianuro y el número de ácaros. Solo el estadío y el tamaño tuvieron un impacto en la secreción de cianuro, con los adultos e individuos más grandes

excretando significativamente más que los juveniles y los individuos más pequeño. Ambos sexos secretan una cantidad similar. Los ácaros no tienen una preferencia significativa entre sexos, estadío, o el tamaño de N. python. Al comparar la ubicación de los ácaros con la excreción de cianuro, los ácaros encontrados en cualquier lugar menos la base de las patas del milpies (ácaros no LL) tienen un mayor impacto en la excreción de cianuro que los ácaros encontrados en la base de las patas (ácaros LL, los ácaros examinados en la prueba de atracción). De las tres morfoespecies no descritas encontradas en N. python, una especie fue usada para la atracción al cianuro de hojas majadas de la enredadera Passiflora y se contaron los ácaros cercanos a esta. Los ácaros no fueron atraídos significativamente al cianuro al compararlo con una gota de agua. Estos resultados anadem a un mayor entendimiento de los factores que influencian las defensas químicas de N. python. Esto además muestra que una especie de ácaro no se alimenta ni es atraída al cianuro de hidrogeno de N. python. Esto además también muestra que los juveniles y milpies más pequeños son más suceptibles a la depredación por tener una menor secreción de cianuro. Los factores adicionales como un gran número de ácaros puden debilitar los juveniles de N. python y su exito en general.

INTRODUCTION

Mites (Acari) occupy a variety of environments due to their plasticity and ability to form symbiotic relationships with both plants and animals, leading Acari to be one of the furthest spread taxa of arthropods, occupying even more spaces than even insects (Krantz & Walter 2009). Mites form mutual, commensal, and parasitic relationships with other organisms. Mutualist mites may protect their host from predators in return for shelter, as in the case of *Viburnum tinus* mites and leaf domatia (Grostal & O'Dowd 1994). Commensal mites are phoretic or feed on non-essential or dead host tissue. Phoretic mites use their hosts for transportation as individual mites' small size does not yield itself for long treks to find food or mates (Paré & Dowling 2012). Commensal mites may also feed on debris found on the cuticle of arthropods (Paré & Dowling 2012). Other mites have parasitic relationships. These mites reduce host fitness in a variety of ways, including those that reduce sensory acuity (Kralj *et al.* 2007), cause deformities and overall weakness (Bowen-Walker & Gunn 2001), or decreasing host defense (Alba *et al.* 2014; Miller 2018). This study further investigates external mite's ability to decrease a host's chemical defense by building upon Miller's (2018) finding that when mites are present on *Nyssodesmus python* (Diplopoda:Polydesmida) there is a significant inhibition of chemical defense.

N. python is a large millipede found in the Neotropics that has variable numbers of external mites (Miller 2018). It defends itself by curling its exoskeleton and excreting a mixture of mandelonitrile, benzaldehyde, benzoic acid, and hydrogen cyanide (HCN; Heisler 1983, Shear 2015). HCN is the gaseous form of cyanide that prevents cellular respiration (Traber et. Al 2007). *N. python* produces HCN like most Polydesmida, by producing mandelonitrile via repugnatorial glands (Shear 2015). The glands of Polydesmida are described to have two compartments; one containing the undissociated cyanogenic compound and the other containing an enzyme catalyst for cyanogenesis (Eisner *et al.* 1963; Shear 2015). Between these compartments is a muscular valve that controls secretion. When the millipede secretes its chemical defense, the mandelonitrile is degraded by an enzyme in the second compartment into benzaldehyde, HCN, and benzoic acid that is then excreted from the ozopore opening. The ozopore does not have muscular aperture control and is always open. This study focused on the HCN excretion as it is easily identified by its strong almond-like smell. The creation and

secretion of chemical defense is well understood but external factors that contribute to the quantity of *N. python's* chemical defense still need to be explored.

Miller's (2018) study resulted in two main findings. One was *N. python's* mites do not have a host sex preference. The other finding was when mites were present on *N. python*, they do not excrete HCN, while millipedes without mites do (Miller 2018). This indicates a parasitic relationship in which the mites inhibit the millipede's chemical defense. However, for both tests only presence or absence of mites was recorded, not quantity or location of mites on individuals. Miller (2018) theorized that the inhibition of HCN in host millipedes was caused by one of three options: (a) the mites upregulated genes to suppress the expression of the enzyme that catalyzes the creation of HCN (like *T. urticae;* Alba *et al.* 2014), (b) the mites block the open ozopore, blocking the HCN, or (c) they feed on the HCN, based on the documented case of *Megaselia* phorid flies feeding on *N. python*'s HCN excretions (Hash 2014). The last two theories indicate that the mites may be attracted to HCN or some smell related to the ozopores. Thus the goal of this research is to build upon Miller (2018) by quantifying the significant parasitic relationship she found between *N. python* and its mites, determining factors that impact *N. python's* cyanide excretion and mite load, such as length, stage, and sex, and finally, testing the theory that mites have an attraction to the HCN excretion.

MATERIALS AND METHODS

Study Site

The primary study site for this research took place in the forest adjacent to the Estación Biológica, Monteverde, Costa Rica. Specimens were collected from paths of the elfin forest, as well as lower trails near streams. *N. python* were found among leaf litter, under large logs, and walking across trail paths. Once an *N. python* specimen was found, sex and stage were determined, initial mite count was taken, then the millipede was placed in a 20 oz Tupperware. The Holdridge Life Zones were Lower Montane Wet Forest and Lower Montane Rain forest at 1,350 to 1,842 meters in elevation (Holdridge 1971). The study took place during the late wet season from October 18th to November 15th.

Study Organisms

N. python is a large forest millipede that feeds on decomposing plant matter and is commonly found in moist areas and leaf litter (Heisler 1983). Sexes are distinguished by the first set of legs on the seventh segment, where only males have a shortened pair of modified legs. They have dimorphic stages, where juveniles are general smaller and mostly brown, lacking the pale-yellow longitudinal strip seen in the adult stage. Their average length is between 6 to 10 cm, females being longer on average. Juveniles and adults have 20 segments. *N. python* has two primary defense mechanisms: a rigid, calcified exoskeleton and repugnatorial glands that produce and secrete HCN through ozopores. The locations and size of the ozopores on *N. python* are undocumented, however the locations of ozopores on other species within Polydesmida occur dorsally, near the edges of the keels (Golovatch 1994; Golovatch *et al.* 2013). Polydesmida also commonly display a gland pattern of bilaterally paired glands in segments 5, 7, 9, 10, 12, 13, and 15 through 19 (Shear 2015), but the locations of the ozopore is species specific (Golovatch *et al.* 2013). These mechanisms are coupled with curling into a spiral and releasing hindgut contents in response to disturbance.

Each specimen was placed in separate 20 oz. Tupperware containers with moist leaves and decaying wood to assure mites would not intermix between specimens. Specimen Tupperware containers were misted every two days. Every five to six days, the *N. python* was moved to another container and the 20 oz. Tupperware was emptied, rinsed, and moist leaf litter was replaced. After cleaning, the millipede was returned to its Tupperware.

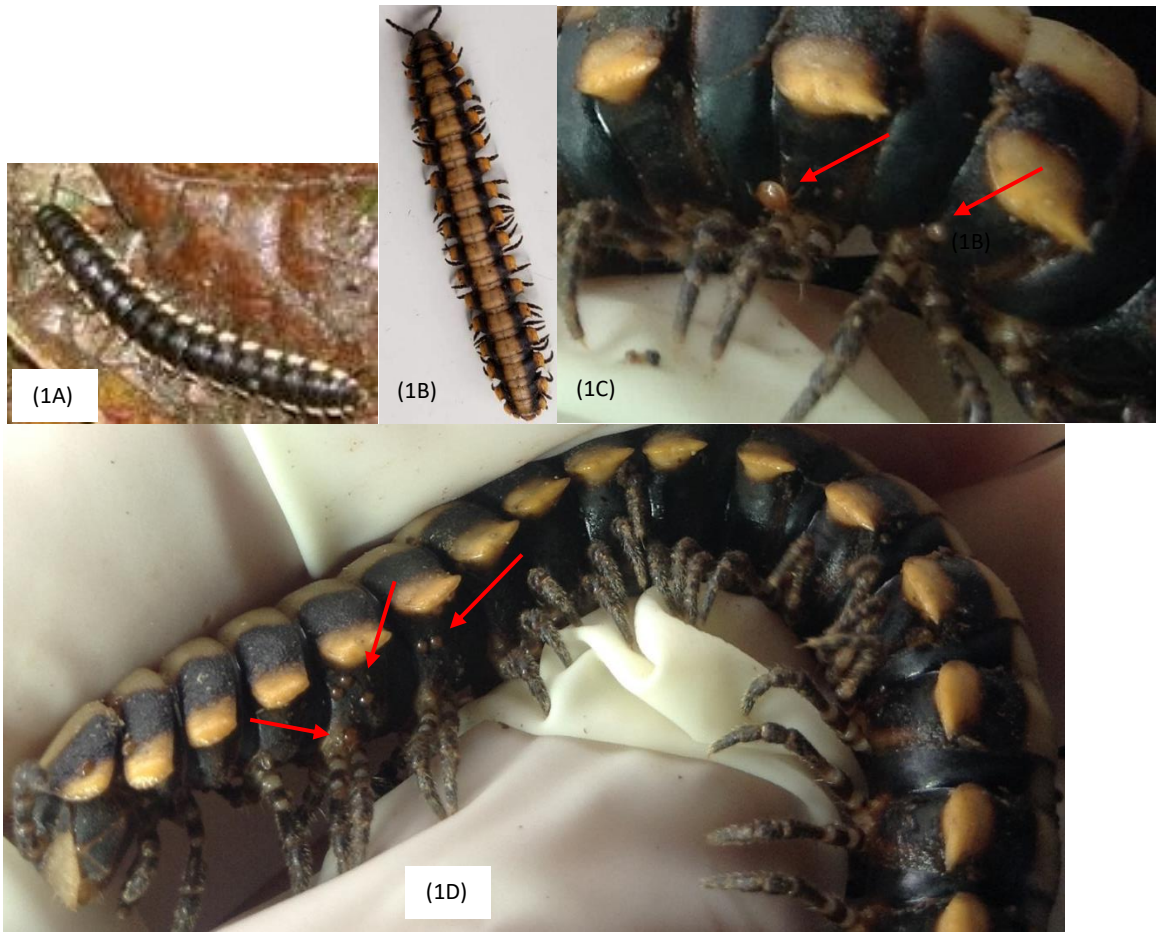

Figure 1*:* N. python *is commonly found in the Neotropics in Central America. Costa Rica's wet forests and rainforest offer a moist environment and plenty of decaying wood and leaves for the millipedes to feed on. The image on the top left (A) is of a juvenile millipede that does not yet have the pale-yellow longitudinal stripe of the adult (B). Juvenile millipedes are typically smaller than adult millipedes, however this depends on sex as females are larger than males, on average. Both adults and juveniles excrete HCN as chemical defense. Three mite morphospecies can be found on both adults and juveniles. Image (C) and (D) display mite morphospecies on an adult* N. python*. Image (A) was found at https://www.wnf.nl/dieren/dierenbieb-ongewervelde-dieren/nyssodesmus-python.htm#animaltab1*

N. python specimen in this study had three undescribed morphospecies of mites. Species were adults because they had eight legs, rather than the six legs present on instars (Krantz & Walter 2009). The morphospecies were defined as A, B, and C (Figure 2). Morphospecies A was small, brown, and mostly appeared on the back or sides of the millipede's exoskeleton. They had four front facing legs and four back legs that were covered by the main body of mite. They appeared mostly stationary. When removed from *N. python*, within 10 minutes their legs stopped moving and they seemed to die. Morphospecies C were large red or white mites that appeared as the most mobile of the morphospecies, mostly found either walking around the millipede or stationary between leg segments on the stomach. They were larger and wider than the other two

mite morphospecies. Morphospecies B was small, orange, and found in crevices where the legs met the exoskeleton. They were mostly stationary except when disturbed. When disturbed they would walk around the millipede lateral and dorsal exoskeleton to return to another leg crevice. These were the primary organisms for the HCN-mite interaction test as they were most likely to block ozopores as they had mobile ability and located close to the millipede's keels.

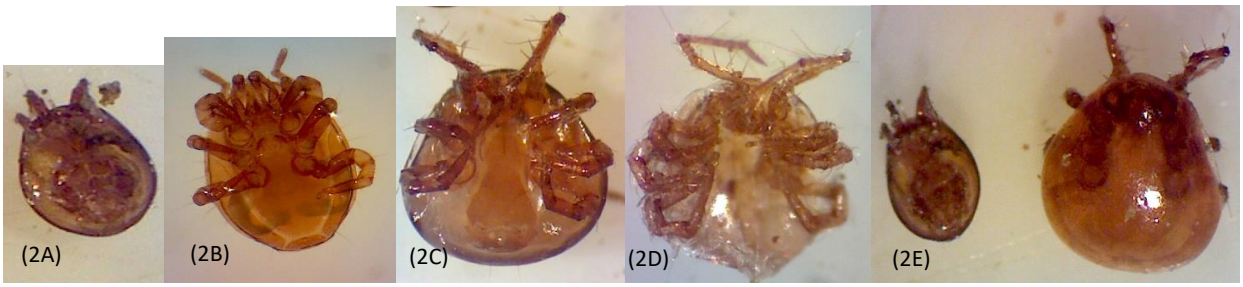

Figure 2*: Three morphospecies of mites were found on* N. python. The total mite load found for each millipede, with the location of each mite was noted before experimentation. Image (A) is the ventral side of morphospecies A. This mite was found one the back and sides of the millipede and mostly stationary. Image (B) is the ventral side of morphospecies B. This mite was found at the base of the millipedes' legs and was also mostly stationary. Image (C) & (D) are the same morphospecies in different colors. They are morphospecies C, found mostly on the stomach of the millipede, and were the most mobile species. The final image (E) is a size comparison between morphospecies A and C.

Experiment 1: HCN Excretion by Millipedes

Sodium picrate solution was made by dissolving 12.5 g of sodium carbonate and 2.4 g of picric acid in 500 mL of distilled water (Taylor 2016; Tava & Annicchiarico 2000). This solution was stored in a bottle then small portions were poured into a petri dish to dip filter paper strips. Filter paper strips of 1x5 cm were soaked in the sodium picrate then dabbed to remove excess. Strips were wet but not dripping when used.

To quantify cyanide excreted, one millipede was transferred from the Tupperware to a Ziploc bag containing five strips of 1x5 cm sodium picrate paper. The mites were counted on each specimen before testing, noting location. Location of mites were categorized as base of legs (LL), lateral and dorsal exoskeleton (B), ventral exoskeleton (S), and other (O) (i.e. directly on leg, antennae, etc.). These were further categorized into two main groups for analysis as LL mites (mostly morphospecies B) and Non-LL mites (other morphospecies of mites). Length and sex of the millipede was also recorded. The specimen was manipulated for four minutes to simulate a predator attacking. This was accomplished by holding the bag by the top and rapidly shaking the bag up and down. *N. python* often curled up during this experiment and to stimulate a predator, the keels of the tail segments and middle segments of the millipede were held through the bag and pulled, trying to uncurl the body (Figure 3). Excretion was noticeable through HCN's strong smell of almonds and because the filter papers changed color. After four minutes of manipulation, the specimen was returned to its Tupperware. The bag with the sodium picrate papers was held at the top and rapidly shaken side to side for one to two minutes to soak more HCN. The bag was then left to sit for 18 to 19 more minutes (always totaling 20 minutes), away from direct sunlight, at room temperature.

Each of the five sodium picrate papers were removed from the Ziploc bag and dipped 30 times into separate test tubes containing 5 mL of deionized water. One paper was dipped into one test tube. The liquid was then transferred to a cuvette, careful to not pour in any dirt or fecal material that may transferred during dipping of the sodium picrate papers. Only a few samples

had dirt or fecal material in their test tubes, and even fewer in the cuvettes. This did not impact readings as the dirt nor fecal material seemed to dissolve into the solution, and spectrophotometer readings were taken in the middle of the cuvette. Dirt or fecal materials sunk to the bottom of the cuvette and did not interfere with the overall reading. The cuvettes were placed in a 200 series spectrophotometer (UV-200-RS, V-200-RS) at 540 nm. The five readings of percent transmission ('x') were measured, averaged, and input the into the equation A=2-log(x) to obtain absorbance ('A'). To obtain milligrams of HCN ('y'), the absorbance ('A') is put into the equation y=0.2476(A)+0.009, from a standard curve made from a serial dilution of KCN (Brito *et al.* 2009).

Experiment 2: Mite attraction to HCN

To test if mites were attracted to the HCN instead of any other *N. python* pheromone, another source of HCN was used to test mite attraction. Young passion vine leaves (Passifloraceae:*Passiflora)* were a readily available source of HCN. When mechanically damaged, passion vine leaves release hydrogen cyanide as a defense mechanism (Jaroszewski 2002). Young to middle age passion vine leaves were collected from the gardens of Estación Biológica and the nearby TA's cabin. These were checked for cyanide content by crushing the leaf in a glass vial with a glass stirrer and adding three drops of toluene. A 1x5cm sodium picrate filter paper was then suspended above the sample, held by the cap and the side of the vial. After 20 minutes the filter paper turned orange, indicating cyanide content and viability as the test's experimental HCN variable.

To prepare for each trial, two to three passion leaves were crushed using a mortar and pestle. The test placed ten specimens of morphospecies B in the center of circular filter paper in a petri dish. One side of the petri dish was dabbed with crushed passion vine leaves and the opposite side had a droplet of distilled water as a control variable. The crushed passion leaves and droplet of distilled water were similar in area. The mites were timed for ten minutes. After ten minutes, mite locations were noted if they were within a centimeter of the passion leaf circle ('Passion'), the water droplet ('Water'), or if they were not near either ('Away'). This was completed ten times with ten mites. After every trial, each mite was checked to assure its legs were still moving, indicating that it was alive and could be mobile. If the mite no longer moved its legs, it was removed and replaced by another mite. Some mites were reused for the trials. Midway through the mite trials, methodology changed to covering the petri dish, leaving a hole for air. The reason was to avoid desiccation by the natural sunlight that occurred in the chemistry room. I do not believe this impacted results because there was not a large decrease nor increase in the amount of mites that died in the trials after covering the petri dish.

Figure 3*: The first two images (A&B) are images of the before and after the cyanide excretion test. The millipede was put into a Ziploc bag with five, yellow 1x5 cm sodium picrate filter paper strips. The bag was then shaken for four minutes and predation was simulated by attempting to uncurl the millipede. Image (B) shows the areas of N. python that were held and pulled in the direction of the red arrows to simulate a predator attempting to uncurl it. When* N. python *excreted HCN, the yellow sodium picrate papers turned to an orange to red color depending on how much cyanide it absorbs. After four minutes, the millipede was removed, and the strips were left in the sealed bag for 20 minutes. The orange color in image (C) indicates cyanide release. Image (D) is a one of the mite trials. Each of the small dots on the filter paper is a mite that started the ten-minute trial in the middle of the paper. The green circle is crushed passion vine leaves and the wrinkled paper directly across from the green circle is where the control, a water droplet, was dropped. Image (B) was found at https://entomologymanchester.wordpress.com/2014/07/11/costa-rica-fieldcourse-2014-large-forest-floor-millipede/.*

RESULTS

This investigation tested the hydrogen cyanide excretion of 26 individual millipedes of varying stages, sexes, lengths, and mite load. There were 18 adults and eight juveniles, 13 females and 13 males, and mite load ranging from 0 to 79. Between the all 26 *N. python* specimens, 210 mites were found.

The locations with the most mites in decreasing order was B (117), LL (62), S (18) and O (13). Thus, the number of LL-mites was 62 and Non-LL Mites was 148. These locations were generally occupied by one of the morphospecies. Morphospecies A was mostly found on the dorsal and lateral areas of the exoskeleton (B), morphospecies B resided at the base of the legs (LL), and morphospecies C were found walking along the body and on the stomach (S).

Morphospecies C were the only mites observed walking off the millipede. Continual captivity likely impact mite counts, and mites should be counted the day of initial capture.

Stage and Sex

Stage significantly impacted the overall hydrogen cyanide excretion (Two-tailed T-test, df=24, t=2.596, p=0.016). Adults secreted significantly more HCN than juveniles, adults averaging double the amount of cyanide produced by a juvenile (Figure 4C; Adults: $\bar{x}$=0.11±0.015; Juveniles: $\bar{x}$=0.05±0.014).

Sex did not significantly impact hydrogen cyanide excretion (Two-tailed T-test, df=24, t=0.91, p=0.37). On average, females secreted more HCN than the males in this trial, however the standard error bars indicate that females and males secrete similar amounts of hydrogen cyanide (Females: $\bar{x}$=0.11±0.018; Males: $\bar{x}$=0.08±0.018).

Mites did not significantly prefer a specific stage nor sex (Two-tailed T-test, df=24, t=, p=0.68; Two-tailed T-test, df=24, t=0.05, p=0.96, respectively). More mites were found on juveniles than adult *N. python*, but the standard error overlaps between the two stages and are thus not significantly different (Adults: $\bar{x}$=7.33±4.41; Juveniles: $\bar{x}$=10.25±3.47). The average number of mites was nearly identical between female and male specimens at $\bar{x}$=8.38±6.12 and $\bar{x}$=8.08±2.33, respectively. Female specimens showed more variability in mite load as standard error is larger than male specimens.

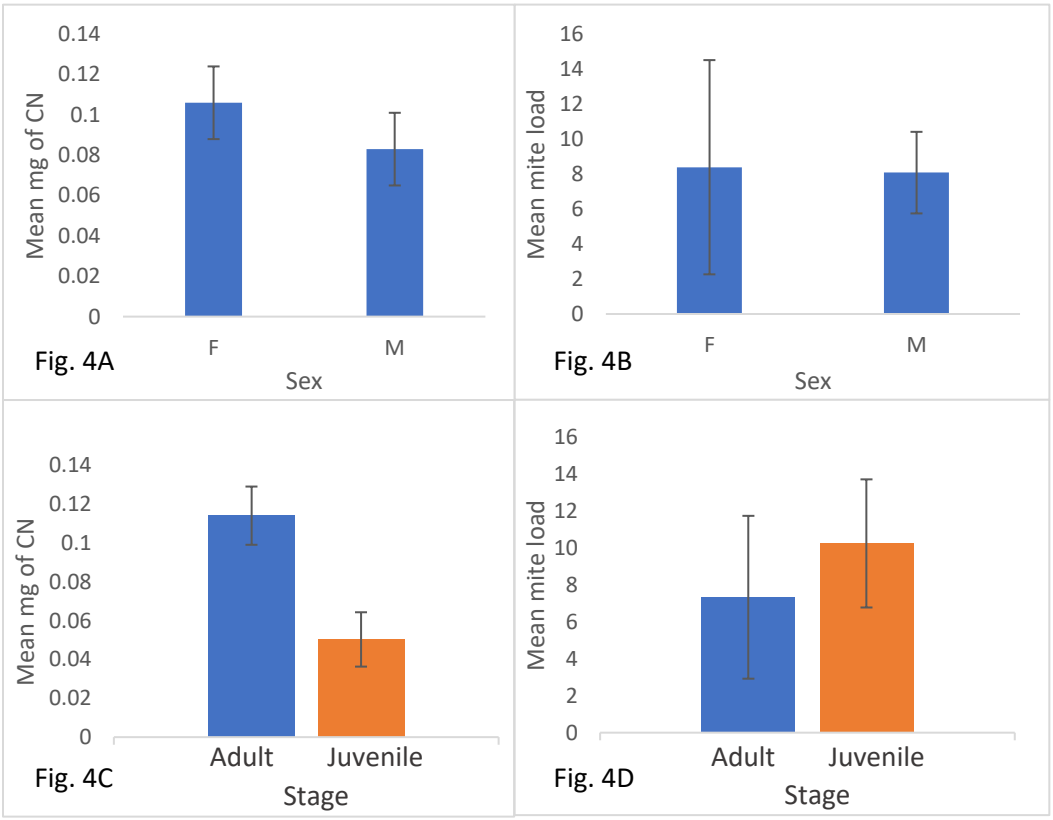

***Figure 4**: Millipedes were sexed and determined which stage they were in upon initial capture. There were 26 millipedes in total. 13 were female and 13 were male. There were 18 adults and 8 juveniles. These graphs show the results of T-tests with standard error. Graphs 4A and 4B compare average cyanide excretion and average mite load of each sex. There was no significant difference between males and females in excretion nor mite load. The was greater variation in female mite load than there was*

in males, as indicated by the standard error bar. Graphs 4C and 4D compare average cyanide excretion and average mite load between stages. 4C shows that adults excrete significantly more cyanide on average than juveniles and 4D shows that mites do not significantly prefer one stage over another, but juveniles trended towards having more mites.

Length and Stage

Length significantly impacted hydrogen cyanide excretion (Regression, R^2=0.13, n=25, F=4.52, p=0.045). The significant positive trend indicates that longer millipedes excrete more cyanide than shorter millipedes. Length did not significantly impact mite load on individual millipedes (Regression, R^2=0.039, n=25, F=0.09, p=0.77). The number of mites on a given millipede cannot be predicted from the length.

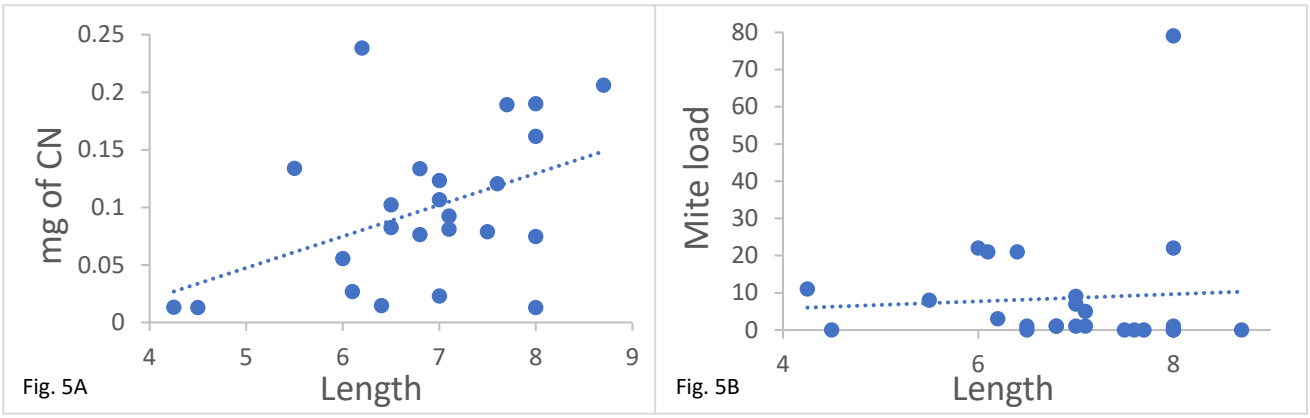

***Figure 5**: Millipedes length was measured and compared against amount of cyanide excreted (5A) and the mite load (5B). Length significantly impacts amount of cyanide excreted in a positive relationship as shown by the trendline. This means that the longer and presumably older a millipede is the more cyanide it will produce. Length did not significantly relate to the number of mites it hosted, as shown by the relatively flat trendline.*

Stage significantly impacts length (One-tailed T-test, df=23, t=3.07, p=0.008). This is unsurprising as length is positively correlated to how long a millipede has been alive and thus juveniles are often shorter than adults. However, length significantly impacts cyanide excretion during the juvenile stage (Regression, R^2=0.56, n=8, F=9.87, p=0.02), but does not impact cyanide excretion during the adult stage (Regression, R^2=0.03, n=17, F=0.58, p=0.46). These regressions R^2-values show that there is much more variability in cyanide excretion as an adult *N. python.* As such, stage and length are related but they cannot replace the other when predicating cyanide excretion in *N. python.*

Mite load

Mite load significantly impacted hydrogen cyanide excretion (Regression, R^2=0.15, n=25, F=5.50, p=0.045). When the outlier with 79 mites was removed, the relationship was still significant (Regression, R^2=0.16, n=25, F=5.76, p=0.025). This shows the more mites a millipede has, the less HCN they excrete.

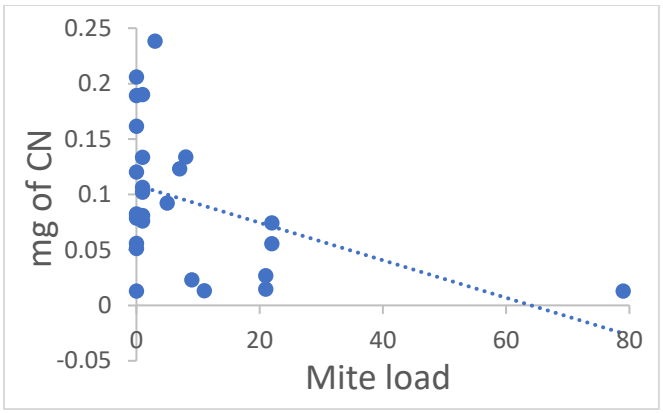

***Figure 6**: Mite load was found by counting to total number of mites on the millipede. Mite load significantly impacted the cyanide excretion in a negative relationship. The trendline shows that as mite load increases, cyanide excretion decreases.*

Mite attraction to HCN

There was a significant difference between mite attraction between the treatments 'Passion' (form of CN), 'Water', and 'Away' (Friedman test, X^2_r=14.15, df=2, p=0.00085). However, this was because most mites were 'Away' and there was not a significant difference between mite attraction to 'Passion' and 'Water' (Friedman test, X^2_r=0.9, df=1, p=0.3428). Meaning that the mites did not prefer 'Passion' over 'Water'. This fits my observations that many mites would wander away or stay in one place, outside of the one-centimeter perimeter of the 'Passion' and 'Water' treatments. This can be seen in Table 1 as only one trial (Trail 2) exhibited more than half of the mites choosing between the 'Passion' or 'Water' treatments. A majority of the trials saw that half or more of the mites were found 'Away' from both treatments.

***Table 1**: This table shows the results of the ten mite trials. Only morphospecies B was tested. The mite trials consisted of placing ten mites in the middle of filter paper in a petri dish and dabbing crushed passion leaves and a droplet of water on opposite sides of the petri dish. After ten minutes, the mite's locations were recorded.*

Trials	Dates	Passion extract	Water	Away
1	12-Nov	3	0	7
2	12-Nov	3	3	4
3	12-Nov	3	0	7
4	12-Nov	0	1	9
5	12-Nov	1	1	8
6	12-Nov	1	0	9
7	13-Nov	5	0	5
8	13-Nov	1	2	7
9	13-Nov	1	1	8
10	13-Nov	3	2	5

Mite location

LL mites, or mites found at the base of legs on the sides of *N. python* (mostly morphospecies B), had a significant impact on the amount of cyanide secreted by *N. python* (Regression, R^2=0.14, n=26, F=4.99, p=0.035). Non-LL mites, mites found anywhere other than the base of the legs (other mite morphospecies), also had a significant impact on the amount of cyanide secreted by *N. python* (Regression, R^2=0.14, n=26, F=5.05, p=0.034). This indicates that both LL and Non-LL mites play nearly equal roles in decreasing amount of cyanide excreted by *N. python*, with Non-LL mites influencing cyanide slightly more.

However, when the outlier was removed from the regression analysis, LL mites lose their significant impact while Non-LL mites increase in significance (Regression, R^2=0.088, n=25, F=3.33, p=0.08; Regression, R^2=0.23, n=25, F=8.09, p=0.009). This shows that Non-LL mites likely decrease the amount of cyanide excretion more than the LL-mites. This is illustrated in Figure 8, as Fig. 8D's data points follow the trendline closer than the other three graphs.

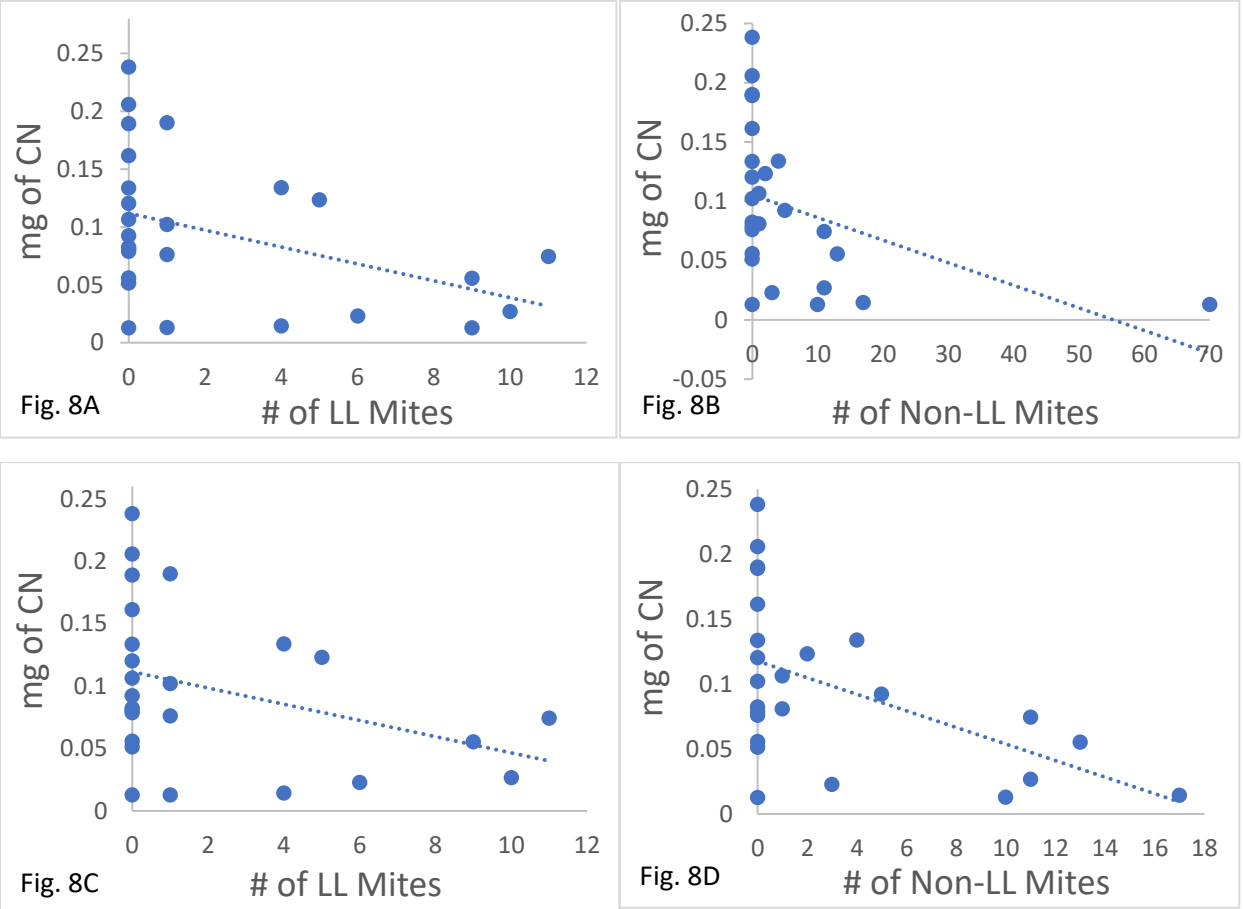

Figure 7: The two categories of mites tested compared to mg of CN excreted by N. python *were LL mites (morphospecies found at the base of the legs, mostly the morphospecies B; main subject of the HCN attraction test) and Non-LL mites (morphospecies not found at the base of the legs). Regression analysis were run with the outlier (8A and 8B) and without the outlier (8C and 8D). With the outlier, there were 62 LL mites and 148 Non-LL mites tested. Without the outlier, 53 LL mites and 78 Non-LL mites were tested.*

Additional Observations

Two albino millipedes were found during this investigation. One millipede did not have mites but had a much slower reaction time than the millipede with mites. The millipede without mites secreted less cyanide than the other, and eventually died after its cyanide trial. This may indicate that weaker millipedes do not have the capabilities to create the enzyme to catalyze the mandelonitrile into HCN.

DISCUSSION

These findings corroborate Miller's (2018) conclusion that mites significantly lower cyanide excretion and that mites do not have a sexual preference for their host. This study adds to her study by quantifying the ectoparasitic mites' impact on the millipede's cyanide chemical defense, testing the external factors of sex, stage and length's impact on amount of cyanide excreted and mite load, and testing mite attraction to HCN. This adds to our overall understanding of factors influencing *N. python*'s chemical defense by showing that older and longer millipedes excrete significantly more cyanide than younger and shorter millipedes, that mites do not have a sex, stage, or length preference, and that non-LL mites likely impact cyanide excretion more than LL-mites. The HCN attraction test found that the morphospecies B likely did not feed on nor was attracted to the millipede's chemical defense.

Mechanisms of parasitism likely vary across mite species. This experiment found that the morphospecies B is not attracted to HCN. Meaning, it is unlikely that the mites feed on the HCN or aggregate on the ozopores due to an attractive scent as Miller (2018) theorized based on the observation of *Megaselia* phorid flies feeding on *N. python*'s HCN (Hash 2014). The mites could still aggregate on the ozopores for other reasons besides attraction to the HCN scent and thus block secretion. This cue could be chemical or physical, such as detecting the mandelonitrile that is present in the glands leading to the ozopore or by simply finding the opening of the ozopore and residing in or near it. *N. python* ozopore size and location must be described to test if mites do indeed block the ozopores. In regards to the HCN attraction test, there were limitations as it was not pure HCN; it was HCN with crushed leaf matter. Regardless of the leaf matter, there was still HCN strongly present as indicated by the initial cyanide presence test.

This experiment counted total mites, noted their locations, and tested HCN attraction of one of the three morphospecies. The regression analysis comparing mite loads and locations an individual (LL-mites and non-LL mites) indicates that the species that was tested for HCN attraction likely has a lesser impact on cyanide excretion when compared to all the other mites. This can be concluded because morphospecies B were only found at the base of the legs on the sides of the millipede (LL mites). Thus, further testing of the two other morphospecies for their HCN attraction would reveal if either of those morphospecies feeds on the HCN.

Beyond investigating HCN attraction of the other two species, studying the interaction of all the species could reveal that the combination of species creates a greater impact than any one of the species. Mites on invertebrates are known to consume debris on the host cuticle, secretions of the host, or feed on the hemolymph (Paré & Dowling 2012). There is a low likelihood that morphospecies B fed on the HCN secretions, but only one of the three morphospecies was tested. The other two species could potentially be involved in feeding on HCN secretions or blocking the ozopores due to attraction to the scent of HCN. The feeding habits of the individual mites must be researched further. If they don't feed on the HCN, debris on the host cuticle and the hemolymph are also possibilities. If all the species feed on hemolymph (hematophagous) then

their mechanism of parasitism would be the overall weakening of the millipede to the point of inability to produce HCN. Thus, the parasites would be like *Varroa destructor,* a widely studied parasitic mite found on honey bees that feeds on hemolymph. These parasitic mites significantly decrease the amount of proteins in the honey bees' hemolymph, decreases body weight, and interferes with organ development (Bowen-Walker & Gunn 2001). If the mites fed on the hemolymph and weakened the millipede enough, it may not be able to synthesize the enzymes needed to catalyze the mandelonitrile into HCN, thus reducing cyanide excretion. The weakened albino millipede observed in my experiment may be an example of how overall weakness (seen through sluggish movement and slow reaction time) inhibits HCN excretion even when it did not have any mites to influence its excretion. Thus, one could infer that, if the mites weaken the millipede enough through hematophagy, then it may inhibit HCN excretion. For further testing, I advise to closely examine morphological characteristics and try to find the families to which the mites belong, as this could potentially reveal feeding habits (Paré & Dowling, 2012).

There are several possible implications this study offers on *N. python* fitness. This study revealed that juvenile millipedes secrete significantly less cyanide than adult millipedes, on average. A T-test also showed that mites did not significantly prefer juveniles over adults on average, however juveniles were observed to have more mites than adults. If juveniles tend to have more mites, their chemical defense is further weakened and leaves them more susceptible to predation. This leads to two cases. Mites are killed along with the millipedes in predation, or the mite would weaken the millipede then find another host to feed on, in which the millipede has a lower chance of survival and the mite survives regardless. If the latter scenario occurred frequently enough, *N. python's* fitness may decrease.

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Distance gradient of vocalization discrimination and aggression in neighborhoods of Rufous-and-white wrens *(Thryophilus rufalbus)*

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ABSTRACT

Communication networks have been observed in multiple taxa. Many territorial animals in communication networks tend to act differentially towards their neighbors given the competitive pressure of the habitat. Many birds in particular have the ability to discriminate between vocalizations of neighbors versus the vocalizations of strangers (neighbor stranger discrimination, NSD). Rufous-and-white wrens (*Thryophilus rufalbus*) vocally defend territories year round and have been shown to exhibit NSD. The focus of this study was to investigate whether *T. rufalbus* demonstrates a gradient of aggression when played back vocalizations from neighbors, neighbors of adjacent neighbors, and strangers. Multiple parameters measured displayed this gradient of aggression in a population of *T. rufalbus* in Monteverde, Costa Rica. Particularly time spent vocalizing (stranger: 57.92 ± 22.18, neighbor-neighbor: 193.83 ± 95.29, neighbor: 409.75 ± 120.69) and number of songs performed while defending (stranger: 1.71 ± 0.29, neighbor-neighbor: 2.14 ± 0.26, neighbor: 2.67 ± 0.28). Other variables measured demonstrated differences between treatments, but weren't as significant, such as bandwidth (stranger: 1732.97 ± 121.40, neighbor-neighbor: 2174.20 ± 204.13, neighbor: 2314.94 ± 126.74). This study demonstrates that *T. rufalbus* displays a gradient of aggression in defensive behaviors to neighbors at differing distances, and strangers. Eavesdropping behavior described in other species of wren may explain this gradient of aggression, because wrens may frequently listen to and identify the vocalizations of neighbors more than a territory away. This study also suggests that this population of *T. rufalbus* exhibits the nasty neighbor effect, based on more aggressive responses to simulations of neighbor intrusions compared to strangers.

RESUMEN

Los canales de comunicación han sido observados en diferentes taxones. Muchos animales territoriales en canales de comunicación tienden a actuar de maneras diferentes hacia sus vecinos debido a las presiones de competencia en el hábitat. Muchas aves en particular tienen la habilidad de discriminar entre las vocalizaciones de los vecinos versus aquellas de extraños (discriminación vecino extraño, NSD por sus siglas en ingles). Los soterrés Rufo y Blanco (*Thryophilus rufalbus*) defiende los territorios vocalmente durante todo el año y muestran NSD. El propósito de este estudio fue investigar si *T. rufalbus* muestra un gradiente de agresión cuando se les tocó una vocalización de los vecinos, vecinos de los vecinos más cercanos, y extraños. Parametros múltiples medidos mostraron este gradiente de agresividad en las poblaciones de T. rufalbus en Monteverde, Costa Rica. Particularmente el tiempo vocalizando (extraño: 57.92 ± 22.18, vecino-vecino: 193.83 ± 95.29, vecino: 409.75 ± 120.69) y el número de canciones cantadas durante la defensa (extraño: 1.71 ± 0.29, vecino-vecino: 2.14 ± 0.26, vecino: 2.67 ± 0.28). Otras variables medidas demuestran diferencias entre tratamientos, pero no fueron significativas, tales como el ancho de banda (extraño: 1732.97 ± 121.40, vecino-vecino: 2174.20 ± 204.13, vecino: 2314.94 ± 126.74). Este estudio demuestra que *T. rufalbus* demuestra un gradiente de agresión en comportamientos defensivos a vecinos a diferentes distancias, y extraños. El comportamiento de espionaje descrito en otras especies de soterrés puede explicar este gradiente de agresión, porque los soterrés pueden frecuentemente escuchar e identificar las vocalizaciones de los vecinos a más de un territorio de distancia. Este estudio también sugiere que esta población de *T. rufalbus* exhibe el vecino del vecino desagradable, basado en las

respuestas más agresivas a las simulaciones de vecinos intrusos comparado con los extraños.

INTRODUCTION

Territoriality has been observed in a variety of species from multiple different taxa (Bourne *et al.* 2005, Heinze *et al*. 1996, Leiser and Itzkowitz 1996, Pratt and McLain 2006). Defending a territory containing mates or resources is incredibly useful. However surveying a territory requires time, actively defending it requires energy, and physical altercations can be lethal (Huntingford and Turner 1987, Neat *et al.* 1998). If the benefits gained outweigh the costs of territory defense then territoriality will be implemented (Brown 1964). Vocalizations are consistently used by a variety of taxa to establish and defend territories (Briefer *et al.* 2007).

Being able to discriminate between signals from neighboring individuals and signals from strangers is valuable (Lovell and Lein 2004). In highly competitive habitats, individuals that consistently encounter each other have to choose between evading, tolerating, or fighting the competitor (Tanner and Alder 2009). Fights are costly to animals; they can result in serious injury to both the winner and loser, they require time and energy, and they increase the risk of predation (Huntingford and Turner 1987, Neat *et al.* 1998). Being able to recognize a neighbor's signal can increase the fitness of both individuals involved, by lowering the chance and frequency of physical altercations (Briefer et al. 2007). This phenomenon, described as "the dear enemy effect" (Fisher 1954), has been explored throughout a multitude of species and taxa (Bourne *et al.* 2005, Heinze *et al*. 1996, Leiser and Itzkowitz 1996, Pratt and McLain 2006). Multiple oscine passerines such as skylarks, sparrows, and wrens display this behavior (Vehrencamp et al. 2014). The dear enemy effect reflects an established mutualism between two individuals of the same species capable of neighbor stranger discrimination (NSD). By contrast, there are situations in which neighbors represent a greater threat than strangers. Potential losses to neighbors may be more detrimental than a loss to a stranger. For example, fluctuating food levels on feeding territories can encourage usurpation by neighbors. In these cases, a greater response to neighbors is expected. This phenomenon is referred to as the "nasty neighbor" effect (Christensen and Radford 2018).

Territorial interactions arise because any territorial individual is equidistant from multiple neighbors and is capable of receiving signals from more distant individuals up to a certain distance (McGregor 2005, McGregor 1993). Groups of territory holders can therefore be considered as an interacting network of signalers and receivers, where many combinations of signalers and receivers are possible (McGregor 1993). In such networks, individuals can obtain information on the quality and motivation of neighboring individuals by eavesdropping on their signaling interactions (Naguib *et al.* 2004). It has been shown in a variety of species that birds learn their songs from eavesdropping on the interactions of other individuals (Beecher *et al.* 2007). The capacity to learn another individuals song could be beneficial in the context of territory defense. Individuals capable of recognizing signalers from more than one territory away will gain an inherent benefit in knowing the location of another individuals territory. Moreover, territory holders with the capacity to determine if a signaler is within their territory will gain multiple benefits. First, they would reduce the amount of energy required in detecting the position of the signaler. Second, they would minimize the potential injury associated with closely approaching the signaler (McGregor 1993). The term range is often used to refer to the distance between signaler and signal receiver. Increasing the range of bird and insect songs results in decreases in signaling amplitude and increases in signal degradation or distortion (McGregor

1993). Either decreases in amplitude or increases in distortion can be used as determinants for range (McGregor 1993). This assists multiple avian species with signal discrimination (McGregor 2005).

Birds consistently live in communication networks where NSD occurs. This idea has been tested across multiple different species of songbirds in the tropics (Battiston *et al.* 2015, Vehrencamp *et al.* 2014, Wei *et al.* 2011). However the extent at which eavesdropping and information gathering comes in to play alongside NSD has not been tested thoroughly. Naguib *et al* (2014) demonstrated that territorial birds pay attention to responses of neighbors to other birds, and that they respond according to what their neighbors do in those interactions with other birds. Hence, birds that exhibit NSD should also be able to discriminate between neighbors, strangers, and neighbors of neighbors at least, if they can estimate distance range and eavesdrop. This has not been tested in any species within the context of NSD to our knowledge.

The rufous-and-white wren (*Thryophilus rufalbus*) is a neotropical bird species with an elevation range that is capped at approximately 1300 meters (Stiles and Skutch 1989). Other species of wren demonstrate the ability to estimate signal range (McGregor 1993), and eavesdrop on heterospecifics (Fallow and Magrath 2010, Magrath *et al.* 2009). The population of *T. rufalbus* that lives in Monteverde, Puntarenas, Costa Rica exists at the maximum of their elevational range and has been shown to demonstrate NSD (Dunn 2006). Dunn (2006) showed that Rufous-and-white wrens in Monteverde respond for longer to simulated intrusions of strangers compared to neighbors, and overall sing more songs. This illustrates that rufous-and-white wrens in this area exhibit the dear enemy effect.

This study aims to evaluate whether *T. rufalbus* can discriminate between neighbors located at different distances and strangers. Calls from multiple individuals in a neighborhood were recorded and played back to simulate territorial intrusions to focal individuals. This was done with the goal to evaluate whether focal territory holders responded differentially to the invasion of adjacent neighbors, neighbors of adjacent neighbors, and strangers. *T. rufalbus* is a great candidate study species because the species exhibits NSD, and wrens show territoriality year round (Hyman 2002). Based off of the results of Dunn (2006), which found evidence for the dear enemy effect in *T. rufalbus,* rufous-and-white wrens should exhibit less aggression towards simulated neighbor intrusions, as compared to neighbors located at further distances and strangers. A gradient of aggression from the three different simulated intrusions is predicted across the variables measured.

METHODS

Study Site

Research was conducted at Bajo del Tigre Reserve in Monteverde, Puntarenas, Costa Rica (10° 19’ 31.5” N, 84° 48’ 58.1” W) at an elevation of approximately 1300 meters. This reserve is dominated by a thick secondary forest and is classified as a tropical premontane moist forest (Holdridge 1966). Research was conducted in October and November of 2018.

Study Species

Rufous-and-white wrens are distributed widely throughout the neotropics. They can be found from southernmost Mexico to Panama’s canal zone, then through northern and eastern Columbia,

to north east Venezuela (Stiles and Skutch 1989). The Rufous-and-white wren is a year-round resident of the mature humid and late-succession forests on the pacific slope of Costa Rica, and prefers sunny edge habitat (Douglas *et al.* 2012). This species of wren is socially monogamous. Typically one male and one female defend a territory together (Mennill 2006, Osmun and Mennill 2011) and build a globular nest (Mennill and Vehrencamp 2008). Rufous-and-white wrens inhabit their territories year round, and begin nesting at the onset of the rainy season in May (Battiston *et al.* 2015). This study was conducted outside of the breeding season, which extends from April to August (Stiles and Skutch 1989).

Territory Location

Rufous-and-white wren territories were discovered by walking along the trails of the Bajo del Tigre reserve as well as on the roads adjacent to this reserve. A generic conspecific song from xeno-canto.org was played from a small Bluetooth speaker every 100m for 3 minutes in order to determine if a responsive individual was present or not. The generic song form xeno-canto was recorded in Santa Rosa, Costa Rica. Territory confirmation was made if an individual responded with a song to the generic playback. If visual confirmation of an individual was made, then the generic playback sequence would be repeated again in order to provoke a response from the individual. This was done over the course of several days. Upon confirmation of a territory, GPS coordinates of the site of response were generated using a GarminX60c model GPS system. Neighbors shared a territory boundary, neighbor-neighbors were two territories away, and strangers were three or more territories away (Fig. 1). The average rufous-and-white wren territory is 1.35 Ha ± 0.10 (Battiston *et al.* 2015). Neighbor boundaries are usually 50m in width and can span into neighboring territories (Battiston *et al.* 2015).

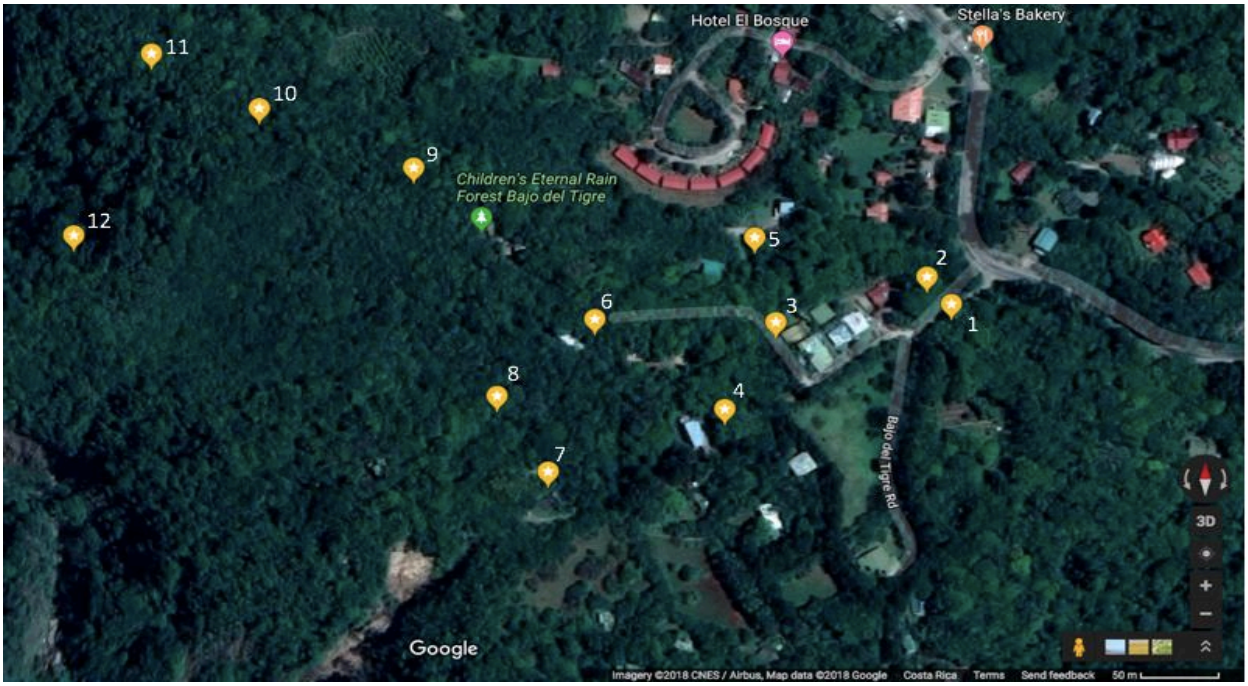

Figure 1: Territories used in the study of rufous-and-white wren in Monteverde, Puntarenas, Costa Rica. Neighbors were one territory away, neighbor-neighbors were two territories away,

and strangers were three or more territories away. For instance, Individual 4 would be a neighbor to 3, a neighbor-neighbor to 5, and a stranger to 11.

Initial Recording of Individuals

Confirmed territorial individuals were presented the generic Rufous-and-white wren again to record its response. After reaching a confirmed territory, the portable speaker was hung roughly 1 meter off the ground from a perch that was off trail. I watched the perch from approximately 5 to 10 meters away. Upon the response of an individual or pair, the playback was immediately stopped, and their full song was recorded using an Olympus LS-12 Linear PCM Recorder, which produces uncompressed waveform recordings.

Preparation of Recorded Songs for Intrusion Simulations

The recorded songs of the 12 individuals were edited in Audacity 2.3.0 to isolate the song portion from each recording and to filter out background noise. The isolated song stimulus was repeated once every five seconds for three minutes. The prepared 3 minute recordings for the 12 confirmed individuals were then downloaded onto an iPhone 6.

Simulated Intrusion of adjacent neighbors, neighbors of neighbors and strangers

Each of the 12 confirmed territories (Fig. 1) were then assigned a playback from an adjacent neighbor (hereafter called just neighbor), a neighbor of a neighbor (hereafter called neighbor-neighbor), and a stranger. Each territory received all three treatments sequentially, in randomized order using a random number generator. The protocol stated above for initial response recording was then followed. After the individual sang its last song or call, a 7 minute grace period was given before stopping the recording, in order to ensure the individual finished its defense. There was a 10 minute waiting period between treatments to reduce the chance of aggravating the individual due to rapid, sequential playbacks.

Quantifying Subjects Responses

Behavioral and acoustic responses were recorded for each of the 36 trials from the 12 territories. Behavioral parameters measured were distance of closest approach to the playback speaker within 10m (cm), latency from the start of the playback to the subjects first song (seconds), and time spent vocalizing (seconds). Acoustic parameters measured were bandwidth (Hz), lowest and highest frequencies (Hz) across all songs produced during the response, and number of song types used during response (Fig. 2). Before initiating playback trials, a perch 10m away from the speaker was marked. If the individual responded without being seen and was outside of the 10m boundary, then it was assigned a distance value of 20m. If the bird did not respond during the treatment, then a value of 420 seconds was assigned for the latency value (the amount of time for the grace period). The acoustic response measurements were taken using Audacity 2.3.0 and Ravenlite 2.0.

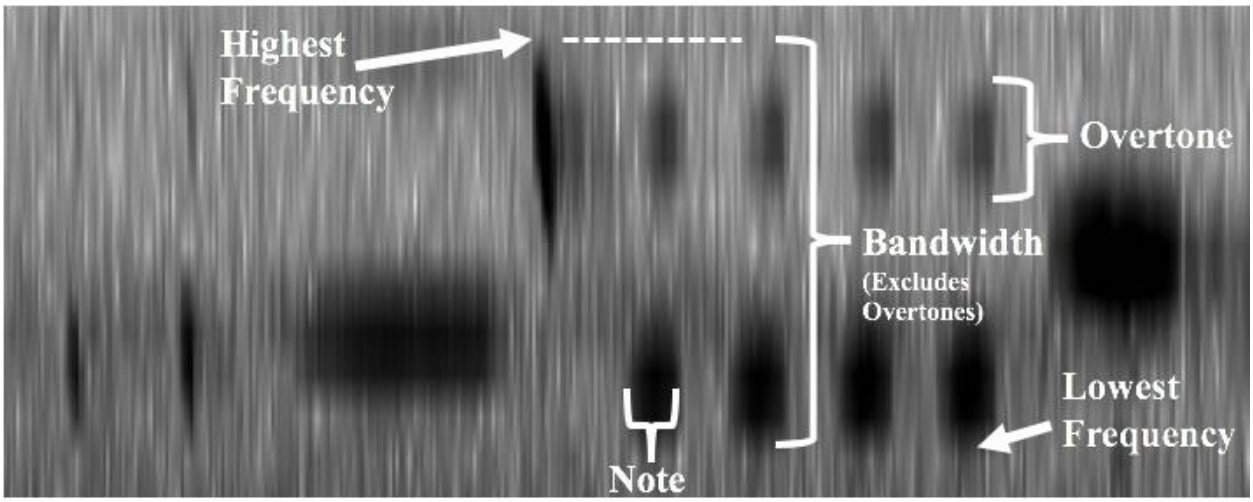

Figure 2: Sonogram of a rufous-and-white wren song (individual 5 from fig. 1) created in Ravenlite 2.0. Highest frequency (Hz), lowest frequency (Hz), and bandwidth (Hz) were measured from sonograms as shown here. Only the fundamental frequencies were measured. Overtones were ignored as they would convolute frequency measurements.

Statistical Analysis

Linear mixed models (LMMs) were used to compare variables individually between treatments while acknowledging the dependency between responses produced by a given individual (e.g. individual was included as a random effect in the model). Variables were log transformed, when necessary, to meet the assumption of normality. P-values correspond to an analysis of deviance conducted with the function "Anova" of the R package "car". Pairwise post-hoc comparisons of means were conducted using Turkey tests with the R package "emmeans". All analyses were conducted in R 3.4.3.

RESULTS

In general, rufous-and-white wrens demonstrated marked differences between behavioral and acoustic responses to the neighbor, neighbor-neighbor, and stranger treatments. In the case of bandwidth, responses to strangers showed reduced bandwidths compared to neighbors and neighbor-neighbors (LMM: chi-square = 17.463, df = 2, p = 0.0001, Fig.3). A similar trend was observed in number of songs, with less songs being performed for simulated strangers compared to neighbor-neighbors and neighbors (LMM: chi-square = 11.064, df = 2, p = 0.0040, Fig. 4). As for latency from start of playback to first response, focal individuals responded much more quickly to simulated intrusions of neighbors compared to neighbor-neighbors and strangers (LMM: chi-square = 6.1715, df = 2, p = 0.0457, Fig. 5). Largely significant differences between time spent responding to the three different simulated intrusions were observed (LMM: chi-square = 42.487, df = 2, $p < 0.0001$, Fig. 6). *T. rufalbus* tended to approach the playback speaker more closely during simulations of neighbor intrusions compared to neighbor-neighbor and stranger intrusions (LMM: chi-square = 7.1573, df = 2, p = 0.02791, Fig. 7). Response time and number of songs displayed the predicted gradient. The lack of significance is likely due to small sample size.

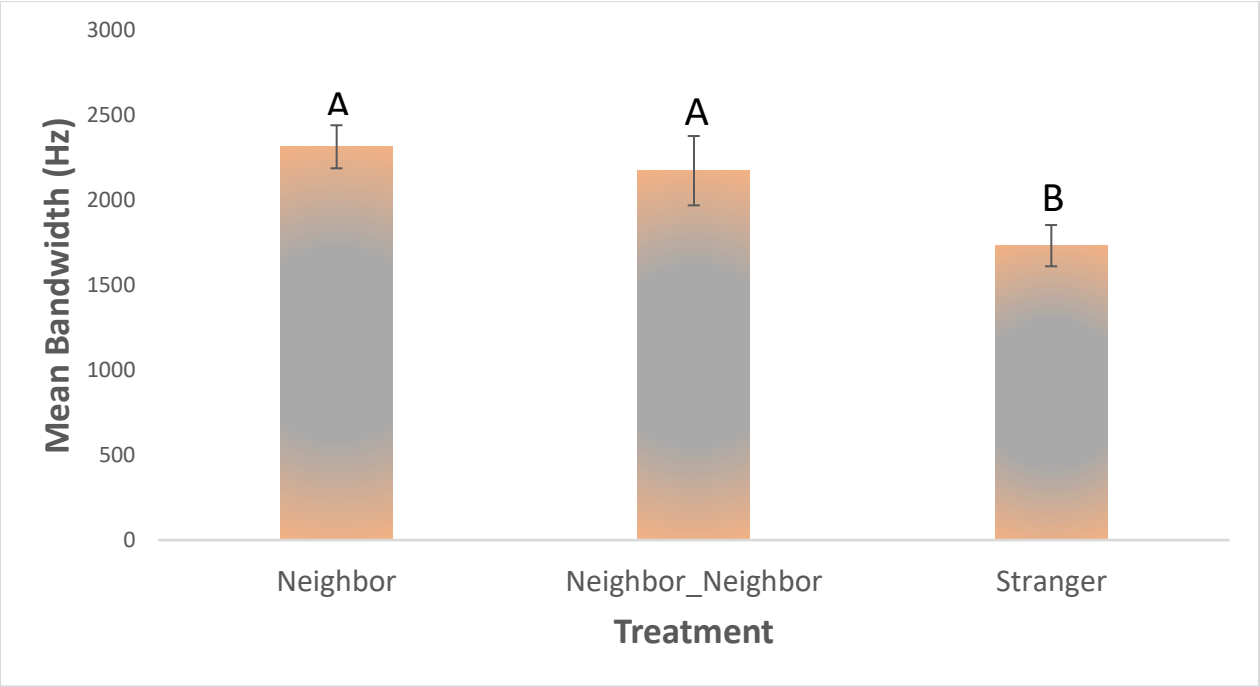

Figure 3: Bandwidth change in responses from *T. rufalbus* in Monteverde, Puntarenas, Costa Rica to simulated intrusions of conspecific adjacent neighbors, neighbor of neighbors, and strangers. Bars with differing letters indicate a significant difference in post-hoc pairwise comparisons between means according to Turkey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model.

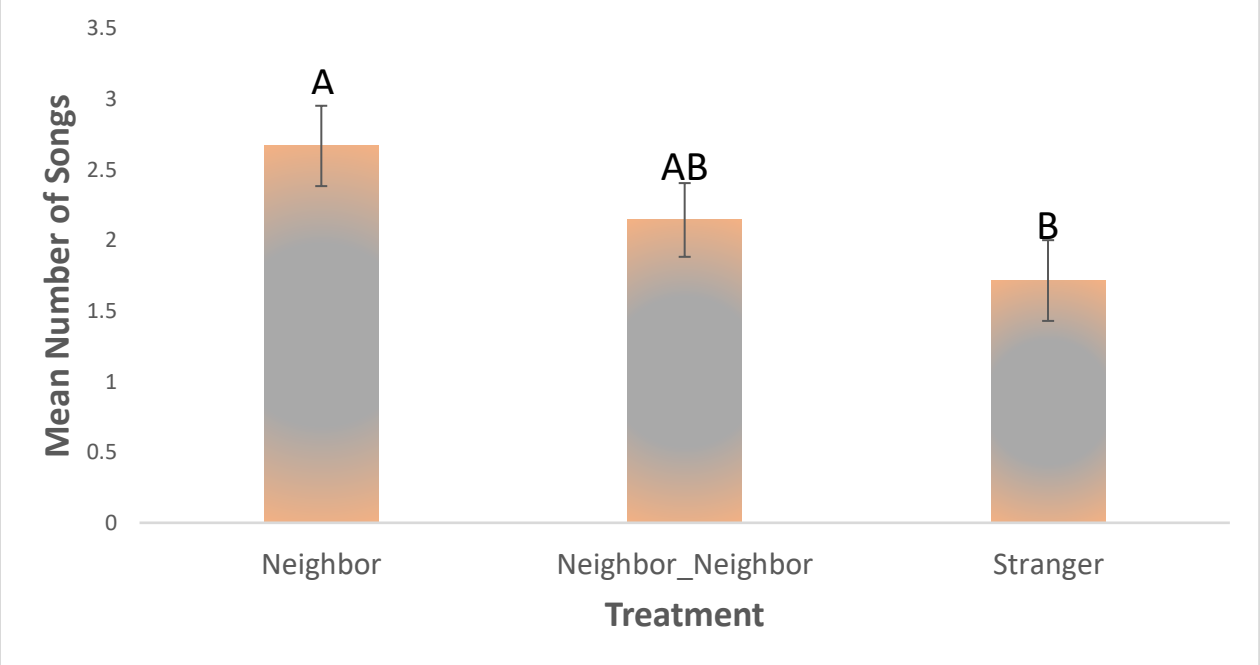

Figure 4: Change in number of songs in responses from *T. rufalbus* in Monteverde, Puntarenas, Costa Rica to simulated intrusions of conspecifics to adjacent neighbors, neighbor of neighbors,

and strangers. Bars with differing letters indicate a significant difference in post-hoc pairwise comparisons between means according to Turkey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model.

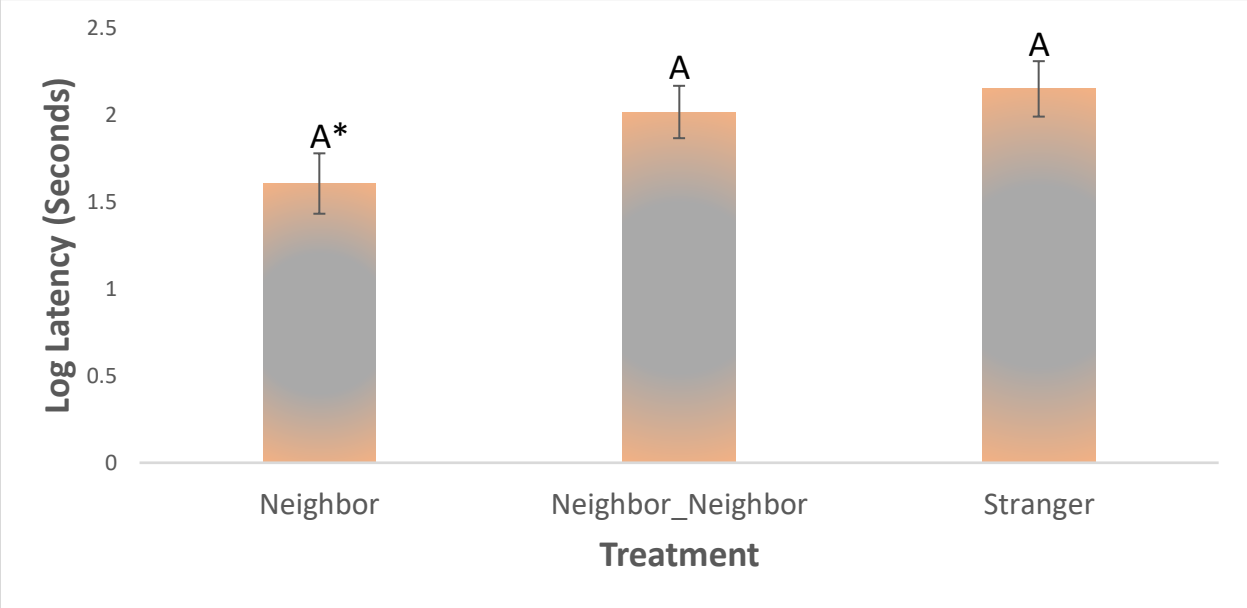

Figure 5: Changes in log of latency from start of playback to subjects first response by *T. rufalbus* in Monteverde, Puntarenas, Costa Rica to simulated intrusions of conspecific adjacent neighbors, neighbor of neighbors, and strangers. Bars with differing letters indicate a significant difference in post-hoc pairwise comparisons between means according to Turkey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model. Post hoc p-values between 0.05 and 0.10 are marked with different letters followed by an asterisk.

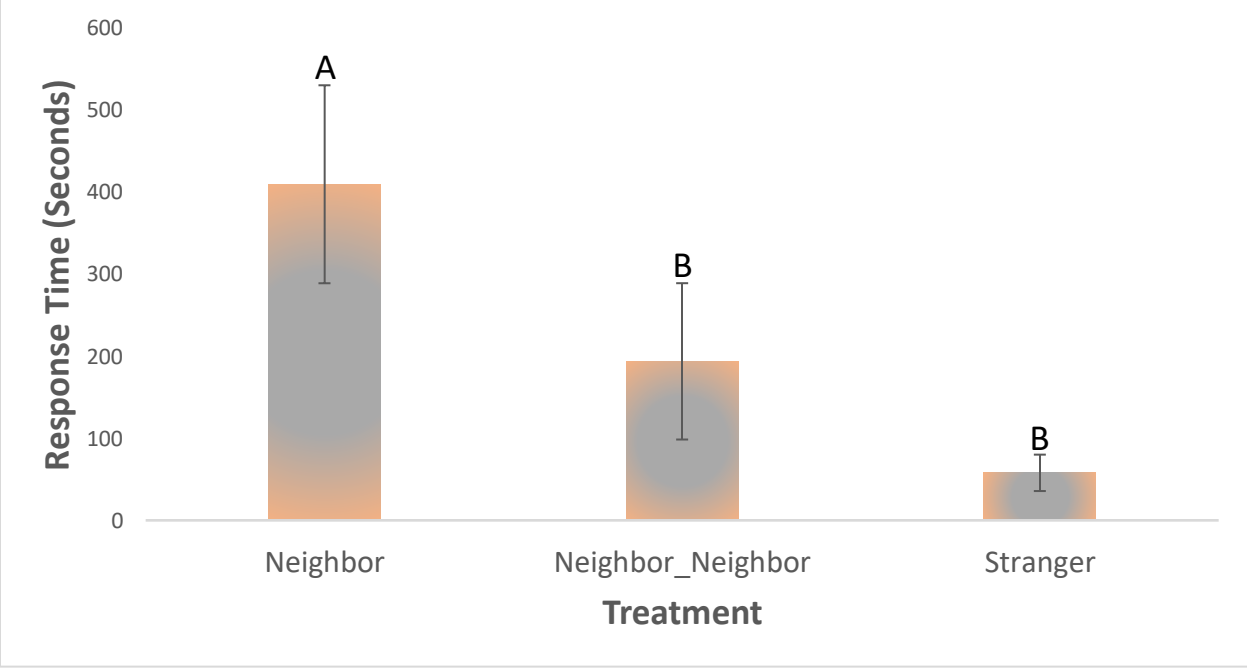

Figure 6: Time spent responding by *T. Rufalbus* in Puntarenas, Monteverde, Costa Rica to simulated intrusions of conspecific adjacent neighbors, neighbor of neighbors, and strangers. Bars with differing letters indicate a significant difference in post-hoc pairwise comparisons between means according to Turkey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model.

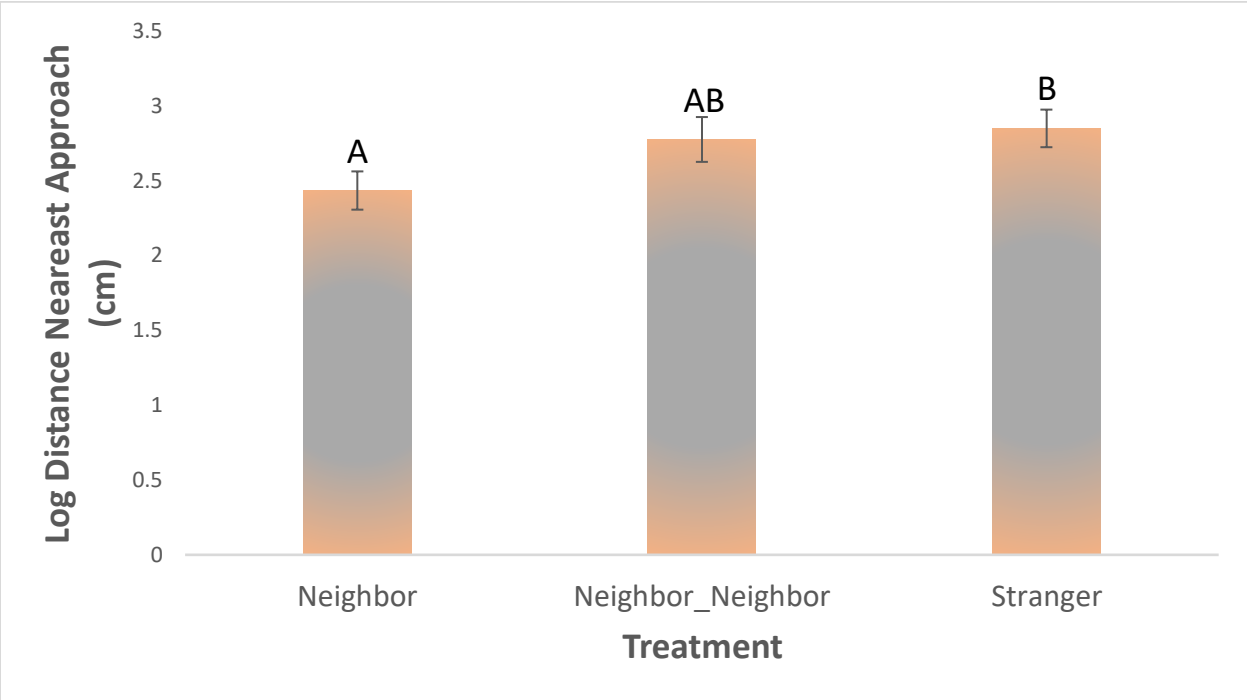

Figure 7: Log of nearest approach distance by *T. rufalbus* in Puntarenas, Monteverde, Costa Rica to simulated intrusions of conspecific adjacent neighbors, neighbor of neighbors, and strangers. Bars with differing letters indicate a significant difference in post-hoc pairwise comparisons between means according to Turkey tests ($p<0.05$) following the detection of significant differences between treatments using a linear mixed model.

DISCUSSION

The individuals tested in this study displayed traits contradicting the results of Dunn (2006). Individuals responded for longer, and at closer distances (Fig. 6, Fig. 7) to simulated intrusions of neighbors and neighbor-neighbors compared to strangers. They also responded with more songs (Fig. 4) to simulated intrusions of neighbors and neighbor-neighbors compared to strangers. More songs in response to neighbor intrusions explains the broader bandwidths observed (Fig. 3). Dunn (2006) found evidence for the dear enemy effect in *T. rufalbus*. However, the variables tested in this study reflect more aggressive behavior towards neighbors. *T. rufalbus* vocalized for longer, responded at a closer distance, and with lower latency (Fig. 5) between start of simulated intrusion and response. Godard (1993) demonstrated that these behaviors reflect aggression. Individuals demonstrating these aggressive traits were seeking out the source of the playback, in an attempt to potentially initiate a physical altercation with the competitor. A variety of factors can potentially describe this behavior. Life history theory predicts that males with more opportunities for reproduction should avoid risk-taking behavior in

order to minimize the cost of current reproduction, whereas competitive environments should favor higher aggression to defend limited resources. Male (the more vocal sex in population of *T. rufalbus*) aggression can be modulated by familiarity with competitors to be either lower (dear enemies) or higher (nasty neighbors) towards neighbors. Competitive environments, as indicated by breeding density, rather than life history, shape geographical variation in levels of aggression (Yoon *et al.* 2012). The discrepancies between this study and Dunn (2006) could be explained by the differences in competition between our sampling sites. While both of our studies were conducted in Monteverde, they were conducted at different places within the area. Differences in roosting or nesting sites could lead to discrepancies between intraspecific competition levels. Gustafsson (1988) demonstrated that a lack of nesting sites results in an increase in intraspecific competition, which could then lead to increased aggression towards neighbors. Previous research has demonstrated that species are not always most abundant at the center of their ranges, and that species-abundance trends are difficult to predict (Dallas *et al.* 2017). It is possible that *T. rufalbus* is more abundant at the sampling site of this study as compared to the site of Dunn (2006). It should also be noted that both the study conducted by Dunn (2006) and this study were conducted in late October and early November, so differences in behavior cannot be described by presence or absence of the breeding season (which is from April to August, Stiles and Skutch 1989). In highly competitive environments, neighbors pose a higher threat to male's paternity, partnership, and the resources on his territory, such as nesting sites and feeding sites (Battiston *et al.* 2015). This population of *T. rufalbus* potentially responds more aggressively to neighbors, reflecting "nasty neighbor" behaviors.

The results of this study support the possibility that rufous-and-white wrens learn the calls of their neighbor-neighbors. Eavesdropping behavior potentially explains how *T. rufalbus* learns to discriminate between adjacent neighbors and neighbors of neighbors, however this study did not explicitly collect data on eavesdropping behavior. In simulations of neighbor-neighbor intrusions into the territories of the individuals sampled, the behavioral and acoustic measurements frequently matched the responses of individuals to the neighbor, and in some cases were less aggressive than the response to the neighbor. The time spent responding by individuals to each of the three treatments, and the number of songs produced as a response, showed a gradient of aggression consistent with this idea, although the difference between means is not statistically significant due to a limited sample size. Individuals on average responded for much longer and with higher bandwidth to neighbors, then neighbor-neighbors, then strangers, suggesting that individuals in the sample area have the capacity to identify the calls of individuals more than a territory away. Within the context of an area dictated by nasty neighbor principles, *T. rufalbus* individuals should respond for longer and with more songs that have a larger bandwidth to neighbors that pose larger threats to territory based resources. Being able to discriminate between neighbors, neighbors of neighbors, and strangers potentially bolsters the territorial defense ability of an individual (Naguib *et al.* 2004). If eavesdropping explains this discrimination behavior, then the eavesdropping individual adherently gains an advantage by gaining information that was not intended for the eavesdropper (McGregor 2005). Information gained via eavesdropping may assist a bird in deciding to expend energy on defensive behaviors. Knowing whether the signal was sent from a neighbor, a neighbor-neighbor, or a stranger will help the bird in making these decisions. It should be noted that Naguib *et al.* (2004) discovered that birds respond to neighbors of neighbors based on how they interacted with other birds. This may introduce variation that makes detecting the gradient of aggression difficult in some

variables. Particularly with latency between start of playback and response of focal individual, variation may arise because birds are waiting to hear a response from another bird.

If this experiment were to be repeated in the future, then a lot more time should be allocated to determining the potential boundaries of each individual sampled. Previous studies showed that other species of wren respond slightly more intensely to a neighbor on the wrong side of the subject's territory than to the same song on the correct side of the territory. Birds tend to identify the source of a signal that comes from a particular direction, and associate that signal with another birds territory, depending on how consistently it hears the same signal from that particular direction (Wiley and Wiley 1977). This indicates that wrens have the capability of differentiating neighboring songs by direction. By effectively mapping out the territory of each individual, then future intrusion experiments will be sure that they are playing back the appropriate individuals on the correct sides of the territory. Studies such as this should also span a much longer time frame, due to the fact that duetting and individual singing strategies change significantly with time of year and breeding stage (Topp and Mennill 2008). Responses of rufous-and-white wrens to duets can be much stronger and more aggressive (Mennill 2006). Mennill (2006) suggested duets play a role in territory defense against conspecific rivals, and, for males, duets may play an additional role in mate guarding and paternity guarding so studies should also attempt to measure change in responsiveness from males to duets versus individual songs.

Ultimately, the results discovered in this study suggest that *T. rufalbus* is capable of discriminating between adjacent neighbors, neighbors of their adjacent neighbors, and strangers. This may suggest that other species that exhibit NSD are also capable of this level of discrimination. This study also suggests that eavesdropping may play a prominent role between conspecifics in territorial avian communication networks.

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Climate change and aquatic insect diversity in a lower montane cloud forest

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ABSTRACT

Recent anthropogenic activity have resulted in increased release of carbon at alarming rates that have altered atmospheric composition and changed natural global landscape. Tropical freshwater ecosystems at higher altitudes are especially vulnerable to the effects of climate change due to high endemism, severely restricted range, sensitivity to abiotic factors, and rapid receive of changes due to climate change. Aquatic insect communities in these habitats are sensitive to abiotic factors but very few studies have evaluated whether climate change has affected community diversity and structure over time. This type of studies are particularly scarce in the tropics. In this study, I sampled aquatic insect abundance in a lower montane wet forest pristine stream in Monteverde, Costa Rica to determine possible changes in community structure in relation to a reference dataset collected in this stream in 2000. Diversity and community composition have changed significantly in the last 18 years. Climate change may have caused these changes due to the increased frequency and magnitude of abnormal weather phenomena such as storms. These findings fit a trend set by previous studies on freshwater ecosystems which states that large scale climatic changes and abnormal phenomena are causing major alterations in freshwater macroinvertebrate populations and communities all over the globe.

RESUMEN

La actividad antropogénica reciente ha resultado en un aumento en la liberación de carbono a tasas alarmantes que han alterado del composición atmosférica y han cambiado el paisaje natural global. Los ecosistemas dulceacuículas tropicales a grandes altitudes son específicamente vulnerables a los efectos del cambio climático debido al alto endemismo, rangos severamente restringidos, sensitividad a factores abióticos, y los cambios rápidos recibidos debido al cambio climático. Las comunidades de insectos acuáticos en estos habitats son sensibles a los factores abióticos pero pocos estudios han evaluado si el cambio climático ha afectado la diversidad de comunidades y estructura a través del tiempo. Este tipo de estudios son particularmente escazos en los trópicos. En este studio, realice un muestreo de la abundancia de insectos acuáticos en una quebrada pristina de un bosque humedo montano bajo en Monteverde, Costa Rica para determinar los cambios posibles en la estructura de la comunidad en relación a una colección de datos en esta quebrada en el 2000. La composición de la comunidad y diversidad ha cambiado significativamente en los últimos 18 años. El cambio climático puede haber causado estos cambios debido al aumento en la frecuencia y magnitud de los fenómenos climáticos anormales como tormentas. Estos hallazgos siguen la misma tendencia encontrada en estudios previos en los ecosistemas dulceacuícolas los cuales dicen que los cambios climáticos a larga escala y los fenómenos anormales estan causando alteraciones anormales en las poblaciones de macroinvertebrados y las comunidades alrededor del globo.

INTRODUCTION

Human activity and irresponsible release of carbon into the atmosphere have altered atmosphere composition, and transformed and fragmented the global natural landscape (Ciais *et al.* 2013). Current climate change has caused an accelerated increase in temperature of 1°C in the last century (Bindoff *et al.* 2013; Corlett 2014; Smith *et al.* 2015; Scheffers *et al.* 2016) as well as

changes to natural weather patterns and increase occurrence of abnormal weather phenomena (Coumou 2012; Bindoff *et al.* 2013; Smith *et al.* 2015). These strong changes in abiotic factors in turn have been linked to changes in community composition and structure, as well as altitudinal and latitudinal shifts in species ranges in existing ecosystems (McLaughlin *et al.* 2002; Parmesan & Yohe 2003; Parmesan 2006; Gattuso *et al.* 2015). Such changes in community composition can have detrimental ecological effects on ecosystems and further down the line due to different responses to global warming, shifts in resource use and dispersal, and genetic adaptations to new temperatures, as well as extinction (Parmesan 2006; Jankowski *et al.* 2012; Geerts *et al.* 2015).

The tropics are a particularly vulnerable area in terms of diversity loss due to the fact that average temperatures have been more stable historically and therefore organisms living in the tropics have narrower thermal ranges (Corlett 2014). Freshwater ecosystems make up less than 1% of earth's surface, yet are biodiversity hotspots especially in the tropics (Strayer & Dudgeon 2010). Tropical aquatic ecosystems are particularly vulnerable to climate change because these habitats are limited geographically so many species do not have where to migrate within their fragmented habitat; water temperature, chemistry, and availability are mostly climate dependent; and many of these systems are already degraded (Woodward *et al.* 2010). These stressors have already caused severe declines in abundance and range of many freshwater species (Strayer and Dudgeon 2010). Ecosystems at higher altitudes are experiencing faster warming and therefore are more at immediate risk (Hassan *et al.* 2005). Tropical freshwater ecosystems at higher altitudes are extremely vulnerable for the reasons described above and could provide warning signs earlier than other ecosystems (Woodward *et al.* 2009; Layer *et al.* 2010).

In these freshwater ecosystems, the benthic community makes up a large percentage of the present biodiversity (Illies 1969) and performs important ecological services (Covich *et al.* 1999). Unexpected changes in freshwater ecosystems are oftentimes caused by changes between benthic organisms and their associated food webs (Goedkoop & Johnson 1996, Lodge *et al.* 1998, Stockley *et al.* 1998) or by abiotic disturbances that alter their community composition (Johnson *et al.* 1998). This suggests that benthic invertebrate communities in freshwater ecosystems at high altitudes in the tropics, such as aquatic insect communities, are a good choice on which to study the effects of climate change. Aquatic insects are abundant, sensitive to abiotic factors, and have a high impact on the rest of the ecological community and therefore are a good indicator of cascading effects to come (Covich *et al.* 1999).

Studies on climate change effects on aquatic insects have been conducted before with varied conclusions. McKee and Atkinson (2000) found that short term temperature increase had minimal effects on size or abundance of mayflies. However the study was conducted in a laboratory. Another study found that effects of temperature fluctuations are species specific, positively impacting warm water organisms and negatively impacting cold water organisms (Heino *et al.* 2009). Another study conducted in the tropics found that climate effects on freshwater ecosystems are large, albeit not very well defined (McIntyre *et al.* 2007). A study conducted in the Australian tropics in an artificial stream mesocosm found that aquatic insects can survive to various species dependent degrees in hypoxic conditions in the short term, however still experiencing sub lethal effects such as delays in phenology (Connolly *et al.* 2004). However studies looking at both temperature and dissolved oxygen effects concurrently at a community level in the wild are scarce (Connolly *et al.* 2004) and could potentially provide much needed information about climate change effects on tropical fresh water ecosystems that can then be applied to many other ecosystems in different biomes.

Field studies on the effect of climate change on freshwater tropical ecosystems require long-term data, which is usually scarce in the tropical countries. The Monteverde Cloud Forest in Costa Rica provides a unique opportunity for such comparison. The area has been studied extensively for the past 30 years (Nadkarni & Wheelwright 2000) and it includes large tracks of old-growth forest that remains largely undisturbed. Isolated streams in this forest offer good opportunities to study the effect of climate change on aquatic insects. Because the streams are isolated, it is highly unlikely that any changes in the ecosystem are caused by humans. The streams are also at a high enough altitude and low enough temperature that there are no fish living in them (Clark *et al.* 2000), so the predator-prey interactions between the aquatic insect community and predators are simplified. This therefore allows for study of climate change effects only, disregarding predator-prey interactions and pollution or other immediately human produced factors. All changes in the ecosystem can be attributed to climate change, which has caused a significant increase in temperature and changes in precipitation and mist levels in the area over the last two decades (Still *et al.* 1999; Pounds *et al.* 1999; Lawton *et al.* 2001; Pounds *et al.* 2006).

This study compares aquatic insect population counts sampled along a cloud forest stream in 2018 with population counts from samples taken in 2000 from the same stream (Houseworth 2000). The main goal is to compare and detect changes in richness and diversity that can then be attributed to climate change over this timespan.

MATERIALS AND METHODS

Site Selection

Research was conducted at five sites along Quebrada Máquina in the vicinity of the Monteverde Biological Station in the Monteverde Cloud Forest, Costa Rica during the wet season in October and November 2018. The creek is located in the Tilarán mountain range in a Pacific tropical lower montane primary wet forest Holdridge life zone at an altitude of about 1500m. Annual rainfall in the area is from 1.85-4m, the mean annual temperature is 12-17°C, and canopy height is between 20-30m (Bolaños & Watson 1993). Data was collected during October 18th – November 13th. Following Houseworth (2000) insects were collected at five sites along the Quebrada Máquina in three different substrates at each site: sand, rock, and sand + rock, for a total of 15 samples of various numbers of individuals. The sites were chosen based on the map and indications provided by Houseworth (2000). Four of the five sites were previously studied by Houseworth (2000) but a fifth site was established upstream from previously studied sites (Figure 1). The fifth site is the farthest site upstream reachable by feet.

Above (N 10°19’06.9” W 084°48’29.5”) is the farthest site upstream, not present in previous research. The site is secluded and located after a dense patch of vegetation and several fallen trees across the creek. *Pristine* (N 10°19’11.5” W 084°48’12.2”) is the most upstream study site in the 2000 study and is a place that is harder to reach by foot and therefore assumed to be minimally disturbed by humans. It is about 50m downstream from the *Above* study site. The site is surrounded by steep forest slopes and therefore hardly accessible. *Quetzal Tree* (N 10°19’11.4” W 084°48’14.9”) is the portion of the creek in the vicinity of what was previously known as the quetzal tree that was located exactly at the intersection between the Yos and Jilguero trails, on the Yos trail side of the stream. The creek is slower at this site as it passes through a relatively flat area. *Between Waterfalls* (N 10°19’02.9” W 084°48’29.6”) is a site

located in between two waterfalls South of the biological station. The portion of the creek that was sampled was about 100 meters downstream from the upper waterfall. The last study site was *After Waterfalls* which is the most downstream site (N 10°18'59.2" W 084°48'29.1"). The sampled areas are about 20m downstream from the waterfall. Vegetation along the creek banks varied between the sites but was overall low.

Speed of creek flow varied between sites. Depth of creek at area sampled varied between 10cm and 40cm between sites and between substrates. The sampling location based on substrate (sand, rock, or sand + rock) was chosen using visual observations where substrate was relatively even in areas of at least 1.5 m^2 and where there was a constant flow of water and no obvious pools. All of the three sampled areas per site were located within the same 10m stretch of creek. After the ideal sampling area for each substrate was identified, insects were collected starting with the substrate farthest downstream followed by middle and lastly most upstream substrate, in order to avoid sampling insects dislodged from an undesired substrate. In total, 15 samples were collected for this study. One sample per each of the three substrates at all five sites. Houseworth had seven samples. One sample per each substrate at the *Quetzal Tree* site, and one sample per each of the four sites *Pristine, Quetzal Tree, Between Waterfalls,* and *After Waterfalls* without regarding substrate.

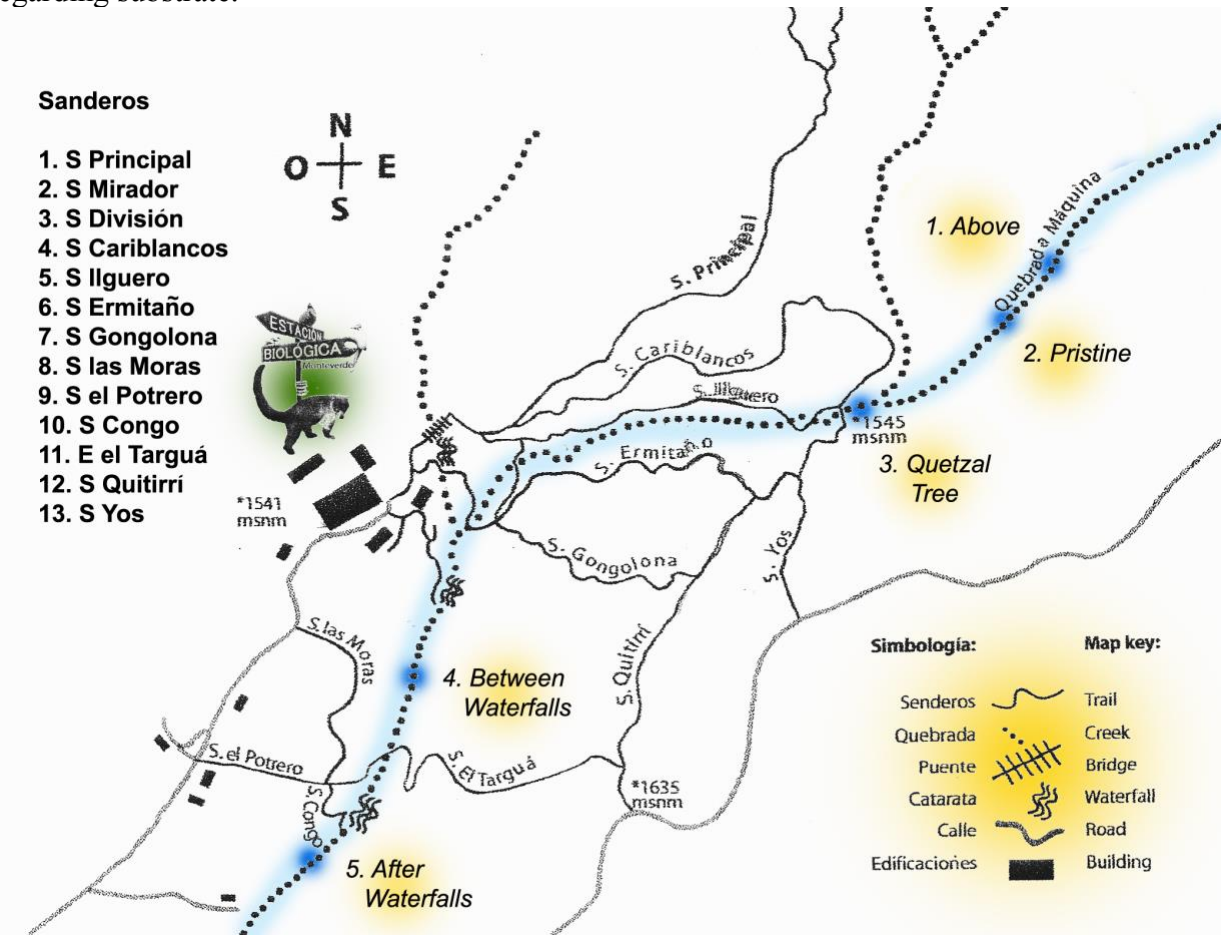

Figure 1. Map of the Quebrada Máquina and the trails surrounding the Monteverde Biological Station, Costa Rica, on the Pacific side of the Tilarán mountain range in neotropical lower montane wet forest. Five study sites were established to sample aquatic insect diversity to compare against a similar study conducted in 2000.

Water temperature and percent dissolved oxygen were also recorded at each site for each substrate using a Vernier LabQuest device and the corresponding temperature and percent dissolved oxygen probes. Coordinates were recorded using a Global Positioning System.

Insect Collection

Insects were collected by holding a kick net into the stream and thoroughly disturbing the whole selected substrate area (about 1.5-2 m^2) with my feet for two minutes for two of the substrates (sand and sand + rock) at each site. The kick net frame dimension are approximately 31x16 cm with a mesh size of 0.125 μm. The kick net was placed directly downstream from the disturbed area so that all the dislodged insects could be collected into the net. The kick net was repositioned often as I moved all along the selected substrate area. For the rock insect collection, instead of disturbing the substrate with my feet, I selected medium sized rocks that could be easily removed from the stream and thoroughly rubbed them with my hands on all sides directly into the kick net to dislodge and collect insects living on said rocks.

After the two minutes, the sample was moved into a small white basin, careful to not lose any of the sample and to remove it all from the kick net. As little substrate as possible was left into the kick net. A small amount of water from the stream was added to the basin until the sample substrate was completely submerged and insects were able to swim. The sample was left alone for a few minutes in order for the water to settle and clear. After the water was clear, the sample was attentively observed and insects were collected using soft tweezers for five minutes for each sample. If needed, a head lamp was used to facilitate observation of the insects while collecting them. The collected insects were placed in vials of 70% ethanol solution for preservation for later identification in the lab. After collecting insects for five minutes, the sample was returned to the creek, and the basin and kick net were properly rinsed for the next sample.

Insect Identification

Collected insects were examined under a microscope and identified to family. Insects that were collected and identified as not aquatic were disregarded. Larvae, adults, and pupae were all counted. Molts or fragments of insects were ignored. Several small aquatic invertebrates such as *Psydothelphysidae*, *Turbellaria*, and *Amphipoda* were also counted after having been identified to class/order.

Statistical Analysis

Due to differences in the number of samples between years, richness/diversity values cannot be directly compared between studies because this metrics are highly dependent on sampling size and sampling effort (Hsieh *et al*. 2016). Rarefaction/extrapolation (R/E) curves (Colwell *et al*. 2012, Chao & Jost 2012) were used to equalize the samples and compare richness and Shannon-Wiener diversity indices from both years without discarding data (Chao *et al.* 2014). Rarefaction analysis was conducted using the R package iNext (Hsieh *et al.* 2016). Rarefied indices are computed with a 95% confidence intervals, CI, (Chao *et al.* 2014). The lack of overlap between the confidence intervals around estimates from different years was considered as statistically significant difference in richness or diversity between years (Chao *et al.* 2014). A Morisita index of similarity was calculated in Past.

RESULTS

Richness

Twenty-one families were collected in 2018 versus ten families in 2000. Extensive overlap between the rarefied/extrapolated richness values indicates no significant difference in family richness in aquatic insects between 2000 and 2018 at any sample number (Figure 2). The asymptotic diversity estimates (Chao *et al.* 2014) are 15.375 ± 6.532 families for 2000 (95% CI: 10.825 - 44.782) and 33.600 ± 11.665 families for 2008 (95% CI 23.696 - 79.896).

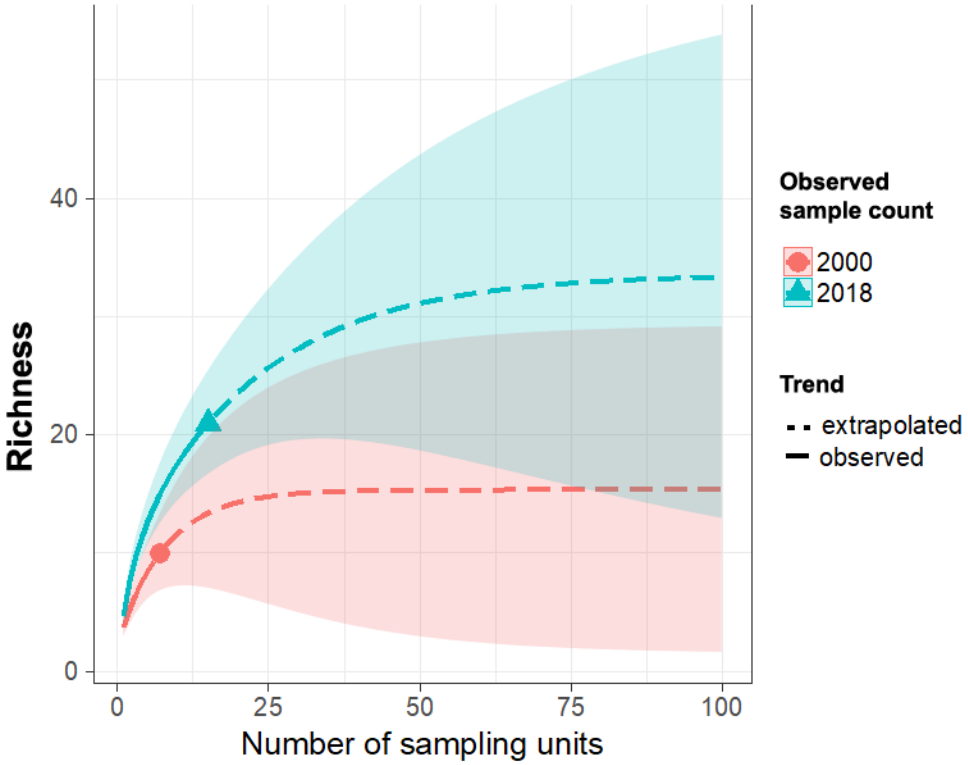

Figure 2. Rarefied Richness estimates of aquatic insect samples collected in 2000 and 2018 with 95% confidence intervals. Observed family count values are 10 in 2000 and 21 in 2018. A total of 7 samples were collected in 2000 while 15 samples were collected in 2018 along the Quebrada Máquina in Monteverde, Costa Rica, in a primary lower montane neotropical wet forest.

Diversity

The curves slightly overlap at a lower sample count, however at a higher sample count there is no overlap making results statistically significant (Figure 3). Shannon Index (H') for the observed families in 2000 is 7.220, lower than the H' for the observed families in 2018 which is 14.452. After extrapolation, asymptotic estimated H' (Chao *et al.* 2014*)* are 9.386 ±1.736 for 2000 (95% CI: 7.22-12.788), and 14.452 ± 2.423 in 2018 (95% CI: 14.452-22.882). Evenness values are 0.469 for 2000, smaller than 0.746 for 2018.

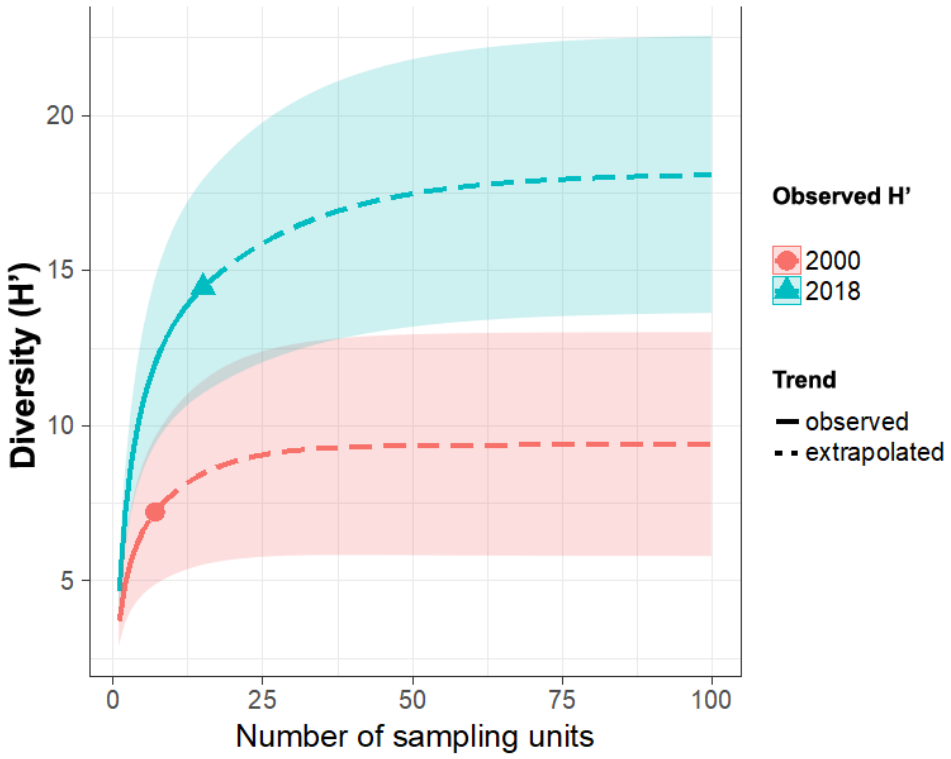

Figure 3. Rarefied diversity estimates of aquatic insect samples collected in 2000 and 2018 with 95% confidence intervals. Observed Shannon Diversity Index (H') values are 7.220 in 2000 and 14.452 in 2018. A total of 7 samples were collected in 2000 while 15 samples were collected in 2018 along the Quebrada Máquina in Monteverde, Costa Rica, in a primary lower montane neotropical wet forest.

Similarity

Community assemblages between 2000 and 2018 are very different (Figures 4 and 5). Most of the families collected were only present either in 2000 or in 2018 (23 out of 27), with 17 (or 63%) having been present only in 2018 (Table 1). *Elmidae* and *Veliidae* counts increased in 2018 compared to 2000, while *Amphipoda* and *Gerridae* counts decreased. The most drastic change is in the count of *Amphipoda*: 119 were collected in 2000, while only one was collected in 2018, even though sampling effort was more than double in 2018. Despite a big difference in sampling effort, the total number of individuals collected is roughly the same (180 in 2000, 173 in 2018).

Amphipoda drastically dominated the community in 2000, this order making up 66% of the collected individuals out of 10 collected orders (Figure 4). In contrasts, *Amphipoda* only makes up 1% of the community in 2018 (Figure 5). The dominating order now is *Diptera* with *Smuliidae* making up 28%, and *Chironomidae* 21%. The third most abundant family was *Baetidae* (*Ephemeroptera*) with 13%.

The Morisita index of similarity between these two communities is 0.0147, which means that only 1.5% families are common at relative abundances between communities. An overwhelming majority of families do not overlap, suggesting that the community structure has changed drastically in the last 18 years.

Table 1. Aquatic insect abundance collected in 2000 and 2018 during the wet season from the Quebrada Máquina in Monteverde, Costa Rica. Abundances by Order and Family are organized to show either and increase, decrease or absence between years.

Order	Family	2000	2018	
Coleoptera	*Elmidae*	3	4	**Increase**
Heteroptera	*Veliidae*	3	5	
Amphipoda		119	1	**Decrease**
Heteroptera	*Gerridae*	2	1	
Ephemeroptera	*Leptophlebidae*	6	0	**Absent in**
Hemiptera	*Naucoridae*	40	0	**2018**
Odonata	*Aeshnidae*	4	0	
Odonata	*Coenagrionidae*	1	0	
Odonata	*Gomphidae*	1	0	
Tricoptera	*Calamoceratidae*	1	0	
Coleoptera	*Ptilodactylidae*	0	3	**Absent in**
Coleoptera	*Staphylinidae*	0	2	**2000**
Decapoda	*Psydothelphysidae*	0	1	
Diptera	*Chironomidae*	0	37	
Diptera	*Simuliidae*	0	49	
Ephemeroptera	*Baetidae*	0	23	
Ephemeroptera	*Leptohyphidae*	0	1	
Hymenoptera	*Ceraphronidae*	0	1	
Odonata	*Calopterygidae*	0	4	
Odonata	*Lestidae*	0	3	
Odonata	*Libelulidae*	0	13	
Odonata	*Polythoridae*	0	1	
Turbellaria		0	3	
Tricoptera	*Glossosomatidae*	0	6	
Tricoptera	*Hydrobiosidae*	0	2	
Tricoptera	*Hydropsichiidae*	0	10	
Tricoptera	*Polycentropodidae*	0	3	
11 Orders	**27 Families**	**180 individuals**	**173 individuals**	

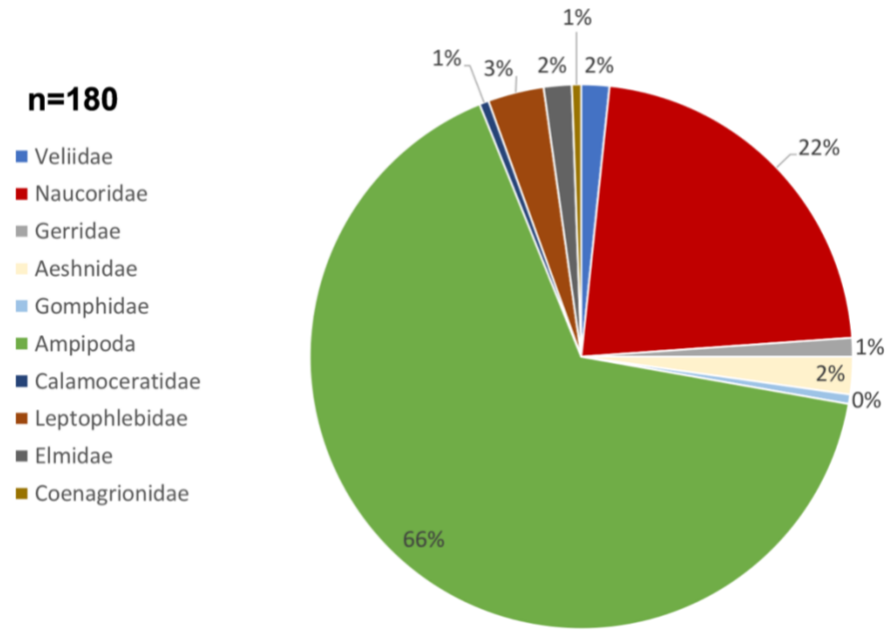

Figure 4. Aquatic insect community collected in 2000 at the Quebrada Máquina in Monteverde, Costa Rica, in a primary lower montane neotropical wet forest, at an altitude of approximately 1500-1550m. Pie slices correspond to percent abundances. Samples size: 180 individuals.

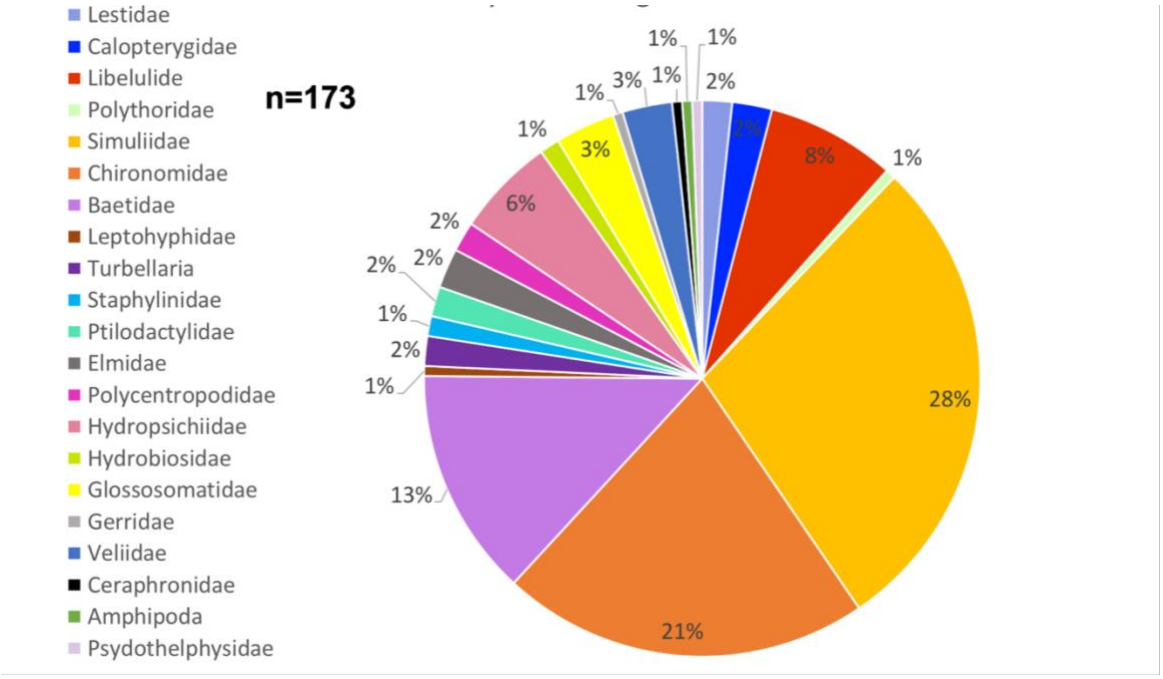

Figure 5. Aquatic insect community collected in 2018 at the Quebrada Máquina in Monteverde, Costa Rica, in a primary lower montane neotropical wet forest, at an altitude of approximately 1500-1550m. Pie slices correspond to percent abundances. Samples size: 173 individuals.

Abiotic Factors

Average water temperature in 2018 was recorded at 17.24 +/-0.003°C (from 150 data points – 10 data points at each substrate at all 5 sites). Average water temperature in 2004 in the same

system of streams was recorded at 16.7°C (Gruber 2004). This represents an increase of 2.4%. Average percent dissolved oxygen present in Quebrada Máquina in 2018 was recorded at 7.11 +/-0.014mg/L (86.05%), lower than the average percent dissolved oxygen recorded in the creek in 2000, which was 8mg/L (96.82%). This is an 11.1% decrease.

DISCUSSION

There is no recorded water temperature from 2000 to compare with the current water temperature of 17.1°C. However when compared to the average water temperature in the same system of streams from 2004 (Gruber 2004), there is a 2.4% increase that can be attributed to climate change since the creek is not disturbed by humans. Dissolved oxygen has decreased in the last 18 years by 11.10% which can be attributed to water temperature increase (Connolly. *Et al.* 2004). Oxygen tolerances for *Diptera*, *Ephemeroptera*, *Amphipoda* and *Odonata* (respective dominant orders in 2018 and 2000) are similar, with tolerance ranges greater than this fluctuation. *Diptera* has the most narrow range, its tolerance limit being 6 mg/L (Hilsenhoff 1988). All of the families/orders collected have tolerances lower than the recorded 7.11 mg/L in Quebrada Máquina. Although the slightly lowered oxygen level could explain the increase in *Diptera*, which actually prefers polluted water with lower oxygen levels (Lencioni *et al.* 2012), it appears that these changes in dissolved oxygen or temperature are not severe enough to directly disrupt aquatic insect life cycles, and do not account for the observed community changes. Nevertheless, community structure and diversity have indeed changed, most probably due to indirect effects of climate change such as weather patterns, which will be discussed below.

Family richness was the same over the years, proportional to sampling effort. Diversity proved to be significantly different because evenness was higher in 2018. This is because in 2000, the community was strongly dominated by *Amphipoda*, while in 2018 there was no dominating family. The most common families in 2018 were either found in very low abundance in 2000 or not at all, while the majority of the families sampled in 2018 are new from 2000.

These differences could potentially be caused by a big storm in Monteverde triggered by Tropical Storm Nate in 2017. The precipitation level associated with the storm was 316mm of rainfall in a single day, which is more than anything previously recorded (Coles 2017). Aside from a record amount of precipitation, the storm caused landslides and flooding, which thoroughly flushed the Quebrada Máquina and "cleaned" it of its macroinvertebrate communities (Parmesan 2000; Coles 2017). After having been flushed down in 2017, macroinvertebrates were found to recover with changes in community structure within the following months (Coles 2017). Insect counts and families present now are different than after the storm in 2017, particularly in *Diptera* counts, which suggests that the community is still recovering and the community structure is not yet stabilized. The storm would also explain lower numbers of individuals collected proportional to the number of samples.

Diversity studies done after 2000 but prior to the 2017 storm within the same system of streams found *Amphipoda* present (Rancourt 2004; Moore 2004; Trefz 2013). Coles (2017) also found *Amphipoda* present, however in lower abundance, which fits with the hypothesis that the change can be attributed to fluctuations in abiotic factors caused by the storm in 2017. Absence of *Amphipoda* could also be explained by changes in abundance or phenology in the plants on the banks of the creek that *Amphipoda* uses in its lifecycle (Wright & Covich 2005) caused by either climate change in general or by the 2017 storm. Further research is needed to support this hypothesis as there is no data on these potential changes in vegetation.

A 2018 study in lowland streams in Costa Rica measuring invertebrate community structure over 15 years found that fluctuations in stream physiochemistry and macroinvertebrate assemblages are the result of large scale climatic phenomena (Gutiérrez-Fonseca *et al.* 2018), which aligns with the findings in this study. Another study done in a tropical stream at 1200m in Puerto Rico over 6 years found changes in shrimp communities and decreased reproductive output as a result of climate changed induced pronounced drought (Covich *et al.* 2003). This supports my findings that larger climate change effects are causing alterations in community structure and lower number of individuals. A study done in temperate freshwater ecosystems in UK over 25 years found that larger climate-change-induced phenomena such as water acidification have a stronger effect on macroinvertebrate communities than do direct climate change effects (Durance & Ormerod 2007), which supports my findings. However, Durance & Ormerod (2007) found that communities were simplified and richness reduced in temperate streams, while my study shows an increase in diversity and no change in richness. This could be explained by higher species diversity in the tropics, with families potentially filling in niches in 2018 left by other macroinvertebrate families that have disappeared since 2000.

In conclusion, aquatic insect community diversity and composition in the Quebrada Máquina in Monteverde, Costa Rica, have drastically changed because of global climatic chain reactions that cause disturbances in weather patterns as well as increased frequency of abnormal events (Bindoff *et al.* 2013; Smith *et al.* 2015). These findings fit a trend set by previous studies on freshwater ecosystems and climate change (Covich *et al.* 2003; Durance & Ormerod 2007; Gutiérrez-Fonseca *et al.* 2018), which states that large scale climatic changes and abnormal phenomena are causing major alterations in freshwater macroinvertebrate populations and communities all over the globe, especially in the tropics.

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Impact of Canopy Bridges on Mammal Movement in a Cloud Forest Eco Reserve

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ABSTRACT

Ecotourism accounts for over 95% of the local economy in some areas of the developing world, including Monteverde, Costa Rica, which receives over 250,000 tourists annually. While positive for humans, its impact on local biodiversity is not often a priority. Canopy bridges in Monteverde are offered at multiple reserves and could be influencing the behavior of local fauna. I compared the canopy bridge trails of Selvatura with the trails at Santa Elena Reserve to see how local mammals use them. Camera traps and visual counts detected 62 individual mammals and a total of 10 different species of mammals in both reserves. Only 1 species of arboreal Mouse Opossum (*Micoureus alstoni*) used the canopy bridges and only at night. Eight other mammal species were detected on Selvatura trails, both between and below canopy bridges, suggesting terrestrial mammals avoid the bridges, as do Howler Monkeys (*Aloutta palliate*) and other large arboreal mammals visually sighted in trees near trails or bridges. Only one species, the Collared Peccary (*Pecari tajacu*), was seen in the Santa Elena Reserve, which lacks canopy bridges altogether. Previous studies show mammal diversity in the Santa Elena Reserve similar to Selvatura, so low richness could be attributed to low numbers of traps and days of trap monitoring. Overall, tourist infrastructure may restrict mammal movements if alternative routes are not available.

RESUMEN

El ecoturismo abarca más del 95% de la economía local en algunas áreas en los países en desarrollo, incluyendo Monteverde, Costa Rica, el cual recibe más de 250 000 turistas anualmente. Mientras es posible para los humanos, el impacto en la diversidad local no es a menudo una prioridad. Los puentes de dosel en Monteverde son ofrecidos en varias reservas y pueden influenciar el comportamiento de la

fauna local. Comparé los puentes de dosel en Selvatura con los senderos en la Reserva de Santa Elena para ver como los mamíferos locales las usan. Cámaras trampa y conteos visuales detectaron 62 individuos y un total de 10 especies diferentes en ambas reservas. Solo una especie de zorricí arboreo (*Micoureus alstoni)* usó los puentes del dosel y únicamente durante la noche. Otras ocho especies fueron detectadas en los senderos de Selvatura, tanto entre como bajo los puentes, sugieriendo que los mamíferos terrestres evitan los puentes, así como los monos Congo (*Alouatta palliata*) y otros mamíferos arborícolas vistos cerca de los puentes en los senderos. Solo una especie, el saíno (*Pecari tajacu*), fue visto en la Reserva de Santa Elena, la cual no posee puentes de dosel del todo. Estudios previos muestran que la diversidad de mamíferos en la Reserva de Santa Elena es similar a la de Selvatura,así que la baja riqueza se puede deber al bajo número de cámaras y días de monitoreo. En general,la infraestructura turística puede restringir el movimiento de los mamíferos si no hay rutas alternativas disponibles.

INTRODUCTION

Eco reserves attempt to protect local biodiversity from the encroaching impacts of human civilization while generating money from ecotourism. Reserve visitation should educate tourists, provide livelihood for local residences and conserve local species (Denman & Denman 2015). Unfortunately, the last piece of the model is often left unstudied. Ecotourism is one of the fastest growing industries globally having experiencing around 9% annual growth (Isaac 2000). As more and more people participate in ecotourism because of its popularity, conservation in eco reserves will become harder. Though ecotourism has the potential to be win:win:win, for the locals:biodiversity:tourists, careful monitoring is still required to assure that the local ecotourism has no negative impacts on the local biodiversity (Denman & Denman 2015).

In Monteverde, Costa Rica ecotourism includes waterfall rappelling, tree climbing, white water rafting and sightseeing on canopy bridges. These canopy bridges cross valleys with deep ravines, giving tourists a chance at seeing the upper canopy at eye level. In Monteverde, four of the most popular eco reserves offer canopy bridge trails, with 23 canopy bridges in total (Solano 2018). As ecotourism increases globally, and with it eco touristic structures like canopy bridges/zip lines, understanding how these structures affect local biodiversity is important to assess the potential impacts ecotourism.

Heavy use/presence of trail infrastructure correlates to lower mammal presence in eco reserves or protected areas (Zhou 2013). Mammals alter their overall behavioral patterns due to this disturbance,

with some diurnal species becoming predominantly nocturnal. Further, the largest mammals were impacted the most by direct disturbances of the trails, such as the physical presence of the trails, while smaller mammals were impacted by the indirect pressures associated with the trail use such as human use of the trails (Zhou 2013). These changes can result in community level changes; animals moving from their natural habitat to habitats were there is no human activity (Buckley 2004). The problems with these shifts in species movements due to tourist infrastructure is laid out by Edington & Edington (1986) who emphasizes the importance of natural balance; the removal or relocation of one species can cause an impact within that community.

Canopy bridges are large artificial structures connected by man made trails and as such could have larger influences on local mammals. Selvatura Park has more and higher canopy bridges than others in Monteverde (Solano 2018). Adamian (2011) is the only known research looking at the influences of canopy bridges on local biodiversity. He found no changes in biodiversity near large structures like zip lines and hanging bridges in Selvatura. However, no camera traps were actually placed on the bridges to observe mammal use of bridges. Additionally, Pendur-Throne (2018) found that Selvatura had a much lower overall mammal abundance but similar species diversity to other reserves in Monteverde with less tourist infrastructure and no bridges, but did not consider the use of bridges by the mammals she studied.

Other than Adamian (2011) there are no other sources of research conducted over the impacts of canopy bridges on local mammals. The main focus of this study is to fill this gap of knowledge and gain an understanding of the impacts canopy bridges might be having on local mammals and potential way these bridges are influencing mammal movement. With 8 canopy bridges included in their trail loop, further study at Selvatura would allow us to see how canopy bridge ecotourism affects local mammal communities in eco reserves.

MATERIALS AND METHODS

This study was conducted at Selvatura Adventure Park and Santa Elena Reserve in Monteverde, Costa Rica for a total of 27 days. Both of these parks are in cloud forests. Santa Elena Reserve is around 310 hectares while Selvatura is around 486 hectares. In Selvatura, there are 8 total canopy bridges interconnected in a trail loop. During this observational study I monitored the animal activity present on 6 of the bridges on the loop as well as the trails between them. All 6 of the bridges differed in age, height and length. The heights of the 6 bridges tested were 3 = 20m, 4 = 31m, 5 = 28m, 6 = 18m, 7 =

25m, and 8 = 20m. The lengths of the bridges were as followed 3 = 90m, 4 = 157m, 5 = 120m, 6 = 57m, 7 = 90m, and 8 = 90m. The bridges ages decreased from the start to the end of the trail loop with bridge 3 as the oldest and bridge 8 as the youngest.

Figure 1. Selvatura Adventure Park located in Monteverde, Costa Rica. Two suspension bridges out of the six total tested that could be impacting mammal activity and diversity in the park. Camera traps setup at both ends of the bridges to mointor mammal activity.

Monitoring of the sites involved 34 camera traps as well as intermediate periodic hour and a half surveying of trails and the bridges at Selvatura. In total I had around 62 hours of visual count surveying. I placed 1 camera trap at either ends of all bridges, 2 underneath each bridge, and 2 - 4 on the trails in between each bridge. I was looking for any land mammals using the bridges or avoiding them. I conducted a visual count along the trails for any land and arboreal mammals using the canopy bridges or trails at Selvatura during the day, but did not do a visual count at Santa Elena Reserve. I used a total of 5

camera traps in Santa Elena and placed them at least 35 meters apart on the Low Trail for a total length of around 175 meters. I monitored the Santa Elena trails to compare trail use of mammals at a location without bridges. I counted mammals as individuals in photos based on timestamp, sizing, and coloration differences based on photo comparisons between cameras.

Figure 2. Two examples of the camera trap locations used to monitor mammal activity on the trails and canopy bridges in Selvatura Park in Monteverde, Costa Rica. Cameras placed on the end of each bridge were aligned with the railing allowing for the monitoring of mammal use along the footpath of the bridge as well as the railing.

I examined 27 days worth of photographs taken by 34 camera traps and identified all of the mammals present in around 15,000 photos. I counted multiple individuals or groups in a single shot as separate individuals. I counted individuals based on differences in times of photos and different patterns of movement in each photo between cameras. I compiled all of the data from the sites on the bridge,

under the bridge, on the trail in Selvatura and on the trail in Santa Elena Reserve [SE] to compare relative mammal abundance.

RESULTS

There was a significant difference in number and species of mammals in the comparison of the trails at the 2 eco reserves as Selvatura had much higher species richness than SE. The bridges had a larger number of total mammals than under the bridges and on the trails at SE. The expected and observed values were both similar for under the bridge and on the trails at Selvatura. The expected values were calculated using the total amount of mammals record with the total amount of hours of camera trap monitoring. The comparison of values was much different statistically at the sites on the bridge and trails at SE, with the expected value much higher for the bridge and the expected value much lower at the trails of SE (Chi-Square = 9.39, df = 3, p = 0.025; Fig 3).

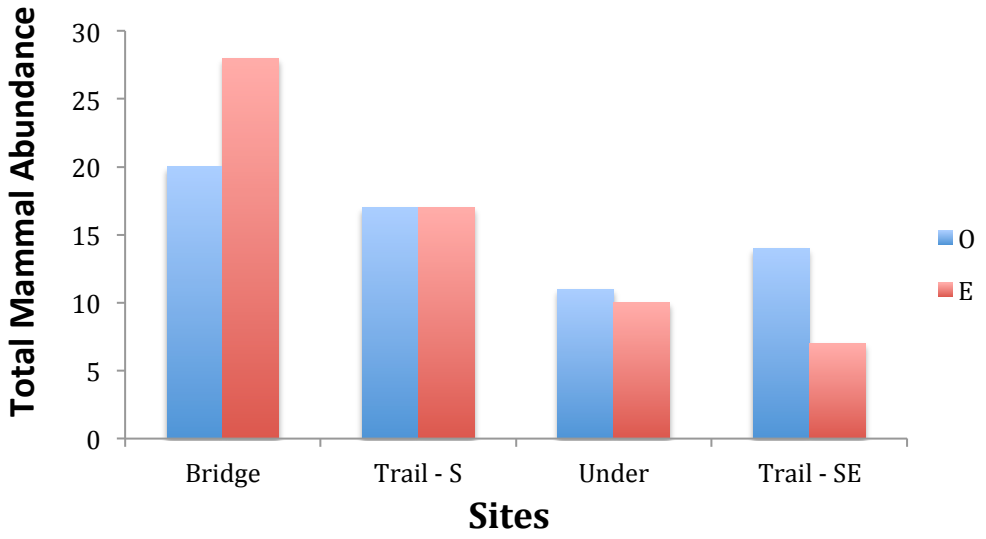

Figure 3. Observed (O) and expected (E) mammal abundance using tourist bridges (Bridge), trails between them (Trail -S), trails directly below the bridge (Under) and trails without bridges in the nearby Santa Elena Reserve (Trail – SE). Selvatura Park and Santa Elena Reserve are adjacent Cloud Forest habitat. Selvatura has high visitation as well as more infrastructures compared to Santa Elena.

The pairwise t – test between the bridges (H = 1.264) and the trails of Selvatura (H = 1.534; t = 3.5157, df = 21.3, p = 0.00202) showed that there is only a small difference in species diversity between the two sites. There was a small difference in species diversity between the bridges (H: 0) and the trails

at Selvatura (H: 1.534; t = -15.116, df = 36, p = 3.64E-17). Additionally there was a large difference in diversity between under the bridges (H = 1.2637) and on the bridges (H = 0; t = -7.2422, df = 11, p = 1.66E-05). There was a large difference in total species diversity between the trails at Selvatura (H = 1.534) and the trails at SE (H = 0.3046; t = 5.278, df = 16.48, p = 6.803E-05). Finally there was also a large difference between under the bridge (H = 1.2637) and the trails at SE (H = 0.30464; t = 3.516, df = 21.3, p = 0.00202).

	Bridge		Under		Trail – S		Trail – SE	
	VC	Camera	VC	Camera	VC	Camera	VC	Camera
Agouti	0	0	0	2	0	7	0	0
Peccaries	0	0	0	4	0	0	0	10
Tayra	0	0	0	1	0	0	0	0
Common opossum	0	0	0	4	0	1	0	0
Mouse opossum	0	20	0	0	0	0	0	0
Coatis	0	0	0	0	2	9	0	0
Ocelot	0	0	0	0	0	1	0	0
Howler	0	0	0	0	6	0	0	0
Capuchin	0	0	0	0	10	0	0	0
Unidentified	0	0	0	0	0	0	0	1

Table 1. Total species recorded and amount of each species compared between all 4 sites. This comparison looks at observed species based on the two methods used – visual counting and camera traps. Overall mouse opossums (*Micoureus alstoni*) have the most recorded sightings with 20 on the bridges but were only found at that location. Coatis (*Nasau nasua*) and capuchins (*Cebus capucinus*) came in second in terms of total sightings, 11 and 10 respectively, but again were only found at one site – trails of Selvatura. Agoutis had the most sightings recorded in more than one site as they were found on the trails and under the bridges of Selvatura.

The only species to use the bridges at Selvatura was the Mouse Opossum (*Micoureus alstoni*) with 20 sightings using camera traps. The most common species spotted going under the bridge were the common opossums (*Didelphis marsupialis*) and peccaries (*Pecari tajacu*) with 4 sightings for each with tayras (*Eira barbara*) being the least common. In the trails of Selvatura the most common species spotted were coatis (*Nasau nasua*), capuchin monkeys (*Cebus capucinus*), agoutis (*Dasyprocta punctate*), and howler monkeys (*Aloutta palliate*); ocelots (*Leopardus pardalis*) were the least common.

Finally on the trails of SE, peccaries (*Pecari tajacu*) were the most common animals spotted on the camera traps with 10 sightings (Table 1).

Between all four sites there were differences in the number of individuals recorded as well as species diversity. Compared to the trails at Selvatura and under the bridges, the number of species of mammals moving across the bridges was smaller how is this possible, if the number of species in the Santa Elena Reserve was one. None of the terrestrial mammals are using the bridges for movement as reported by the data. Peccaries (*Pecari tajacu*), tayras (*Eira barbara*), agoutis (*Dasyprocta punctate*) and common opossums (*Didelphis marsupialis*) all had reported movement under the bridges but were never spotted on the bridges themselves. The only species using the bridges for movement was the mouse opossum (*Micoureus alstoni*), which is an arboreal mammal.

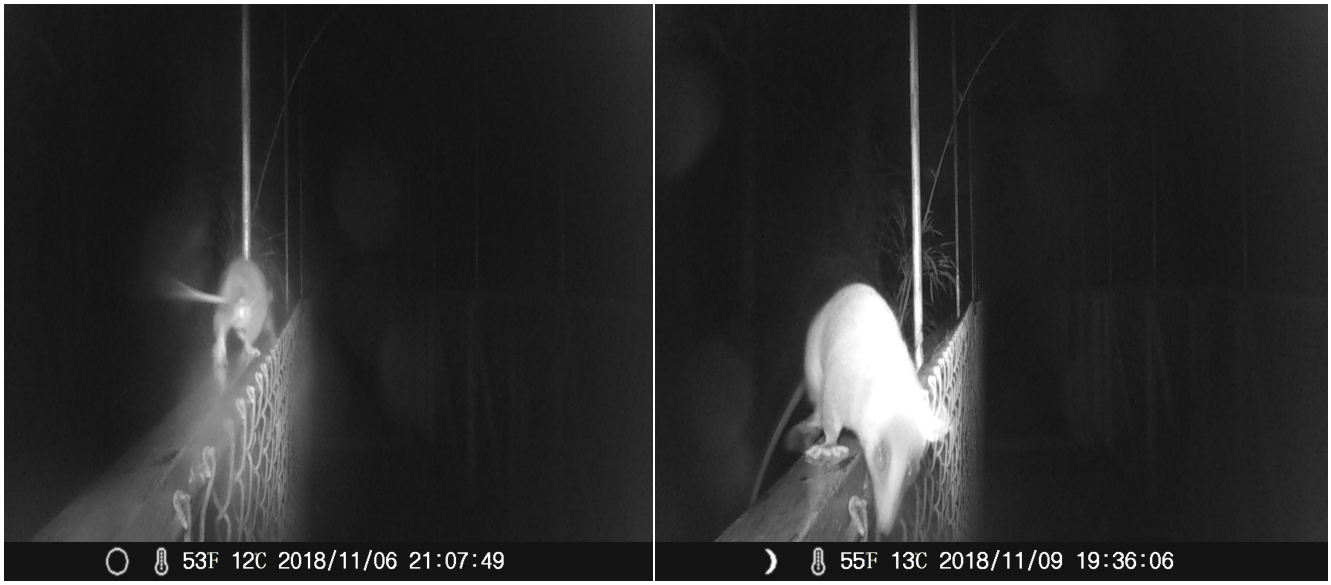

Figure 4. Two mouse opossum (*Micoureus alstoni*) individuals found using camera traps on the same canopy bridge. Both pictures taken at separate times at night and separate days. These pictures show possible use and movements of the mouse opossums (*Micoureus alstoni*) as they appear to move across the railings of the bridge.

DISCUSSION

Based on the findings it is suggested that only arboreal mammals are inclined to use the bridges and when used they will use the railings but not the walkway. As shown from the data any influences on movement or impacts by canopy bridges on local mammals would be arboreal species specific. In Nigel

et al (2011) and Gregory (2017) it is shown that arboreal mammals are inclined to use bridges or bridges over gaps or obstacles. This correlates with my data, as the most sighted species to use the bridges were the arboreal, and nocturnal, mouse opossums, which used the canopy bridges almost nightly. Additionally, it is possible that the mouse opossum (*Micoureus alstoni*), and potentially other arboreal mammals, are utilizing the bridges to cross over the gaps in the canopy similar to bridges across fragmented zones (Gregory 2017), (Nigel et al 2011). Monkeys however had no sightings on the bridges by visual counts and camera traps. Weirdly there was a higher reported sighting of howlers (*Aloutta palliate*) and capuchins (*Cebus capucinus*) near the bridges and trails by visual counts. Lack of sightings of monkey species using the bridges could be due to chance or camera placement as some guides at the park mentioned their own personal sightings of monkeys on the bridges in the past. The lack of sightings at night is due to capuchins (*Cebus capucinus*) and howlers (*Aloutta palliate*) being diurnal (Dennis 2012). Lack of use during the day could be related to the amount of tourist traffic on the bridges (Zhou 2013).

When comparing the overall use of species under the bridges to the bridges themselves we see a stark difference. Many more species of mammals are going under the bridges rather than using them. All of the animals using the bridges are arboreal and all going under are terrestrial with the exception of the tayra. Even though the bridges are incorporated into the trail paths and are set up to act as trails, no terrestrial mammals were observed using the bridges as a path during this study. This shows us that the terrestrial mammals are more inclined to go under the bridges, down into the ravines and cross the rivers as opposed to using the bridges footpath. This could be because canopy bridges are more open and perceived to increase predation risk. Along with this the overall nature of the bridges, being high off the ground, could deter use by terrestrial mammals. Ultimately we can infer that the bridges do not impact the movements of terrestrial mammals that significantly as they do not use them.

Based on the results of Adamian (2011) and Pendur - Thorne (2017) Selvatura and Santa Elena should be similar in species diversity. Specifically, based on Pendur - Thorne (2017) Santa Elena should have had the larger mammal richness. I found that Selvatura had higher mammal species richness even though it has more tourist infrastructure present. Based on the findings of Zhou (2013) one possible reason for this is due to the setup of Selvatura's trial as one loop rather than Santa Elena's trails setup as multiple, expansive, and branching loops. That said, only 5 cameras monitoring the trails in Santa Elena, compared to the 10 on the trails in Selvatura, could have skewed the data set of reported mammals there.

Additionally, the cameras set up at Santa Elena were not present and monitoring the trails as long as the cameras on the Selvatura trail.

In conclusion I have found that canopy bridges could have influences on arboreal species mammal movement. Arboreal mammals were the only species interacting with the canopy bridges in Selvatura; terrestrial mammals did not use bridges. I found that trails with canopy bridges don't differ significantly from trails without in terms of species diversity. Lastly, trail structure may play a larger part in species richness in a reserve rather than the amount infrastructure present. Further studies could investigate the impact canopy bridges have specifically on arboreal mammals in terms of their movements, or lack of, on the canopy bridges.

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Global and Local Environmental Awareness in Rural Costa Rican Elementary Schools

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Abstract

Environmental education promotes awareness and instills stewardship. Because human environmental impacts occur at all scales, students should be aware for both local and global issues. To investigate the gap between environmental awareness on global and local scales, 35 fifth and sixth grade students were assessed using a written quiz and related activity in three private elementary schools in Monteverde, Costa Rica. These schools differ in their level of parental education, environmental education curriculum, and hours devoted to environmental education. The main statistical findings are: (1) students significantly answered more global than local questions correctly regardless of school (2) schools differed significantly in the number of correct answers overall, and (3) schools differ significantly in their understanding of global and local environmental issues. Students from schools with more frequent environmental education and a higher level of parental education tended to score better on all questions. Local schools are likely adopting global environmental education resources but fail to capture much of the local environmental challenges in their environmental education curricula. While all local schools studied should strengthen their environmental education curricula for local issues, schools with less-educated parents and less frequent environmental education are particularly encouraged to strengthen their curriculum for both global and local environmental challenges.

Resumen

La educación ambiental promueve el conocimiento e inculca la administración. Debido a los impactos ambientales humanos que se producen a todas las escalas, los estudiantes deben tener en cuenta tanto los problemas locales como los globales. Para investigar la brecha entre la conciencia ambiental en las escalas globales y locales, 35 estudiantes de quinto y sexto grado fueron evaluados mediante una prueba escrita y la actividad relacionada en tres escuelas primarias privadas en Monteverde, Costa Rica. Estas escuelas difieren en su nivel de educación de los padres, planes de estudios de educación ambiental, y las horas dedicadas a la educación ambiental. Los principales resultados estadísticos son: (1) los estudiantes respondieron significativamente más a preguntas globales que locales correctamente, independientemente de la escuela, (2) las escuelas difieren significativamente en el número de respuestas correctas en general, y (3) las escuelas difieren significativamente en su comprensión delas preguntas globales y locales del medio ambiente. Los estudiantes de las escuelas con la educación ambiental más frecuentes y un mayor nivel de educación de los padres tendieron a obtener mejores resultados en todas las preguntas. Las escuelas locales están adoptando probablemente recursos globales de educación ambiental, pero no logran captar la mayor parte de los problemas ambientales locales en sus programas de educación ambiental. Mientras que todas las escuelas locales estudiadas deben fortalecer sus programas de educación ambiental para los problemas locales, las escuelas con los padres con menor nivel educativo y la educación ambiental menos frecuentes se alientan especialmente para fortalecer su plan de estudios para los desafíos ambientales globales y locales.

Introduction

Human alteration of Earth's ecosystems is substantial and expansive (Pecl *et al.* 2017). As the global population of 7.6 billion people continues to increase, so do the negative impacts of

anthropocentric pressures on the local and global environment (Malhi *et al.* 2014). An informed and engaged public is essential to confront these environmental challenges (Sachs 2012). The most effective way is through formal education (Palmer 2002), as people are less likely to support what they do not understand (Jiménes *et al.* 2017). Environmental education allows students to engage in real world issues (Blum 2008) by linking classrooms to complex environmental problems (Blum 2008), acquire skills they need to be creative problem solver, and become powerful advocates and stewards for environmental protection and conservation (Taylor 2016). For this to be most effective, environmental education must increase awareness of environmental issues both locally and globally.

As a result of increasing human encroachment, tropical rainforests are disappearing rapidly (Malhi *et al.* 2014). Tropical rainforests have been reduced to half their original size during the past century, and the rate of deforestation is only accelerating (Kim & Sexton 2015). This heightened rate of deforestation has caused major losses in biodiversity and increased habitat fragmentation in the tropics (Jiménes *et al.* 2017). Human domination in the tropics has also created more water contamination, solid waste, and air pollution, which is a major contributor to climate change (Pecl *et al.* 2017). As a close connection with the immediate environment creates students more prone to protecting and conserving (Tugurian & Carrier 2017), in tropical developing countries environmental education is even more imperative (Rome and Romero 1998).

Environmental education curricula are more global than local in focus. You might expect UNESCO to be more global in scope as it is a United Nations project that serves a global community (UNESCO 1994). Regional curricula, like the US Next Generation Science Standards, also serve a wide geographical area, so naturally takes a global and regional approach, where local environmental issues are not really addressed (NGSS 2017). Even in Costa Rica, a tropical developing country known for its biodiversity protection and alternative energy use, the national curriculum focuses nearly entirely on broad global issues (MEP 2018). Where, then, do local environmental issues fit into the EE curriculum? They might rely on local knowledge of teachers to infuse local issues with global topics, but risk losing the local perspectives if teachers are not prompted or trained to do so.

In this study, I assess global versus local knowledge of environmental issues in grades five and six in Monteverde, Costa Rica using both a written and activity assessment. Straddling the continental divide, Monteverde is home to seven Holdridge life zones and hundreds of diverse animal and plant species. Environmental protection and conservation are also paramount areas of study in Monteverde since the local economy relies heavily on ecotourism (Martín 2004). These facets make Monteverde an ideal location to study the link between global and local environmental education, since local environmental issues are extremely relevant to the future of the tropical forests, and subsequently, the economy. Grades five and six were chosen as the sample size because they are the highest available grades to study in three local elementary schools in Monteverde. Five and six years of education also allows the students to accumulate environmental knowledge from grade to grade. The study question is as follows: Do students in elementary schools know more about global environmental issues than local environmental issues in Monteverde, Costa Rica.

Methods

Monteverde Schools

This study was conducted at three different private schools in Monteverde, Costa Rica: The Adventist School (AS), Cloud Forest School (CFS), and Monteverde Friends School (MFS). Three classes with a mix of fifth and sixth grade students, a sum of 35 students total, were surveyed for global and local environmental knowledge during the third week of November, 2018. The CFS and MFS follow a traditional United States school calendar, meaning the students are finishing up their first semester of their fifth or sixth grade year. Whereas, the AS follows a traditional Costa Rican school calendar, meaning the students are finishing up their second semester of their fifth or sixth grade year. All three schools are bilingual. Observationally, there were various degrees of English language knowledge across all three schools. Students at CFS and MFS appeared to know more English than students at AS. To informally gauge how bilingual each school was, the relative use of the two languages in conversation with and between students was observed. Also, the number of students who chose the English and Spanish version of the written assessment was noted.

Adventist School: The Adventist School, known locally as La Escuela Adventista, is a private school which was founded in 1991. They are a small, religious school with a student body of local Costa Ricans. The teachers at AS do not teach environmental science to their students, but rather, the head of environmental education at the Monteverde Cloud Forest Reserve does. Once a year, students receive a kinesthetic learning experience from the Monteverde Cloud Forest Reserve which lasts approximately 6 hours. For example, students go out on the trails and search for certain species or collect water samples from local streams. The students then subsequently learn about the environment by attending three workshops per year at the preserve taught by the head of environmental education. The AS class that was surveyed had a total of 11 students, six of the students were fifth graders and five of the students were sixth graders. All were local Costa Rican students and residents of Monteverde. One fourth of the student's parents had professions that reflected an educational level of a high school diploma or higher.

Cloud Forest School: The Cloud Forest School, known locally as the Centro de Educación Creativa, is a private school that was founded with environmental education as a pillar of their curriculum. The CFS have developed a three-tiered environmental curriculum for the fifth and sixth grade students that focuses on 1) land stewardship; 2) local environmental education; and 3) a global course of socio-environmental studies. The CFS students have environmental science class every Thursday for an hour and a half year round which consists both of kinesthetic learning in the field and auditory learning in the classroom. Their goal is to graduate bilingual individuals with strong roots in environmentalism, meaning the students will care and take action to protect the environment after they graduate. The CFS class that was surveyed had a total of 10 students, all from the sixth grade. All were local Costa Rican students and residents of Monteverde. A little over half of the student's parents had professions that reflected an educational level of a high school diploma or higher.

Monteverde Friends School: The Monteverde Friends School, known locally as La Escuela de Los Amigos, is a private school that was founded over 65 years ago by Quakers from the United States. The MFS fosters environmental understanding and ethics by incorporating environmental education into their science curriculum. The MFS students spend one semester of their year studying environmental science. The students have 45 minutes of environmental science class

every day for half of the year. They also do some informal teaching about how their actions affect the environment as a part of Quaker values. Their student body consists of both local Costa Rican students and students from visiting families who live outside of Costa Rica (mostly from the United States). The MFS class that was surveyed had a total of 14 students, six of the students were fifth graders and eight of the students were sixth graders. Nine of the students were local Costa Rican's and residents of Monteverde, while, the other five students were originally from the United States. Roughly half of the student's parents had professions that reflected an educational level of a high school diploma or higher.

Written Quiz Assessment

School curricula were obtained and environmental topics, particularly those related to problems about the environment, were noted. The Costa Rican public school science curriculum, Ministerio de Educación Pública (http://www.dgec.mep.go.cr), was acquired. Using this curriculum and other objective sources (Burt 2016, Nichols 2017, Citizen 2018, Kinhal & Ketcham 2018, Luleva, 2018, Marks 2018, Rinkesh 2018, Staff 2018, Wright & Henson 2018), a list of the six most frequent and most emphasized environmental problems was created: air pollution, climate change, water pollution, loss of biodiversity, habitat loss, and solid waste. Using this list as a framework, an age appropriate quiz was created containing six questions about global environmental issues and six questions about local environmental issues. The quiz was reviewed by a local fifth/sixth grade teacher in the area to assure that all vocabulary, questions, and concepts were age appropriate. Each of the twelve questions assessed one of the six chosen environmental issues on both a global and a local scale. The quiz was made in English (see Appendix 1) and translated in Spanish (see Appendix 2). In this way, each student had the opportunity to take the quiz in their native language. Each question was configured in multiple choice format with three choices, and only one correct answer. Students were asked by me to take the quiz independently and remain silent until all the quizzes were finished. The teacher was present when the quiz was administered at MFS, but not at CFS or AS. All of the students at AS and CFS took the quiz in Spanish, whereas, nine students took the quiz in Spanish and five students took the quiz in English at MFS.

Activity Assessment

An activity was created that evaluated awareness of the same topics covered by the written quiz. The activity consisted of twelve questions, six questions about environment issues on a global scale and six on those same issues but a local scale (see Appendix 3). Each question elicited a true/false response. The activity was also reviewed by the same local fifth/sixth grade teacher in the area to assure that all vocabulary, questions, and concepts were age appropriate. The activity was conducted outside in a grass field at CFS and MFS, and on a pavemented area at AS. Students were divided into groups of three to five students depending on class size. Each group had their own set of two hula hoops. One hula hoop was the 'true' hoop and the other hula hoop was the 'false' hoop. Signs that stated "TRUE" and "FALSE" were placed behind each hoop to designate which hoop was 'true' and which hoop was 'false'. A line made out of flagging tape was staked into the ground which designated the starting position of the students. The hoops were placed side by side one another, roughly 15 meters away and parallel to the starting line. A question was asked while all of the students stood on the starting line. After the question was

repeated twice and the word ‘go’ was said, the students ran to the hoop they thought was the correct answer. The number of students who stood in the ‘true’ and in the ‘false’ hoops was tallied. This process was repeated for all twelve questions.

Results

The main statistical findings are: (1) students significantly answered more global than local questions correctly regardless of school (2) schools differed significantly in the number of correct answers overall, and (3) schools differ significantly in their understanding of global and local environmental issues.

Global vs Local

Students significantly answered more global questions correctly than local questions when all schools were pooled (ANOVA $F = 57.55$, $df = 1$, $P < 0.0001$). Global questions for all schools combined had a per student average of 4.4+/- 0.24 standard error while local had 2.54+/- 0.15 (Figure 1). This trend was also significant in a paired design where a given student’s answers for global vs. local were compared for the 35 students tested (Repeated Measures ANOVA $F = 68.08$, $df = 1$, $P < 0.0001$).

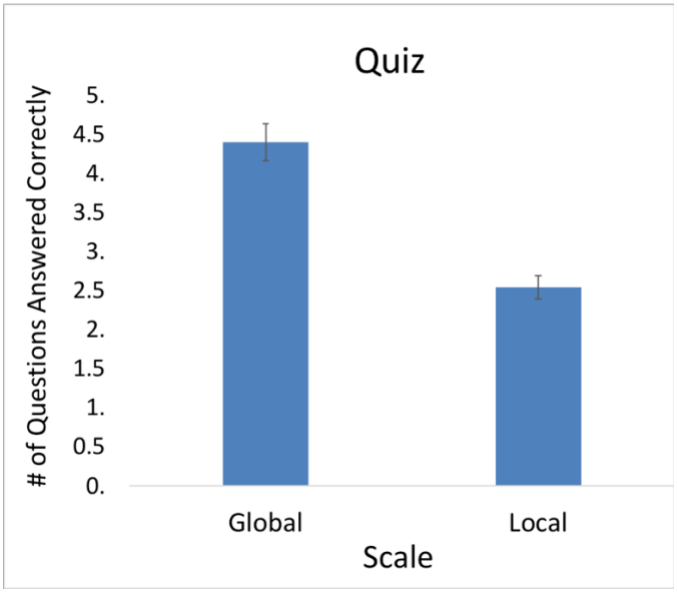

Figure 1. Quiz scores assessing environmental knowledge of 35 fifth and sixth grade students from three elementary schools in Monteverde, Costa Rica (AS= 11, CFS= 10, MFS= 14). Each student was given a quiz that consisted of twelve multiple choice questions, six for environmental issues at a global scale and six of the same at a local scale. Bars are based on the mean number of correct answers per school regardless of scale out of 6 possible. Error bars represent standard error.

Students also significantly answered more global questions correctly than local questions correctly on the activity assessment, regardless of school, according to the results of the ANOVA

(F = 14.99, df = 1, P= 0.00054). The means for scale based on proportions are: global = 0.82+/- 0.056 standard error and local = 0.50+/- 0.07. The proportion of global questions answered correctly, with a mean of 0.82 questions, was higher than the proportion of local questions answered correctly, with a mean of 0.50 questions (Figure 2).

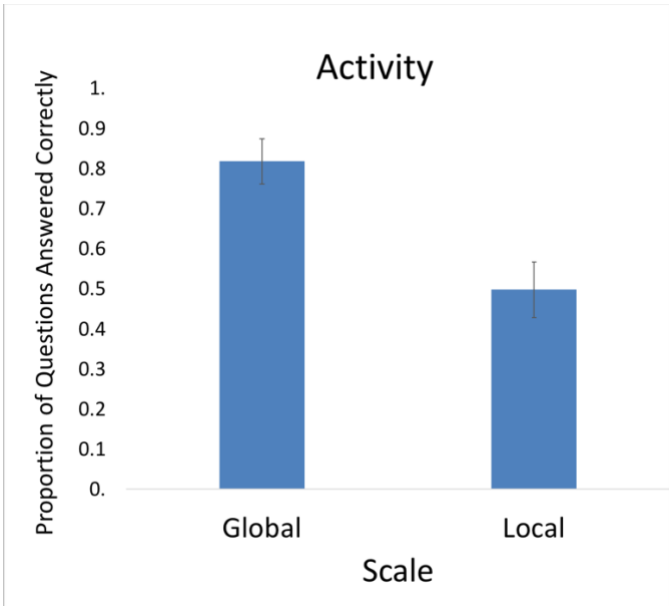

Figure 2. This study assessed environmental knowledge of 35 fifth and sixth grade students from three elementary schools in Monteverde, Costa Rica (AS= 11, CFS= 10, MFS= 14). Students participated in an assessment activity that consisted of twelve true/false questions, six which pertained to global environmental issues and six that pertained to local environmental issues. Bars are based on the mean proportion of correct answers per school regardless of scale. Error bars represent standard error.

School

Schools differed significantly in the number of correct answers for subsections of the written quiz (local or global, but with subsection scores considered collectively) (ANOVA F = 8.18, df = 2, P= 0.00069). The mean number of correct questions answered out of 6 points is reported: AS = 2.77+/- 0.24 standard error, CFS = 4 +/- 0.32, and MFS = 3.64+/- 3.64. AS had the lowest number of questions answered correctly with a mean of 2.77 questions out of a possible 6, followed by MFS with a mean of 3.64 questions. Lastly, CFS had the highest number of questions answered correctly with a mean of 4 questions (Figure 3). Both CFS and MFS differed from AS significantly, but CFS and MFS did not differ from each other significantly (Fisher LSD test at $p < 0.05$).

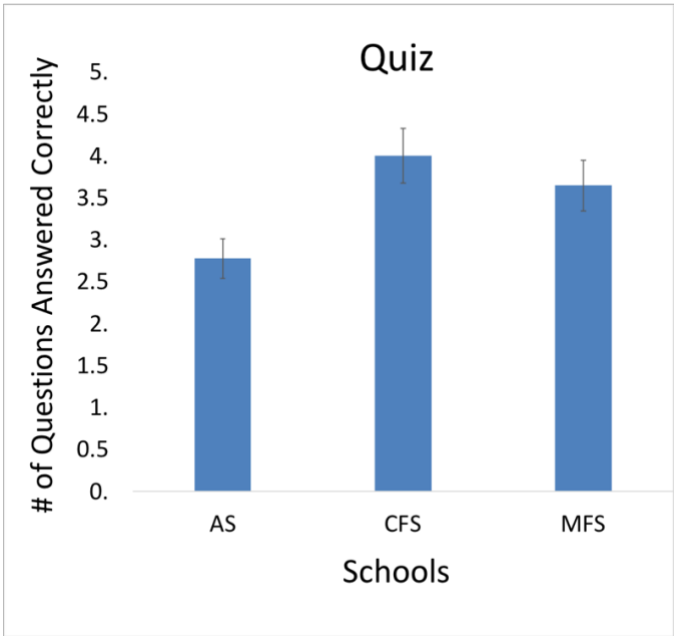

Figure 3. Quiz scores assessing environmental knowledge of 35 fifth and sixth grade students from three elementary schools in Monteverde, Costa Rica (AS= 11, CFS= 10, MFS= 14). Each student was given a quiz that consisted of twelve multiple choice questions, six for environmental issues at a global scale and six of the same at a local scale. Bars are based on the mean number of correct answers per school regardless of scale out of 6 possible. Error bars represent standard error.

Schools differed significantly in the proportion of correct answers when local and global subsections of the activity were considered as a single group (ANOVA $F = 4.14$, $df = 2$, $P = 0.026$). The means for the schools based on proportions are: AS = 0.50+/- 0.087 standard error, CFS = 0.69+/0.09, and MFS = 0.78+/- 0.07. AS had the lowest proportion of questions answered correctly with a mean of 0.50 questions, followed by MFS with a mean of 0.69 questions. Lastly, CFS had the highest proportion of questions answered correctly with a mean of .78 questions (Figure 4). MFS differed from AS significantly, but CFS and MFS, as well as, CFS and AS did not differ significantly from each other (Fisher LSD test, $p > 0.05$).

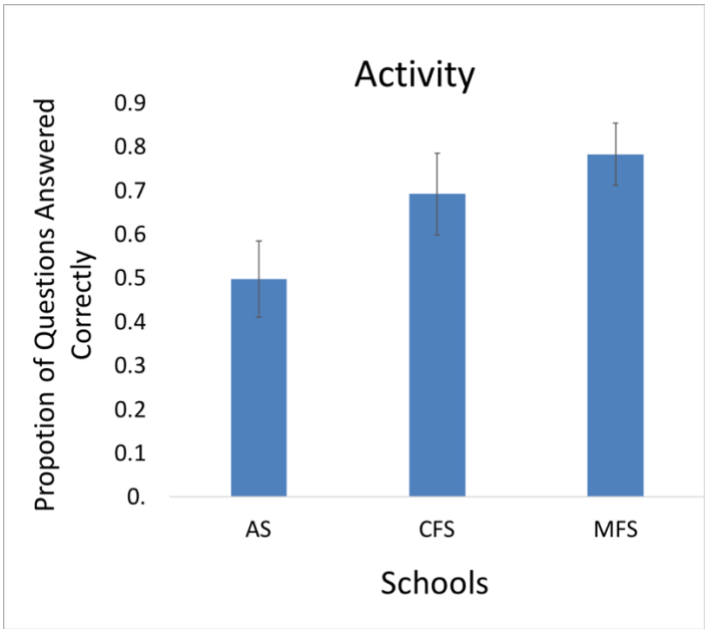

Figure 4. This study assessed environmental knowledge of 35 fifth and sixth grade students from three elementary schools in Monteverde, Costa Rica (AS= 11, CFS= 10, MFS= 14). Students participated in an assessment activity that consisted of twelve true/false questions, six which pertained to global environmental issues and six that pertained to local environmental issues. Bars are based on the mean proportion of correct answers per school regardless of scale. Error bars represent standard error.

Scale and School Interaction

All three elementary schools significantly answered more questions about global than local environmental issues correctly at a $p<.05$, according to the results of the Sign Test. Further, schools differed significantly in their understanding of global and local issues, according to the results of the ANOVA ($F = 4.20$, $df = 2$, $P = 0.01932$). The number of global questions answered correctly varied between schools. AS had the lowest number of global questions answered correctly with a mean of 3.18+/- 0.42 questions. MFS had a slightly higher number of global questions answered correctly with a mean of 4.86+/- 0.27 questions. CFS had the highest number of global questions answered correctly with a mean of 5.1+/- 0.31 questions. The number of local questions answered correctly did not differ statistically between schools: AS 2.36+/- 0.15, MFS 2.43+/- 0.29, CFS 2.9+/- 0.28 (Figure 5). However, AS was statistically lower in mean global question scores (Fisher LSD test, $p > 0.05$).

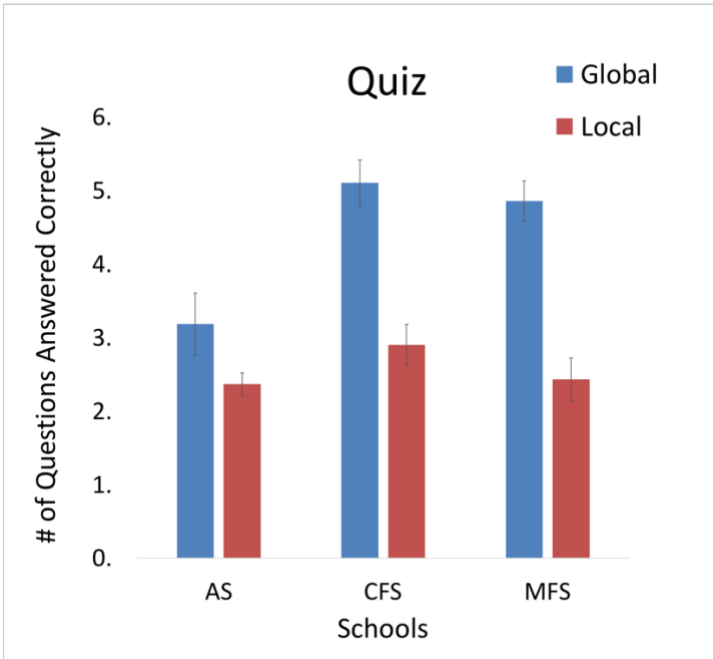

Figure 5. Quiz scores assessing environmental knowledge of 35 fifth and sixth grade students from three elementary schools in Monteverde, Costa Rica (AS= 11, CFS= 10, MFS= 14). Each student was given a quiz that consisted of twelve multiple choice questions, six for environmental issues at a global scale and six of the same at a local scale. Bars are based on the mean number of correct answers per school regardless of scale out of 6 possible. Error bars represent standard error.

Results from the activity yielded no significant difference in schools and their understanding of global and local problems, according to the results of the ANOVA ($F = 0.42$, $df = 2$, $P = 0.66$). All three schools had a similar mean proportion of global questions answered correctly: AS 0.71+/- 0.12 standard error, CFS 0.82+/- 0.11, MFS 0.93+/- 0.046 (Figure 6). The proportion of local questions answered correctly had a greater variance between schools. AS had the lowest proportion of local questions answered correctly with a mean of 0.29+/- 0.043. CFS had a slightly higher proportion of local questions answered correctly with a mean of 0.57+/- 0.14. MFS had the highest proportion of local questions answered correctly with a mean of 0.64+/- 0.11. In regards to the global scale, none of the three schools were significant from each other (Fisher LSD test, $p > 0.05$). In regards to the local scale, AS significantly differed from MFS (Fisher LSD test, $p > 0.05$). CFS and MFS, as well as, AS and CFS did not significantly differ from each other. Additionally, MFS significantly answered more questions about global than local environmental issues correctly at a $p<.05$, according to the results of the Sign Test. AS and CFS did not significantly answer more questions about global than local environmental issues correctly at a $p<.05$, according to the results of the Sign Test.

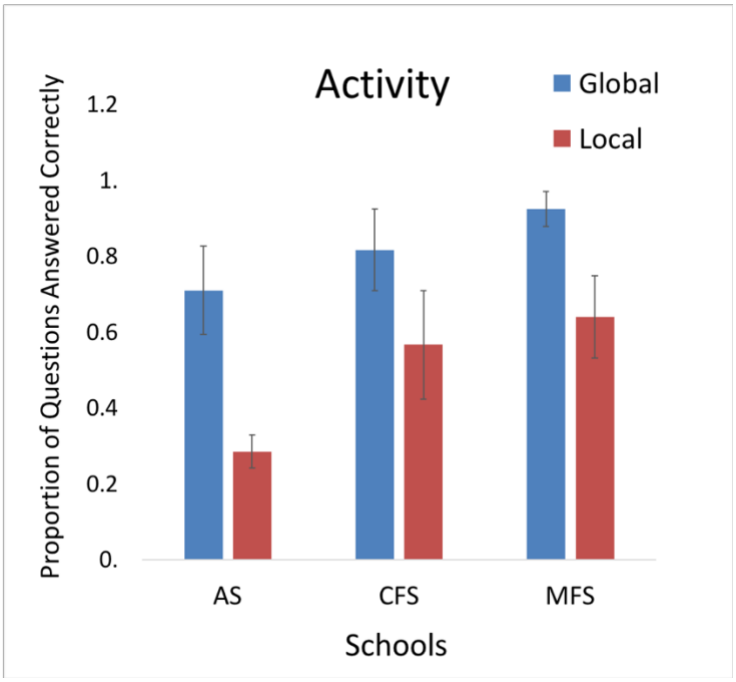

Figure 6. This study assessed environmental knowledge of 35 fifth and sixth grade students from three elementary schools in Monteverde, Costa Rica (AS= 11, CFS= 10, MFS= 14). Students participated in an assessment activity that consisted of twelve true/false questions, six which pertained to global environmental issues and six that pertained to local environmental issues. Bars are based on the mean proportion of correct answers per school regardless of scale. Error bars represent standard error.

Question Observations

There were some questions that students consistently missed and other questions that students consistently answered correctly (Table 1). Across all three schools, the majority of students knew that Earth's climate is warming and that national parks protect habitats. The students also knew that the golden toad was the species that went extinct in Monteverde. It appears that the students have a good understanding of global climate change and loss of biodiversity on both scales. On the other hand, it seems like students do not have a firm understanding on water pollution, habitat loss, and solid waste management in the context of Monteverde. This is because the majority of the students thought that the main source of water pollution in Monteverde came from hotels and houses and that the main cause of habitat loss in Monteverde today was cutting down trees for wood. The students also thought that most of the trash in Monteverde ends up in the ocean or gets recycled.

Table 1. A list of questions and their respective scales that students consistently missed and consistently answered correctly from both the written and activity assessment. In order for a question to make the list, the question had to be answered correctly or incorrectly by three fourths or more of the students at all three schools. The number of students needed to meet this requirement is as follows: AS=8 out of 11, CFS=7 out of 10, and MFS= 10 out of 14.

List of the Questions Most Frequently Answered Incorrectly	List of the Questions Most Frequently Answered Correctly
Quiz Question # 9 (local)	Quiz Question # 2 (global)
Quiz Question # 11 (local)	Quiz Question # 5 (global
Quiz Question # 12 (local)	Quiz Question # 10 (local)
Activity Question # 2 (local)	Activity Question # 1 (global)
Activity Question # 10 (local)	Activity Question # 3 (global)
Activity Question # 12 (local)	Activity Question # 5 (global)
	Activity Question # 9 (global)

Discussion

All three private elementary schools assessed in the Monteverde area have different ways of teaching environmental education. AS students have environmental science class four times a year at the Monteverde Cloud Forest Reserve, CFS students have environmental science class on a weekly basis for an hour and a half, and MFS students have environmental science class every day for 45 minutes but only for one semester of the year. Studies indicate that more frequent school instruction increases student knowledge, awareness, and subsequently, assessment scores (Fulford & Daigle 2007). AS, which has the least frequent environmental science instruction, had the lowest number of questions answered correctly for the written quiz. MFS, which has more frequent environmental science instruction than AS but less than CFS, had a significantly higher number of questions answered correctly than AS, but was statistically equivalent to CFS. CFS, which has the most frequent environmental science instruction, had the highest number of questions answered correctly, but not different statistically from MFS. The activity assessment told a similar story. The only difference in regards to the activity was AS and CFS did not differ statistically from one another.

Student learning is not all from school curricula. Studies suggest that higher levels of parental education are associated with higher student knowledge and performance in school (Muller 2018). Therefore, another possible explanation for the gradient of knowledge of global and local environmental issues across all three schools could be parents' education level. Roughly half of the students' parents at CFS and MFS had professions that reflected an educational level of a high school diploma or higher. Some of these professions included nature guide, elementary school teacher, biologist, and university professor. Whereas, only one fourth of the student's parents at AS had professions that reflected an educational level of a high school diploma or higher. Both CFS and MFS had a significantly higher number of questions answered correctly than AS on the written assessment, although, CFS was not different statistically from MFS. These results suggest that parents' education level also has a positive correlation with the number of questions answered correctly for the written quiz. Because CFS and MFS had a higher number of parents who received a formal education, students are likely drawing from sources of knowledge from their schools, as well as, from their parents. Schools with less-educated parents and less frequent environmental education are particularly encouraged to strengthen their curriculum for both global and local environmental challenges.

Students generally lack more knowledge about what is happening in their immediate environment as compared to the world around on a global scale (Figure 1 & 2). A careful review of the curricula agrees that global environmental issues tend to be emphasized more than local environmental issues (UNESCO 1994, NGSS 2017, MEP 2018). Therefore, it is reasonable to think that there would be more student knowledge around global environmental issues compared to local. The only school curriculum that was obtained out of all three elementary schools surveyed was the CFS due to feasibility. The CFS environmental education curriculum was not consulted when making the list of the six most prevalent environmental issues in order to prevent bias. Although, the EE does help tell the story. The CFS EE curriculum addresses local environmental issues. Students in the third bimester of their third grade year learn how human activities can cause both positive and negative changes in the cloud forest ecosystem of Monteverde and the effects of these changes. Some of these causes and effects include deforestation, solid wastes, black and grey water, smoke from transportation/factories/fires, chemical substances from agricultural/domestic/industrial use which cause illnesses and death of different life forms, deterioration of the natural landscape, alterations in the air/soil composition, and deterioration of water sources. Moreover, the CFS does equally emphasize both global and local environmental issues in their EE. CFS students having the highest number of questions answered correctly for both global and local scales on the written assessment (Figure 5) is most likely a reflection of their strong EE.

Schools differed for global questions answered correctly only if the questions were in writing. During the activity assessment, students sometimes demonstrated the "follower" effect. This is when the class as a whole perceives certain individuals to be most knowledgably, so the rest of the students "follow" the knowledgeable students. In the context of the activity, sometimes the majority of the students ran to the hoop that the perceived more intelligent students ran to. Students were divided up into small groups and given their own sets up hoops to interact with in attempt to diminish the "follower" effect, yet the effect still persisted to some extent. Observationally, students at MFS displayed the strongest follower effect. This most likely explains why MFS had a higher proportion of questions answered correctly than CFS on the activity assessment (Figure 6).

Knowing this information is influential for the future conservation of tropical forests in Monteverde. Nowhere is there a need for local environmental education greater than in rural tropical areas like Monteverde (Şekercioğlu 2012). Conservation efforts are vital in maintaining ecosystem health and biodiversity in tropical forests (Burlingame 2000). There is a system of private reserves of over 24,000 hectares in Monteverde (Burlingame 2000). If school curricula are not providing students with the information they need to learn about local environmental issues, local residents will not only lack awareness but also engagement and knowledge to address them. Elementary schools in Monteverde must take on the task of educating students about local environmental problems concerning air pollution, climate change, water pollution, loss of biodiversity, habitat loss, and solid waste management so that students can become advocates for the conservation of their local tropical forests. Results from Table 1 suggest that more emphasis should especially be placed on water pollution, habitat loss, and solid waste management in local school curricula. Similar studies should be carried out in different schools throughout the world, especially schools in the tropics, and at different grade levels to see if comparable trends exist.

Not only is education imperative in extending knowledge to students, but the quality of education is extremely important and greatly contributes to the success of students in the

classroom and beyond (Hungerford & Volk 1990). The frequency of environmental education and parents' education level can influence students' knowledge. Also, having strong environmental science curriculums that equally emphasize both global and local environmental issues in elementary schools can encourage students to practice environmental responsible behavior and advocate for the conservation of global and local environments. This is important for the future because we live on a human dominated planet. The global population rate is showing no signs of slowing down any time in the next decade (Samir & Lutz 2017). Therefore, current environmental problems will likely persist in the future, and it is probable that they will become more consequential and extensive. The students of today will become the decision makers of tomorrow hence why education is invaluable in extending knowledge in regards to both global and local environmental issues.

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Appendix 1

NAME_____ GRADE_____

Directions: Read each question carefully and circle the option you think is the correct response. There is only ONE correct response for each question.

GLOBAL ENVIORNMENTAL PROBLEMS

1) What is the main cause of air pollution on Earth?

A. gases from farms
B. ash from volcanoes
C. gases from fossil fuels*

2) What is happening to the Earth's climate?

A. it is cooling
B. it is warming*
C. it is staying the same

3) What is the main cause of fresh water pollution on Earth?

A. waste from farms
B. waste from industries*
C. waste from sewage

4) What is the biggest threat to wild animals?

A. habitat loss*
B. air pollution
C. water pollution

5) Which protects habitats ?

A. mining
B. city development
C. national parks*

6) The amount of trash being produced in the world is:

A. decreasing
B. increasing*
C. staying the same

LOCAL ENVIRONMENTAL PROBLEMS

7) What is the main cause of air pollution in Monteverde?

A. gases from farms*
B. ash from volacnos
C. gases from fossil fuels

8) What is happening to Monteverde's climate?

A. it is getting colder with more mist
B. it is getting warmer with less mist*
C. it is staying the same

9) What is the main source of water pollution in Monteverde?

A. farms*
B. factories
C. hotels and houses

10) What animal species used to live in Monteverde but is no longer found here?

A. the speckled lizard
B. the spotted salamander
C. the golden toad*

11) What is the main cause of habitat loss in Monteverde today?

A. building more stores and roads
B. cutting down trees for wood
C. more agricultural lands*

12) Where does the most of the trash end up in Monteverde?

A. oceans
B. landfills*
C. recycling

Appendix 2

NOMBRE_____ GRADO_____

Instrucciones: Lea cada pregunta cuidadosamente y encierre en un círculo la opción que usted cree es la respuesta correcta. Solo hay UNA única respuesta correcta para cada pregunta.

PROBLEMAS MEDIOAMBIENTALES GLOBALES

1) ¿Cuál es la causa principal de la contaminación del aire en la Tierra?

A. gases procedentes de granjas o fincas
B. cenizas de volcanes
C. quema de combustibles fósiles*

2) ¿Qué está pasando con el clima de la Tierra?

A. se está enfriando
B. se está calentando*
C. se mantiene igual

3) ¿Cuál es la causa principal de contaminación del agua de los ríos y quebradas en la Tierra?

A. residuos agrícolas
B. residuos industrials*
C. alcantarillado

4) ¿Cuál es la mayor amenaza para los animales silvestres?

A. la pérdida de habitat*
B. la contaminación del aire
C. la contaminación del agua

5) ¿Qué protege el hábitat de las plantas y animales?

A. compañías mineras
B. el desarrollo de la ciudad
C. los parques nacionales*

6) ¿La cantidad de basura que se produce en el mundo está?:

A. disminuyendo
B. aumentando*

C. se mantiene igual

PROBLEMAS MEDIOAMBIENTALES LOCALES

7) ¿Cuál es la causa principal de la contaminación del aire en Monteverde?

A. gases procedentes de granjas o fincas*
B. ceniza de volcanes
C. gases procedentes de los combustibles fósiles

8) ¿Qué está pasando con el clima de Monteverde?

A. se está enfriando, y hay más niebla
B. se está calentando, y hay menos niebla*
C. se mantiene igual

9) ¿Cuál es la principal fuente de contaminación del agua en Monteverde?

A. granjas o fincas*
B. fábricas
C. hoteles y casas

10) ¿Qué especies de animales vivían en Monteverde, pero ya no se encuentra aquí?

A. el lagarto manchado
B. la salamandra común
C. el sapo dorado*

11) ¿Cuál es la causa principal de la pérdida de hábitat en Monteverde hoy en día?

A. construir más tiendas y carreteras
B. tala de árboles para madera
C. aumento de tierras agrícolas*

12) ¿Donde termina la mayoría de la basura producida en Monteverde?

A. en los océanos
B. en botaderos*
C. en el reciclaje

Appendix 3

GLOBAL & LOCAL ENVIORNMENTAL PROBLEMS

AIR POLLUTION

1) Most of the air pollution in the **world** comes from coal, oil, gas. (T) _____ F_____

2) Most of the air pollution in **Monteverde** comes from coal, oil, gas. T_____ (F) _____

CLIMATE CHANGE

3) The **Earth** is already already experiening climate change. (T) _____ F_____

4) **Monteverde** is already experiencing climate change. (T) _____ F_____

WATER POLLUTION

5) Many people on **Earth** do NOT have access to clean drinking water. (T) _____ F_____

6) Many people in **Monteverde** do NOT have access to clean drinking water. T_____ (F) _____

BIODIVERSITY

7) The number of animals and plants in the **world** is increasing. T_____ (F) _____

8) The number of animals and plants in **Monteverde** is increasing. T_____ (F) _____

HABITAT LOSS

9) The number of trees cut down in the **world** is increasing. (T) _____ F_____

10) The number of trees cut down in **Monteverde** is increasing. T _____ (F) _____

WASTE

11) Most of the trash on **Earth** gets recycled. T_____ (F) _____

12) Most of the trash in **Monteverde** gets recycled. T_____ (F) _____