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Variación Altitudinal de Recompensas Nutritivas en el Mutualismo *Acacia hindsii*-*Pseudomyrmex* spp.

Tesis que presenta

Biol. Ernesto José Mendoza Monge

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Directores de Tesis:

Dr. Martin Heil

Dr. Alejandro Blanco Labra

Dedicatorias

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ABSTRACT

Mutualisms are associations among organisms from different species that benefit all involved partners. In the obligate *Pseudomyrmex-Acacia* defensive ant-plant mutualism, *Acacia* plants provide the ants with nutritional food bodies, extrafloral nectar and nesting space, while the ants defend the plant against herbivores and pathogens. Ant-plant mutualisms are restricted to the lowland tropics, in this case ranging from southern Mexico to Central America and from 0 to ca. 1200 m a.s.l.. In general, mutualisms tend to become less strong, frequent and diverse with increasing latitudes and altitudes. A commonly proposed explanation for the weakening or complete breakdown of mutualisms close to the borders of their area of distribution are lower or no investment by the host or lower or no reciprocating behavior by the partner, causing the species to shift to mutualism-independent strategies or even the local extinction of one or all species involved. In this study, we used the mutualism of *P. ferrugineus* ants with *A. hindsii* plants to investigate the colonization of the plants and the defensive activity of the ants with increasing altitude and correlate putative changes in these indicators of mutualism functioning with the quantity or quality of the food rewards. Surprisingly, even in the fringes of their altitude tolerance, the *A. hindsii-P. ferrugineus* mutualism remained stable. In fact, whereas different species of ants – including non-mutualistic species – colonized *A. hindsii* at low altitudes, all plants were colonized by the highly efficient mutualistic ant, *P. ferrugineus*, at the highest altitude. We observed lower concentrations of soluble solids in extrafloral nectar at higher elevations, but this effect applied only during dry the season, whereas the concentrations of soluble solids increased with altitude after the wet season. We could not find any effect of altitude on the quantity or protein content of the food bodies produced, on the defensive responses of the ants to plant damage or on ant body size. Feeding experiments with the generalist herbivore caterpillar *S. frugiperda* revealed that some type of chemical deterrent in the *A. hindsii* leaves tended to diminish at the highest altitude, a pattern that also contradicts the hypothesis that the plants tend to shift to mutualism-independent, direct defenses at higher altitudes. In summary, at all altitudes tested, the larvae were equally well fed and the ants maintained a stable defensive service. We conclude that the altitudinal limitation of the *Acacia-Pseudomyrmex* mutualism is not likely to be caused by a weakening or loss of the mutualistic relationship with its ant defenders. Future studies will have to explore alternative explanations for the limitation of mutualisms to low altitudes. Based on the results of this study, we propose that changes in the composition of vegetation or other factors that make higher altitudes unsuitable for the host plant are most likely to explain the biogeographical limitation of this - and perhaps other - mutualisms.

RESUMEN

Los mutualismos son asociaciones entre organismos de diferentes especies que benefician a todos los participantes de la interacción. En el mutualismo obligatorio hormiga-planta *Pseudomyrmex-Acacia* las plantas *Acacia* proveen a las hormigas con cuerpos nutritivos, néctar extrafloral y espacio para las larvas, mientras las hormigas defienden a la planta contra herbívoros y patógenos. Los mutualismos hormiga-planta están restringidos a las tierras bajas de los trópicos, en este caso encontrándose desde el sur de México hasta Centro América y entre 0 y 1200msnm. En general, con altitudes o latitudes elevadas los mutualismos disminuyen su intensidad, abundancia y diversidad. Las explicaciones más frecuentemente propuestas para la debilitación o ruptura total de un mutualismo en los límites de su área de distribución son una disminución en la producción de recompensas por el hospedero o una disminución en la calidad de los servicios proporcionados por el socio, con la consecuencia que ambas especies invierten más en estrategias independientes del mutualismo. En este estudio se cuantificó la colonización y defensa de plantas *Acacia hindsii* por hormigas mutualistas de la especie *P. ferrugineus* y se buscaron explicaciones para la esperada disminución del mutualismo en las altitudes más elevadas al nivel de la calidad o cantidad de las recompensas nutritivas. Sin embargo, encontramos que aún en los límites de su tolerancia altitudinal, el mutualismo *Acacia-Grupo Pseudomyrmex ferrugineus* no se rompe. Al contrario, a menores altitudes, hubo colonización de las plantas por diversas especies de hormiga, incluso hormigas no-defensivas, mientras a mayores elevaciones todas plantas encontradas estaban colonizadas por la hormiga mutualista más eficiente: *P. ferrugineus*. A elevaciones más altas se observaron menores concentraciones de sólidos disueltos en el néctar extrafloral durante temporada seca, sin embargo, los mismos valores incrementaron con la altitud después de temporada húmeda. No encontramos efectos de la altitud en la producción de cuerpos nutritivos, su contenido protéico, el tamaño del cuerpo de las hormigas o su respuesta defensiva al daño de la planta. Todo en conjunto indica que las larvas están siendo bien alimentadas en todas las altitudes y que el mutualismo funciona de forma independiente de la altitud. Adicionalmente, un experimento de alimentación con la oruga herbívora generalista *S. frugiperda* reveló la existencia de un metabolito disuasorio en las hojas de *A. hindsii*, el cual disminuye su concentración o eficiencia en el sitio más alto. Todos estos resultados contradicen a la hipótesis que el mutualismo se debilita o tiende a perderse en las altitudes más elevadas y que las plantas por lo tanto empiezan a reemplazarlo por una defensa directa, independiente del mutualismo. Concluimos que la planta no abandona su inversión en el mutualismo y que el mutualismo sigue funcionando en las altitudes más elevadas y que la ausencia de este mutualismo de las altitudes más grandes no se debe a cambios en la relación mutualista. Futuros trabajos deberán explorar explicaciones alternativas de la ausencia del mutualismo *Acacia-Pseudomyrmex* y de muchos otros mutualismos de las altitudes elevadas. En base de los resultados de este estudio proponemos que cambios en la composición de la vegetación u otros factores ambientales limitantes de la planta son la más probable causa de este patrón general.

1. INTRODUCTION

Mutualistic associations occur when two or more organisms from different species interact and when all partners involved benefit from the interaction. Mutualisms are normally established between very different partners. Because practically all higher plants and animals and diverse groups of microorganisms engage in one or more mutualisms, mutualisms are considered one of the most common types of interaction among organisms (Bronstein *et al.* 2004). Well known examples of mutualisms include plant-fungus (mycorrhizae), plant-bacteria (nodule forming bacteria and legumes) (Wilson *et al.* 2009), plant-animal (pollination and seed dispersal) (Galetti *et al.* 2008; Potts *et al.* 2010; Terborgh *et al.* 2008), and plant-animal or animal-animal defensive/cleaning mutualisms (Grutter 1999). According to the endosymbiotic theory for the origin of plastids and mitochondria, even the eukaryotic cell represents the result of this type of interaction (Margulis 1993). This close cooperation between different organisms is sustained by a system of reciprocal services and rewards: usually, the partners exchange a reward that is relatively cheap for them to produce in order to obtain a service that they hardly could obtain on their own (e.g., plants trade carbohydrates and essential amino acids to obtain transportation services from animals or access to the products of metabolic processes or other traits that only exist in microorganisms). Although the specific rewards and services may vary depending on the interacting species, their exchange always should benefit the net fitness of all partners (Bronstein *et al.* 2006; Heil & McKey 2003).

1.1. Ant-Plant Mutualisms

Among mutualisms, those established between ants and plants are among the most well-known and studied systems. These mutualisms can be classified as facultative or obligate depending on the dependency of the partners on the interaction. Facultative ant-plants (myrmecophiles) attract non-specific ants from the vicinity, generally using extrafloral nectar (EFN) as a reward, in order to harness their aggressive nature as a defense mechanism (Heil 2008; Bixenmann *et al.* 2011; Del-Claro *et al.* 2016; Josens & Roces 2000; Heil & McKey 2003). In this scenario, the ants do not depend on EFN secreted by the plant, but they can gain additional energy or nutrients when they opportunistically feed on it. By contrast, obligate ant-plant mutualisms are much more specific and invariably include the housing of an ant colony by host plants. Here, the ant and plant species have developed much tighter interactions, and in most cases neither of them can successfully survive without the presence of the other one (Janzen 1966; Janzen 1967; Herre *et al.* 1999; Heil *et al.* 2001a; Heil & McKey 2003). Plants that provide hollow structures in which ant colonies can nest (Janzen 1966, 1967; Risch & Rickson 1981; Heil & McKey 2003) are called myrmecophytes; however, most of the obligate myrmecophytes also provide nourishment in the form of EFN, cellular food bodies (FBs), or both.

1.2. Mutualism Instability

Although mutualisms are ubiquitous, several general considerations predict that mutualisms should represent unstable interactions. On the one hand, most mutualisms are generally transmitted horizontally: this means that the mutualism has to be re-

established at each generation, putting at risk the survival of both organisms in case they fail to find each other and causing a general conflict among the fitness interests of the partners (Herre *et al.* 1999). Another source of possible instability consists of other organisms that profit from the rewards without offering any services in return. These so-called 'exploiters', 'cheaters' or 'parasites' of mutualism would put pressure on the exploited partners because their investment would not yield any benefits (Bronstein 2001; Ferriere *et al.* 2002; Jones *et al.* 2015). Several authors even argue that 'cheaters' should have an evolutionary advantage over the mutualists because they do not invest in reciprocating, and that mutualisms would naturally convert into parasitism, if there were no counter-balancing mechanisms (Bronstein 2001). In the case of ant-plants, it is thought that the plant diverts resources to produce food rewards instead of chemical defenses, because the ants that feed on these food rewards form an important part of the plant's overall defense system. In this scenario, a 'cheater' would consume the plant's resources without offering any defense, leaving the plant susceptible to herbivore damage, infection or other factors that would impede its survival to maturity (Kautz *et al.* 2012; Clement *et al.* 2008).

On the other hand, while mutualistic relationships in nature are ubiquitous and diverse, they thrive mostly within the tropics and there are most abundant and diverse in the lowland 'eutropical' ecosystems (Boucher *et al.* 1982; Herre *et al.* 1999; Kiers *et al.* 2010). This pattern may be linked to the observed latitudinal gradient in species diversity: most studies found lower species richness and diversity outside of the tropics than within and increased values at lower latitudes. The same phenomenon is also observed in altitudinal gradients, with higher species diversity values in lowlands (Janzen *et al.* 1976; Stevens

1989, 1992; Sanders 2002; Schemske *et al.* 2009; McCain & Grytnes 2010). Evidently, a high number of species interacting in an environment that does not change drastically during the year enhances the probability of the emergence of mutualistic associations. However, several authors also consider that this environmental stability can translate in a low tolerance for change (Boucher *et al.* 1982; Sachs & Simms 2006; Kiers *et al.* 2010). Thus, the risk of co-extinction increases when the mutualistic partners cannot adapt to altered environments or alternatively, at least one species might shift to a mutualism-independent strategy in the outer ranges of its area because the dependence on a (probably less flexible) mutualist might result in a greater disadvantage than the benefits that the mutual cooperation provides. Thus, according to Kiers *et al.* (2010), mutualistic partners faced with environmental changes to which the 'normal' partner cannot adapt might break down the mutualism and develop an independent way of life. They might also recruit new partners or, in the worst case scenario, face co-extinction with the partner, as mentioned earlier.

There are examples of ant-plants diminishing or abandoning mutualistic features when growing in an environmental context where the mutualism is no longer needed, or possible. The most prominent case is that of *Cecropia* plants on Hawaii and some Caribbean islands. Whereas these *Cecropia* plants usually harbor mutualistic *Azteca* ants, on some Caribbean islands where *Cecropia* seeds took root but no ants and no natural specialist herbivores were present, the plants lost their FB producing structures (trichilia) (Janzen 1973). Another instance of mutualistic plant growing without its defensive ants is that of *Acacia* plants brought to the state of Florida in the United States of America, to Cuba and to the Netherlands, where they were able to grow and survive in

harsher condition than in their native environment without the need for ant protection (Janzen 1974). This effect is consistent with the geographic mosaic theory of coevolution, which states that organisms coevolve in the specific geographic context in which they live. Thus, if a mutualism is not favorable in the present environment, it is possible for the organisms to break the mutualism (Thompson 1999, 2009; Thompson & Cunningham 2002).

In spite of these hypotheses, evidence shows mutualisms have existed for millions of years, they have evolved between different groups, and re-appeared several times during Earth's history even among the same taxonomic groups, showing that mutualisms could be more stable than theory predicts (Ward 1991; Sachs & Simms 2006; Gómez-Acevedo *et al.* 2010; Chomicki *et al.* 2015).

1.3. *Acacia-Pseudomyrmex ferrugineus* group

The myrmecophytic *Acacia* (recently, the name *Vachellia* has been proposed) plants belong to the family Fabaceae and the sub-family Mimosoideae. Myrmecophytism has independently evolved among African and American Acacias; however, the present study focuses on the American clade of *Acacia* myrmecophytes, which can be found from the coastal areas of central Mexico down to northern Colombia. The myrmecophytic acacias are shrubs or trees from 1 m to 30 m tall, and all species present structures specialized for mutualistic interactions: hollow stipular thorns called domatia that serve as nesting spaces for the ants, extrafloral nectaries at the base of the leaves' petioles or the leaf rachis and modified leaflet tips forming nutritious FBs (Figure 1), which in this genus have

been called “Beltian bodies” (Janzen 1966, 1967, 1974). Together, these structures ensure the survival of the ant partners by covering their needs for a protected nesting space and nutrition: the thorns are very tough and naturally hollow, the EFN is rich in carbohydrates and the FBs are rich in protein and lipids (Heil *et al.* 2004; Heil *et al.* 2005; Heil 2008; González-Teuber *et al.* 2012).

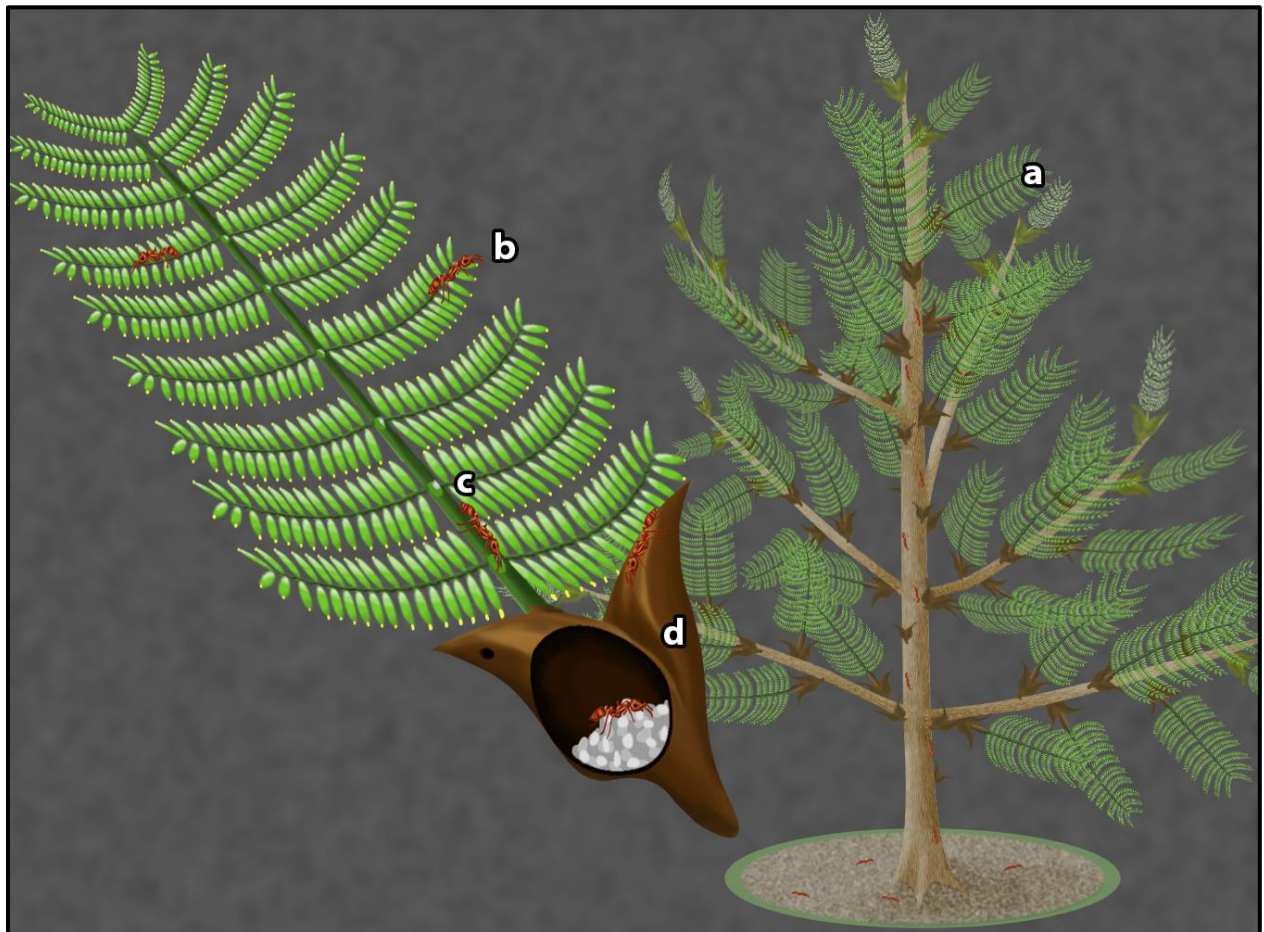


Figure 1. In exchange for continuous defense against herbivores, competing plants and pathogens (a), *Acacia hindsii* plants provide *Pseudomyrmex* spp. ants with nutrient rich food bodies from their leaflet tips (b), extrafloral nectar produced by nectaries distributed along the leaves' rachis (c) and hollow stipular thorns (domatia) that the ant colony uses as living space and nurseries (d).

In fact, the ants are highly dependent on the plant's rewards. Janzen (1966) kept *Pseudomyrmex ferrugineus* colonies under laboratory conditions by replacing the *Acacia*

plant and its domatia with glass tubes and the EFN with honey. However, the FB could not be replaced and fresh FB bearing leaves had to be provided continuously to the colony. The FBs are the main source of protein for the ants' larvae and in fact, the only solid food source that workers of the obligate plant-ant *P. ferrugineus* accept. In exchange for these rewards, the ants offer their services as defenders against herbivores as well as fungal and bacterial infections (Janzen 1966, 1967; González-Teuber *et al.* 2014). The ants patrol the surface of the plant and, when encountering danger, up to 50% of the adult workers can come out in defense of their host plant (Janzen 1967).

This system is a particularly suitable model for studies into the mechanisms that provide stability to mutualisms because of the following characteristics. First, *Acacia hindsii* in particular has been reported to have an altitudinal range going from sea level to 1000 meters above mean sea level (msl) (Janzen 1974), allowing for the study of possible changes in the mutualism in response to environmental change with increasing altitudes: changes that in many respects are similar to those that occur increasing latitude, but much more tractable for comparisons due to the much lower distances (Stevens 1992; Sanders 2002; Anderson & Johnson 2008; McCain & Grytnes 2010). Second, the macroscopic and surface-based nature of the mutualism makes it easy to identify living ants and plants in the field and to quantify the presence, abundance and activity of the ants as well as the quantity and quality of the food rewards. Thus, partners can be identified and the rewards and services can be studied relatively easily in a non-invasive or minimum-invasive manner. This situation makes the central parameters of the mutualism much more tractable than, for example, in endosymbiotic or subterranean mutualisms or all types of mutualisms that include microorganisms. Third, *Acacia hindsii*

is common in the study area, allowing for sampling of sufficient individuals for statistical analysis from all study sites within few days, a time span that allows for the meaningful comparison among the data despite, e.g., changing weather conditions. Heil *et al.* (2001) determined that quantitative changes in the production of FBs and nectar by myrmecophytic *Macaranga* plants entailed changes in the structure and activity of the hosted *Crematogaster* ant colonies, suggesting that the interaction between plant and ant could be impaired by an inadequate nutrient supply by the plants to the ants. Since plants can stop the production of food rewards in order to use the resources elsewhere (Janzen 1973; Folgarait & Davidson 1995; Fáveri & Vasconcelos 2004) and since the ants are highly dependent on the plant (Janzen 1967), it appears logic to hypothesize that food reward production might be affected when the plant grows in a harsh environment, such as their highest natural altitudinal limit, and that the resulting negative effects on the ants might have drastic consequences for the stability of the mutualism.

2. HYPOTHESIS

The amount or quality of food rewards will negatively vary with altitude and more *Acacia hindsii* plants will be unoccupied at higher locations.

3. OBJECTIVES

3.1. Main Objective:

Determine if the quantity or quality of food rewards in *Acacia hindsii* vary with altitude and investigate the effect of this variation on the functioning or maintenance of the *Acacia hindsii*-*Pseudomyrmex* spp. mutualism.

3.2. Specific Objectives:

- Measure the proportion of occupied *Acacia hindsii* and determine the colonizing *Pseudomyrmex* species at different altitudes.
- Quantify the FB production by *Acacia hindsii* plants growing at different altitudes.
- Quantify the total protein content of FBs collected at different altitudes.
- Compare the amount of soluble solids present in extrafloral nectar secreted by *Acacia hindsii* plants growing at different altitudes.
- Compare the defensive activity of *Pseudomyrmex* ants colonizing *Acacia hindsii* plants growing at different altitudes.

4. MATERIALS AND METHODS

4.1. Study Site

The study was conducted in the rural area surrounding the city of Puerto Escondido in the Pacific coast and in the “Sierra Madre Sur” mountains in the state of Oaxaca, Mexico during two field seasons in 2015: during the dry season (April-May) and after the rainy season (November-December).

An extensive search was made alongside the main mountain road (no. 131, the direct connection between Puerto Escondido and Oaxaca) from sea level to approximately 1200 meters above mean sea level (msl). Five study sites were selected during the dry season, based on the availability of suitable *Acacia hindsii* plant groups in terms of number and accessibility of individuals (Figure 2A). The sites' altitudes were: 0 msl (15°55'42.40"N, 97°09'15.60"W), 450 msl (16°00'2.00"N, 97°04'14.70"W), 750 msl (16°06'59.80"N, 97°03'52.00"W), 850 msl (16°08'22.60"N, 97°03'16.50"W) and 1000 msl (16°09'13.00"N, 97°04'24.70"W); location and altitude were determined using a Foretrex® 401 GPS (Garmin Ltd.; Schaffhausen, Switzerland). During the second field stay after wet season, three sites were selected based on previous experience at 0 msl (15°55'42.40"N, 97°09'15.60"W), 450 msl (16°00'27.10"N, 97°03'56.20"W) and 1000 msl (16°09'13.00"N, 97°04'24.70"W) (Figure 2B).

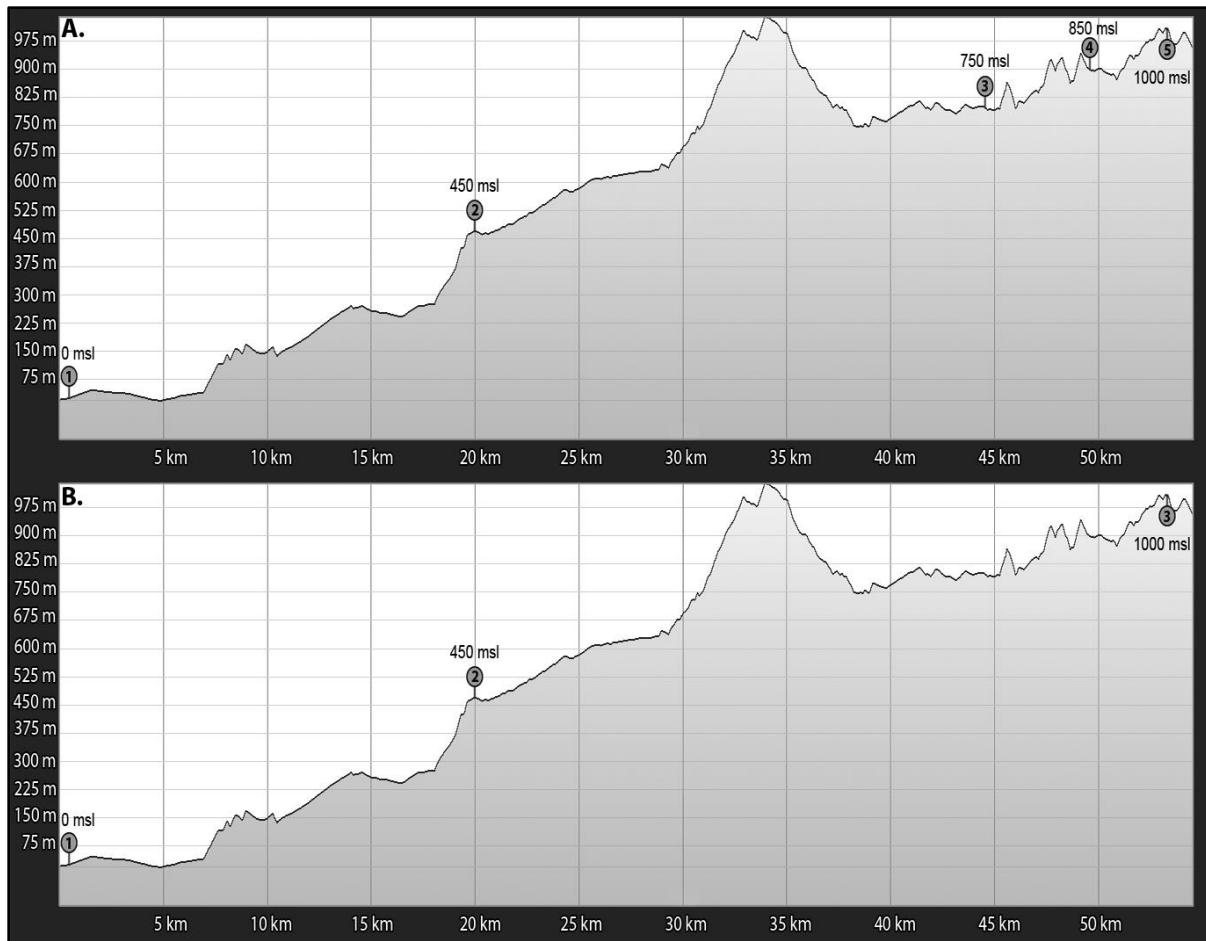


Figure 2. Elevation profile showing the location of selected study sites for sampling during the dry season (A) and after the rainy season (B).

4.2. Ant-Plant model

The study area comprises the entire the altitudinal range reported for *A. hindsii* (Janzen 1974) and the most frequent mutualist ant species in the region are *Pseudomyrmex ferrugineus*, *P. mixtecus* and *P. peperi* (Ward 1993).

Plants belonging to the *A. hindsii* species were identified according to Janzen (1974). The ants were identified following Ward (1993), and the species identification for several

voucher individuals was confirmed by P.S. Ward (personal communication, February 11, 2016).

4.3. Ant Occupancy

During the first field stay (dry season), transects were established starting at each of the selected study site and following the highway for 3 km up the mountain (Figure 3A). All *A. hindsii* plants ranging from 1 to 3 m, growing within 5 m from the road and being physically accessible were selected for the determination of their occupancy by ants.

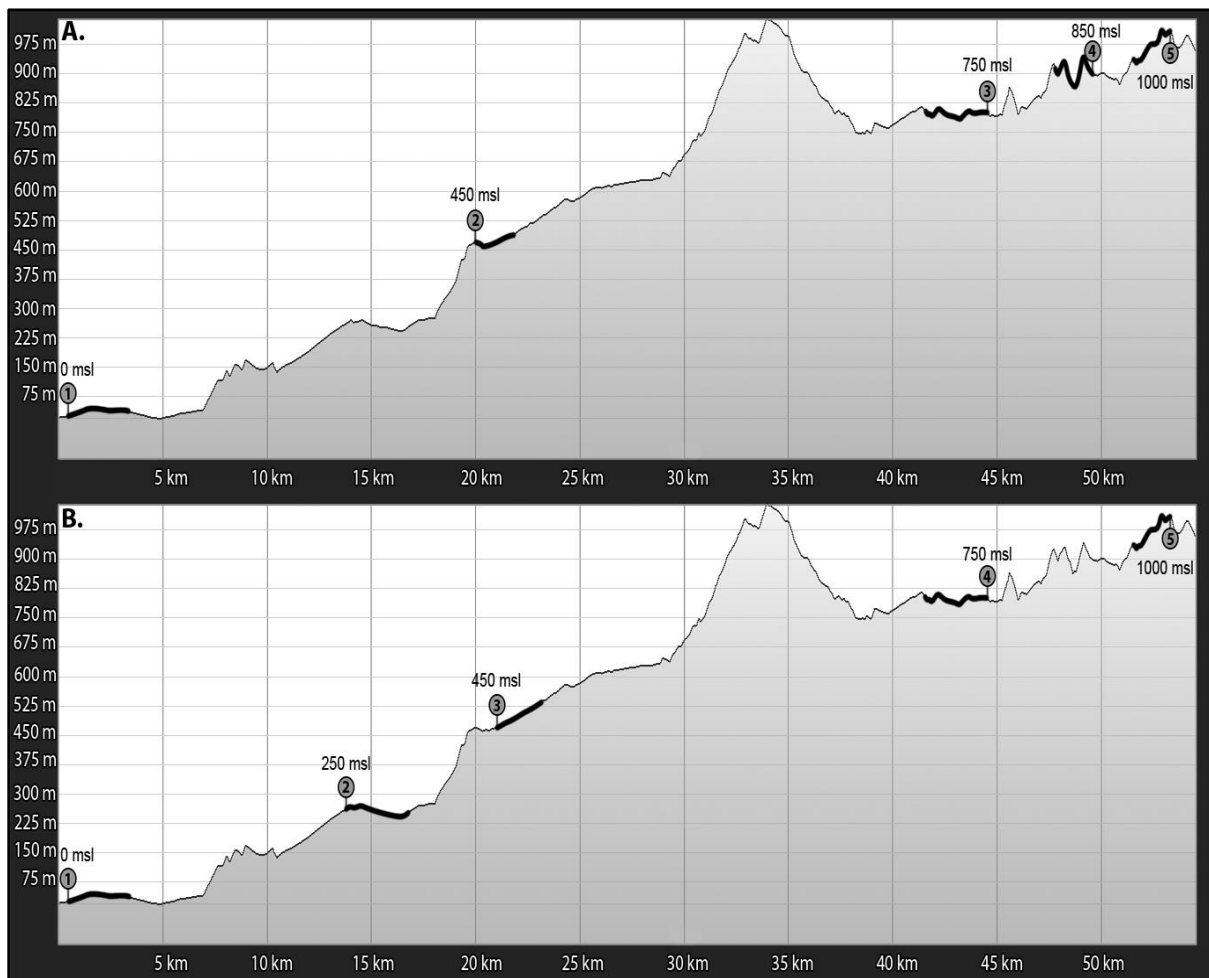


Figure 3. Elevation profile showing the location of transects during dry season (A) and after rainy season (B).

Each plant was lightly shaken and its surface was observed for up to three minutes to ascertain the presence and species of resident mutualistic ants. Plants were then classified according to the hosted species. Ants were considered to reside in the plant when they were seen patrolling the plant's surface, entering or leaving the hollow thorns and visibly belonged to any of the aforementioned mutualistic local species from the *Pseudomyrmex ferrugineus* group. In order to confirm identification, Ant samples (three ants per plant) were taken and transported to the laboratory in 2 mL microtubes containing 70% ethanol. Plants on which no ants could be observed, plants that had only a new colonizing queen on the surface and plants that hosted ants not belonging in the *P. ferrugineus* group were classified as unoccupied. The same survey was performed during the second field stay (after wet season) along transects starting at 0 msl, 250 msl, 750 msl and 1000 msl (Figure 3B).

4.4. Ant Activity

Ant activity was measured during the second field stay (after wet season) on each nine plants per site between 1.5 and 2.5 m in height and growing in full sunlight according to a method used by Heil (2009). Two lines delimiting a distance of 10 cm were marked with pencil on the trunk of the plant, worker ants entering the marked area were observed from an approximate distance of 30 cm to 50 cm and counted with the aid of a hand-held tally counter for the next 2 min, ants that exited and immediately re-entered the area were ignored.

In order to obtain a standardized estimate of the ants' defensive activity, we made use of the observation by Janzen (1966, 1967) that the ants intensively recruit to mechanical damage of their host even in the absence of natural herbivores. A 3-5 cm long, 2 mm deep wound was performed in the middle of the marked area using the tips of pruning scissors. Ants entering the pencil-delimited area were counted again for 2 min, ants that exited and immediately re-entered the area were ignored. To avoid interference caused by alert pheromones from treated ants in neighboring plants, measurements were made over two days (4 plants on the first day and 5 on the second) and the plant individuals that were used during the same day grew at least 1 m apart from each other.

4.5. Ant Body Length

After wet season, ten ants were collected from each of 5 plants from the lowest and the highest sampled sites (0 msl, 1000 msl). Ants from the same plant were stored in a labeled 2 mL microtube containing 70% ethanol. The length of the ants was measured individually from the tip of the closed mandibles to the end of the abdomen using a Leica GZ6 stereoscope with 10x magnification (Leica Camera, AG.; Wetzlar, Hesse, Germany) and noted in millimeters. The mean ant length per plant per altitude level was calculated.

4.6. Collection of Food Bodies

A minimum of eight plants between 1.5 and 2.5 m in height and growing in full sunlight was selected at each site. One branch per plant was selected, it had to be growing at ca. 1 m from the ground, with a minimum of three mature leaves. From each of the selected

branches ants and thorns were mechanically removed and Tangle trap® (Tanglefoot Co.; Grand Rapids, MI, USA) was applied to the base of the branch to avoid re-entry by the ants. In order to easily detect newly grown leaves, the branch was marked near visible leaf buds and before the terminal leaf. The branch was then enveloped by a polyester mesh (1 mm) hand-crafted bag to reduce herbivore damage and exclude exogenous FB consumers. The bag had to cover at least three of the already existing leaves and had to allow space for the branch and additional leaves to grow. After 2.5 weeks, newly produced leaves and the attached FBs were collected, placed individually in tagged paper bags and dry preserved in a polystyrene container containing silica gel beads (Karal, S.A. de C.V.; Leon, Gto., Mexico) hermetically sealed using duct tape. FBs were manually separated from leaflets in the laboratory and counted using a hand tally counter. Dried leaves (after FB removal) from each plant were pooled and weighed. FBs collected were pooled as well according to the plant of origin, stored in 1.5 mL microtubes and weighed. The tubes were, in turn, kept inside the aforementioned container with silica beads.

4.7. Quantification of total Protein content of Food Bodies

FBs collected from each three plants at 0 msl, 450 msl and 950 msl during both the dry and the wet season were ground using liquid nitrogen and tungsten pellets in a Tissue Lyser LT (Qiagen N.V.; Hilden, North Rhine-Westphalia, Germany). An aliquot of five milligrams of dry FB tissue per individual plant was recovered in 1.5 mL microtubes and used for analysis.

Protein extraction was performed according to Wang *et al.* (2006). Ground tissue was re-suspended in 1 mL of 10% TCA-Acetone, vortexed for 30 s and centrifuged using an Eppendorf centrifuge 5424 (Eppendorf AG.; Hamburg, Germany) at 16,000 g for 3 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in 1 mL of an 80% Methanol and 0.1 M Ammonium Acetate solution, vortexed for 30 s and centrifuged at 16,000 g for 3 min at 4 °C. The supernatant was discarded again and the 1.5 mL microtube was then filled with 1 mL of 80% Acetone, vortexed for 30 s until the pellet dissolved and then centrifuged at 16,000 g for 3 min at 4 °C. After discarding the supernatant, the tube was incubated in a Stuart SBH130D Block Heater (Bibby Scientific Ltd.; Stone, Staffordshire, UK) at 30 °C for one and a half hours, until all acetone residues had visibly evaporated. Consecutively, 800 µL of a 1:1 Phenol-SDS pH 8.0 buffer was added, the sample was vortexed, incubated for 5 min and then centrifuged at 16000 g for 3 min at 4 °C. The upper (phenolic) phase was recovered and transferred to a new 2 mL microtube, to then add 1.5 mL of an 80% Methanol and 0.1 M Ammonium Acetate solution. After storage at -20 °C for 12 h, followed by centrifugation at 16,000 g for 5 min at 4 °C, the supernatant was discarded and the pellet was washed once with 1 mL of 100% Methanol and once with 1 mL of 80% Acetone, then allowed to dry for one and a half hours on the block heater at 30 °C.

Quantification of total proteins was made using the BCATM (Bicinchoninic Acid) Protein Assay kit (Pierce Biotechnology, Inc.; Rockford, IL, USA) that follows the methodology proposed by Smith *et al.* (1985). For each sample, the proteins in the pellet were re-suspended in 500 µL of a 0.5% SDS/0.01 M Tris solution. An aliquot of 10 µL was placed in a microplate well and 200 µL of BCA working reagent were added. The microplate was

shaken for 30 s, then incubated at 45 °C for 2 h and cooled down at room temperature for 2 min to consecutively quantify absorbance at 562 nm using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Inc.; Hercules, CA, USA). The protein content was determined by comparison with a seven-point calibration curve ($y = 1369.5x - 44.264$, $R^2 = 0.9816$) consisting of a series of bovine serum albumin dilutions ranging in concentration from 125 µg/mL to 2 mg/mL (Figure 4). Measurements were carried out in technical triplicates, mean total protein concentration values were calculated and used to obtain mean total protein mass values per milligram of sample.

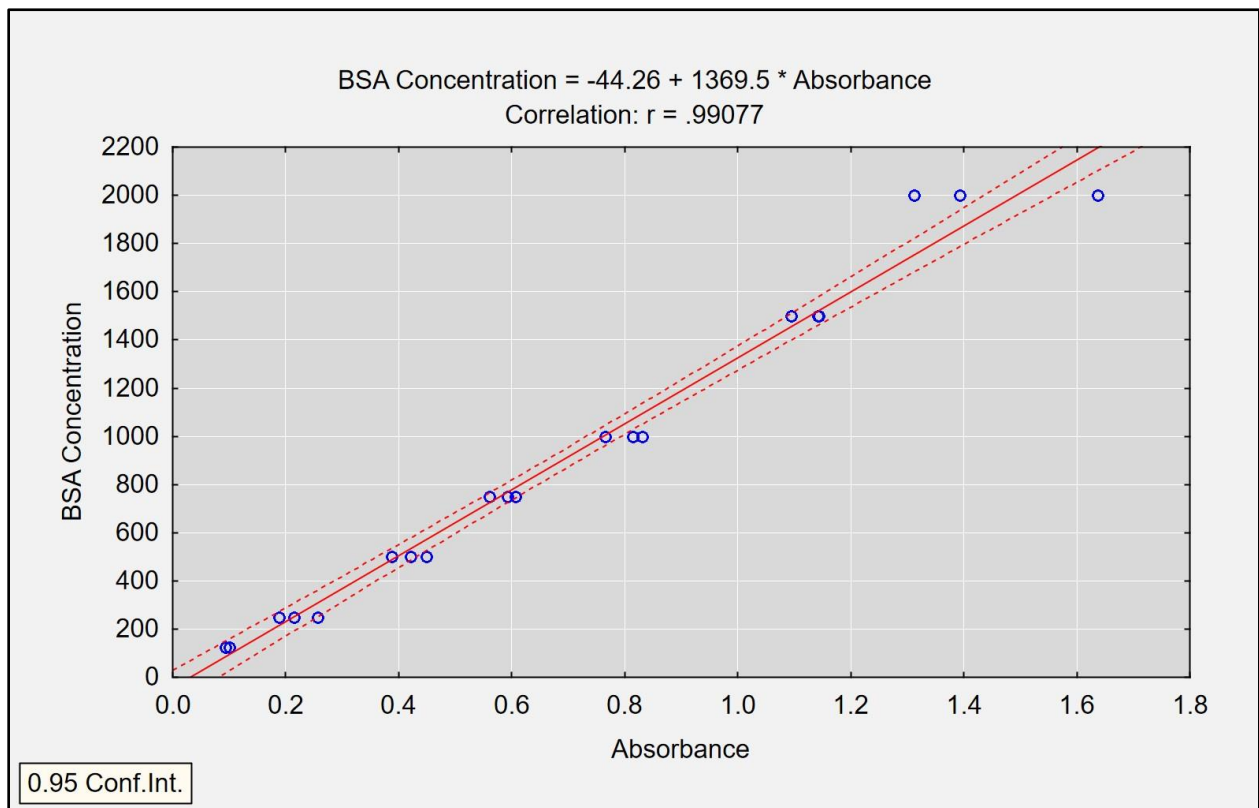


Figure 4. Bovine serum albumin calibration curve used to quantify total protein content in *A. hindsi* FBs. Solid line represents linear fit, dotted lines represent 0.95 confidence interval, circles represent absorbance measured in triplicates.

4.8. EFN Soluble Solids

For the measurement of EFN soluble solids during the dry season, a second branch on each of the eight plants that had been selected for the collection of FBs was isolated as described previously. During the second field trip after the wet season, a branch on each of nine plants per study site was isolated. EFN was collected 24 h later following Stephenson (1982) by applying and recovering 20 μL of water twice with a 10-100 μL Rainin Classic Pipette PR-100 micropipette (Mettler-Toledo, Intl.; Greifensee, Zürich, Switzerland) to each nectary of three randomly selected leaves per branch. Recovered volume was determined with a graduated capillary and the concentration of soluble solids was quantified using a hand-held temperature compensated Atago 1E refractometer (Atago, Co., Ltd.; Tokyo, Japan). Soluble solid mass was calculated for each leaf using the concentrations and volumes measured, the value for the mass was then standardized using the dry mass of the corresponding leaf. The mean value from the three leaves was calculated for each sampled plant at every altitude level.

4.9. Direct Chemical Defenses of the Plant

In order to detect a possible change in overall direct chemical defenses in the leaves of *Acacia* plants growing at three different altitudes (0 msl, 450 msl, 1000 msl), three mature leaves per plant from five plants per site were collected concurrently with the dry season FB collection, following the same procedure previously described: the collected leaves were placed in labeled paper bags and stored in a polystyrene container hermetically sealed with duct tape where they were dry preserved with silica beads.

A feeding experiment was conducted in the laboratory five months later using the generalist herbivore *Spodoptera frugiperda*. Sixty *S. frugiperda* two-week-old larvae were randomly assigned to four groups of fifteen caterpillars each. Larvae were kept individually in a translucent cylindrical plastic container with a volume of approximately 100 cm² and a perforated lid for ventilation. The first group served as control and was fed with an artificial diet modified from Greene (1976) (see Table 1 for composition). The other three groups received the same food to which 9mg of the ground dry leaves from *A. hindsii* collected at 0 msl, 750 msl and 1000 msl had been added during its preparation (Table 1). The dried leaves were pooled according to altitude, the FBs (when present) were removed manually, the leaves were ground with a porcelain mortar and pestle immediately before being added to the food mixture.

Table 1. Recipe for 100g of artificial diet used for *Spodoptera frugiperda* larvae in the feeding experiment. Ground leaves stem from *Acacia hindsii* growing at 0msl, 750msl and 950msl (treatment groups), the control group received the same diet excluding the leaves.

Ingredients	Quantity
Distilled water	456.00 mL
Agar	10.98 g
Corn Grits	12.84 g
Wheat Germ	3.21 g
Yeast	3.43 g
Ascorbic Acid	0.45 g
Benzoic Acid	0.78 g
Nipagin	0.11 g
Ground Leaves	0.09 g

All larvae were weighed at the beginning of the experiment. Food was provided on days 1, 3, 6 and 10 of the experiment. Each larva received one food cube weighing 1.2 g during the first two feedings, this was increased to 2 g cubes per larva starting on the

sixth day in order to keep food in excess at all times. New food, remnants of old food and the larvae themselves were weighed when food was replaced and at the end of the experiment. The experiment was carried out over 14 days, until the first larvae in the control group achieved the pupal stage.

4.10. Statistical Analysis

Every experiment performed yielded data that did not follow a normal distribution, it is because of this reason that all analyses were made using non-parametric tests. All statistical analyses were performed using the software STATISTICA version 10 (Statsoft Inc.; Tulsa, OK, USA).

The data on FB weight and number of FBs produced was tested for correlation with altitude using Spearman ranks correlation test, total sample size (N) is reported for these analyses.

Analyses of data from multiple groups (altitude levels), such as: FB production (mass and amount), mean total FB protein content, the amount of nectar soluble solids, activity of the ants, mass gain and amount of food consumed in the feeding trial with *S. frugiperda*, were performed using the Kruskal-Wallis ANOVA test and post-hoc Dunn's test with Bonferroni adjustment for multiple comparison of groups when significant differences ($p < 0.05$) were detected in the main ANOVA.

Since ant sizes were measured in only two altitude levels, the differences among the groups were tested using the Mann-Whitney U test.

Each group's sample size (n) is reported for analyses that compare different groups.

5. RESULTS

5.1. Ant Occupancy

During the dry season at the lowest elevation, 62% of the *A. hindsii* plants were occupied by *P. ferrugineus*, 16% by *P. mixtecus*, and 22% were unoccupied individuals. Among plants growing alongside the second transect, 52% of hosted *P. ferrugineus* colonies, while *P. mixtecus* and *P. peperii* inhabited 9% and 11% of the *A. hindsii* plants, respectively, and 28% of plants being unoccupied. On higher locations, only *P. ferrugineus* could be found, with no unoccupied plants at the highest elevation (n=14 for all groups, Figure 5).

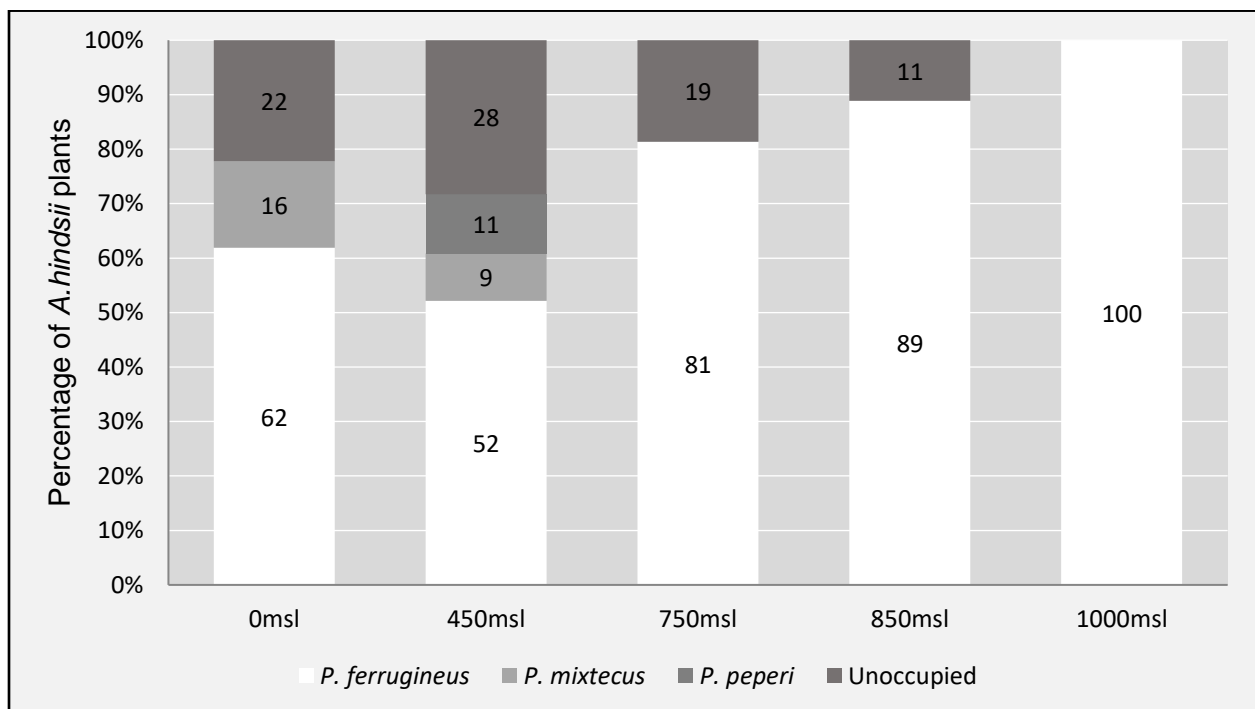


Figure 5. Increasing colonization of *A. hindsii* plants by colonies of the mutualist *P. ferrugineus*. Data shows percentage of plants growing alongside a 3 km transect in high altitudes during the dry season (May 2015). Sample size n=14 plants per transect, ant species are indicated by different grades of grey and altitude is given in meters above mean sea level.

After wet season, *P. ferrugineus* was found inhabiting 60% of *A. hindsii* plants at sea level, while 6% of the plants hosted *P. mixtecus* colonies and 34% were unoccupied. Among all *A. hindsii* plants growing along the second transect 51% hosted *P. ferrugineus* colonies, 29% hosted *P. peperii* and 11% maintained *P. mixtecus*, while 9% were not inhabited by mutualistic ants. Similarly, on the third transect, 80% of plants hosted *P. ferrugineus* while *P. mixtecus* and *P. peperii* colonies were found on 3% and 11% of the plants, respectively, and 6% of the *A. hindsii* plants at this location were unoccupied. At the two highest elevations, only *P. ferrugineus* was found colonizing *A. hindsii*, a small proportion of plants remained unoccupied (n=14 for all groups, Figure 6).

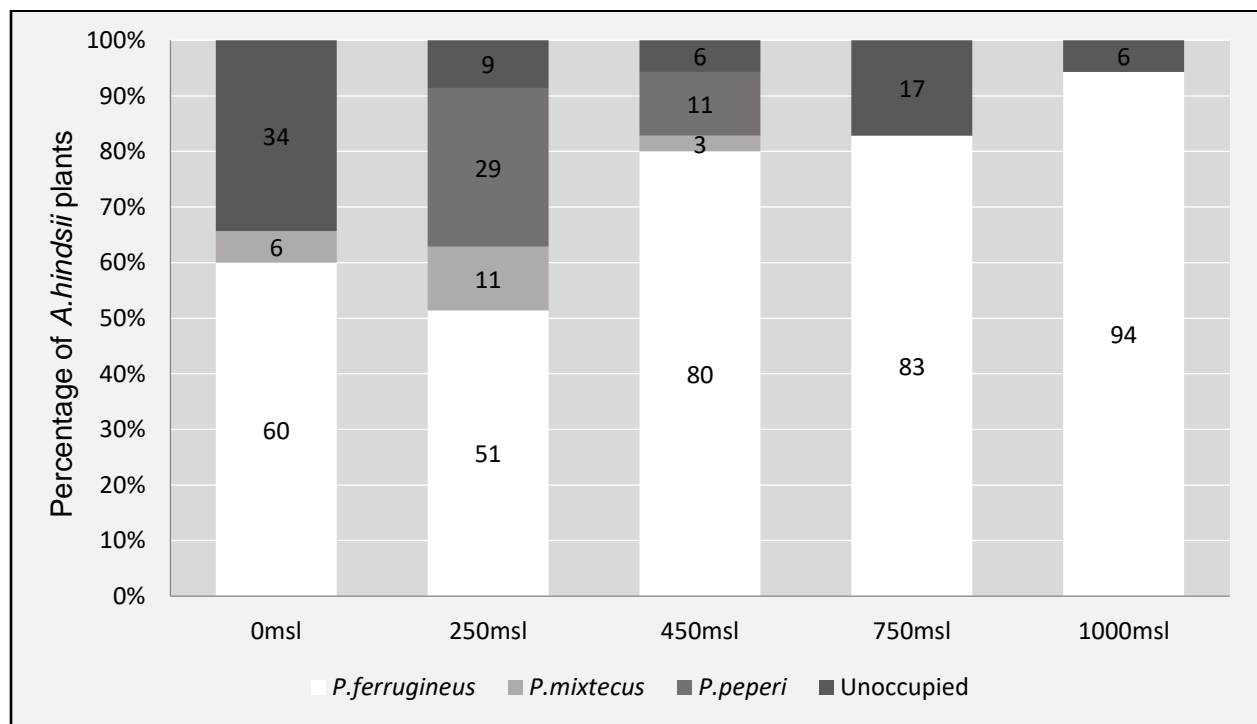


Figure 6. Increasing colonization of *A. hindsii* plants by colonies of the mutualist *P. ferrugineus*. Data shows percentage of plants growing alongside a 3 km transect in high altitudes after the rainy season (December 2015). Sample size n=34 plants per transect, ant species are indicated by different grades of grey and altitude is given in meters above mean sea.

5.1. Ant Activity

The basal number of ants observed on the surface of undamaged plants did not differ significantly among the three altitudes tested. At all sites, the number of *P. ferrugineus* ant workers that responded to experimentally inflicted plant damage was significantly higher than this basal number ($p < 0.01$, according to Kruskal-Wallis ANOVA followed by post-hoc Dunn's test, $n=9$ plants for all groups). Moreover, the number of ants that responded to damage was significantly higher at sea level than at 450 msl ($p < 0.05$, according to post-hoc Dunn's test, $n=9$ plants for all groups) (Figure 7).

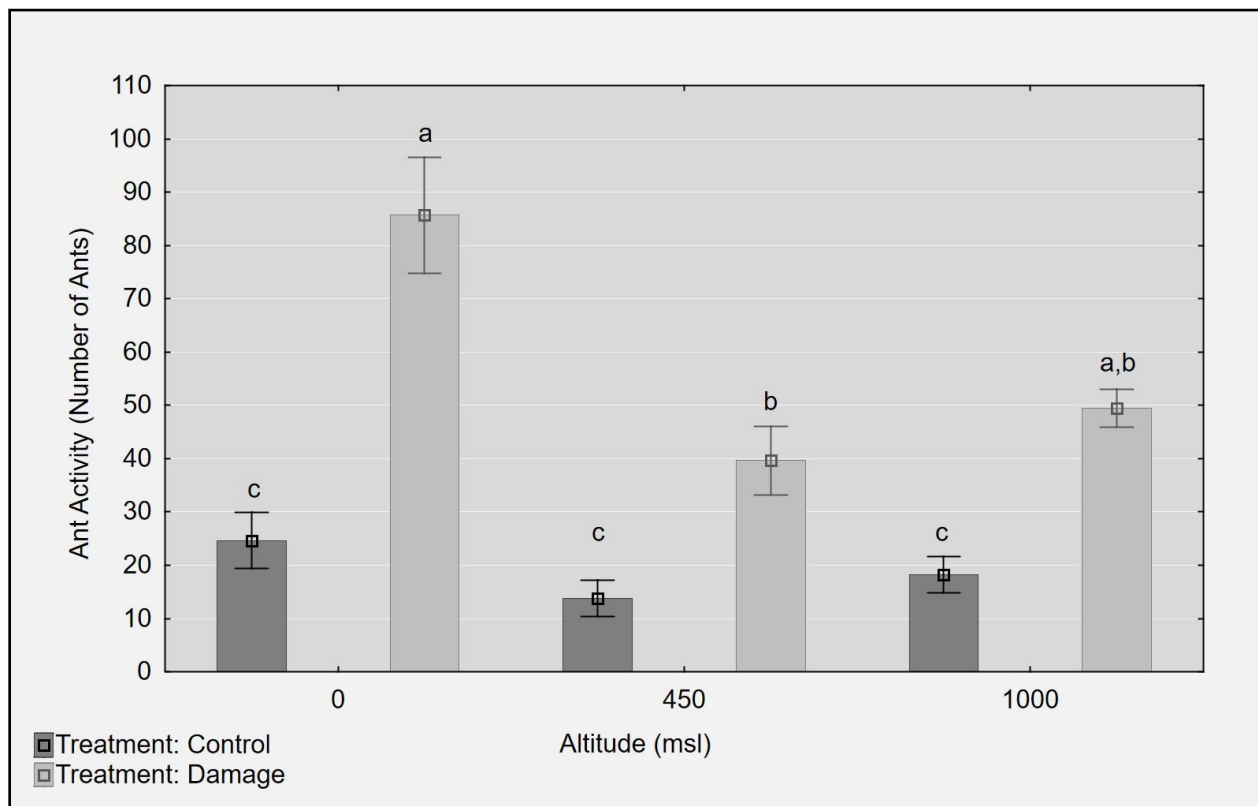


Figure 7. *Pseudomyrmex ferrugineus* ants on plants at sea level respond most strongly to experimental damage. Bars represent mean numbers of workers that entered a defined area on the plant surface in the undisturbed 'basal' stage and in response to experimental damage. Sample size $n=9$ plants per site, whiskers correspond to standard errors; different letters indicate significant differences among groups.

5.2. Ant Body Length

No significant difference ($p = 0.31$, according to Mann-Whitney U test, $n=5$ sampled plants for both groups) in mean ant body length could be detected when comparing ants from sea level to those from the highest altitude (Figure 8).

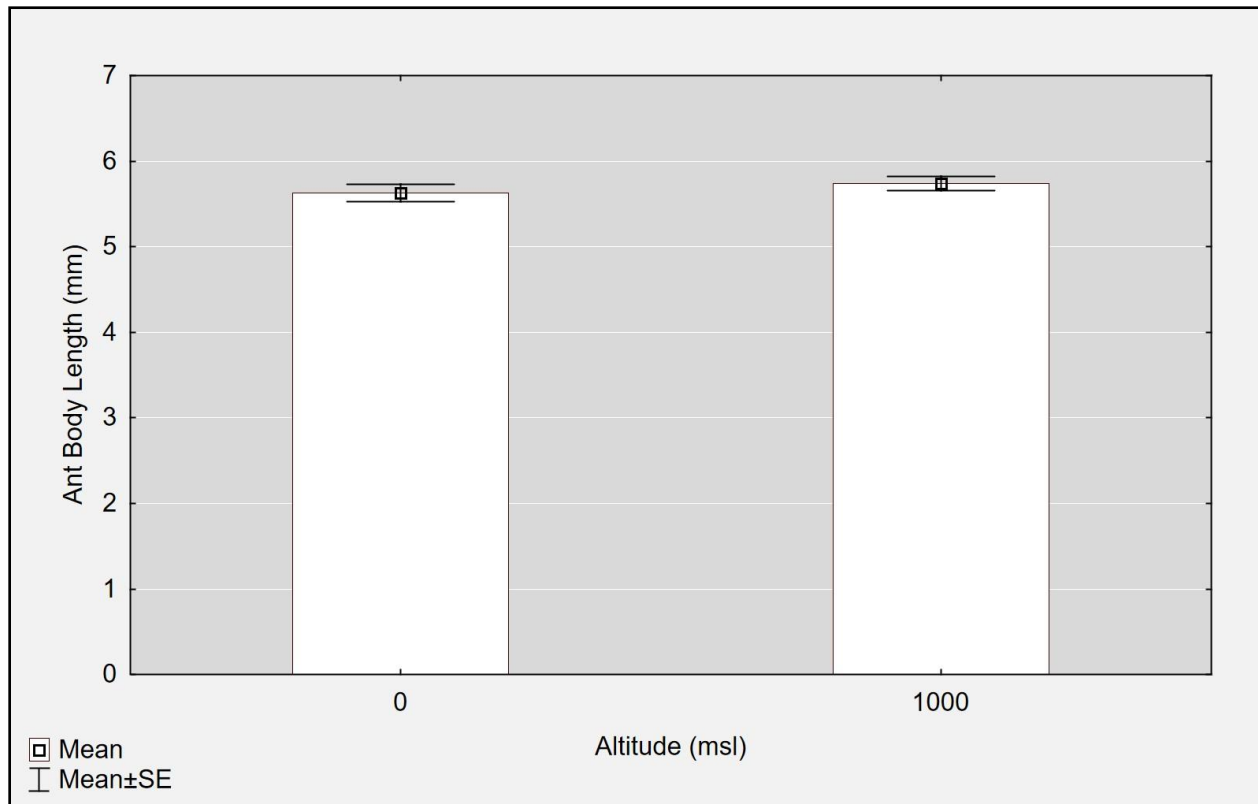


Figure 8. Mean body length of *Pseudomyrmex ferrugineus* workers was not affected by altitude ($n=5$ plants samples per site).

5.3. Food Bodies

The production of FBs varied between sites and season. During the dry season, a tendency towards decreasing mass and number of FBs produced by plants at increasing altitudes became obvious. However, no significant correlation could be detected between

the mass ($p = 0.677$, according to Spearman's rank correlation, $N=21$ total sampled plants) or the number of FBs ($p = 0.655$, according to Spearman's rank correlation, $N=21$ total sampled plants) and the site's altitude. However, there is high variation within each sampled site as reflected by the standard error (Figure 9).

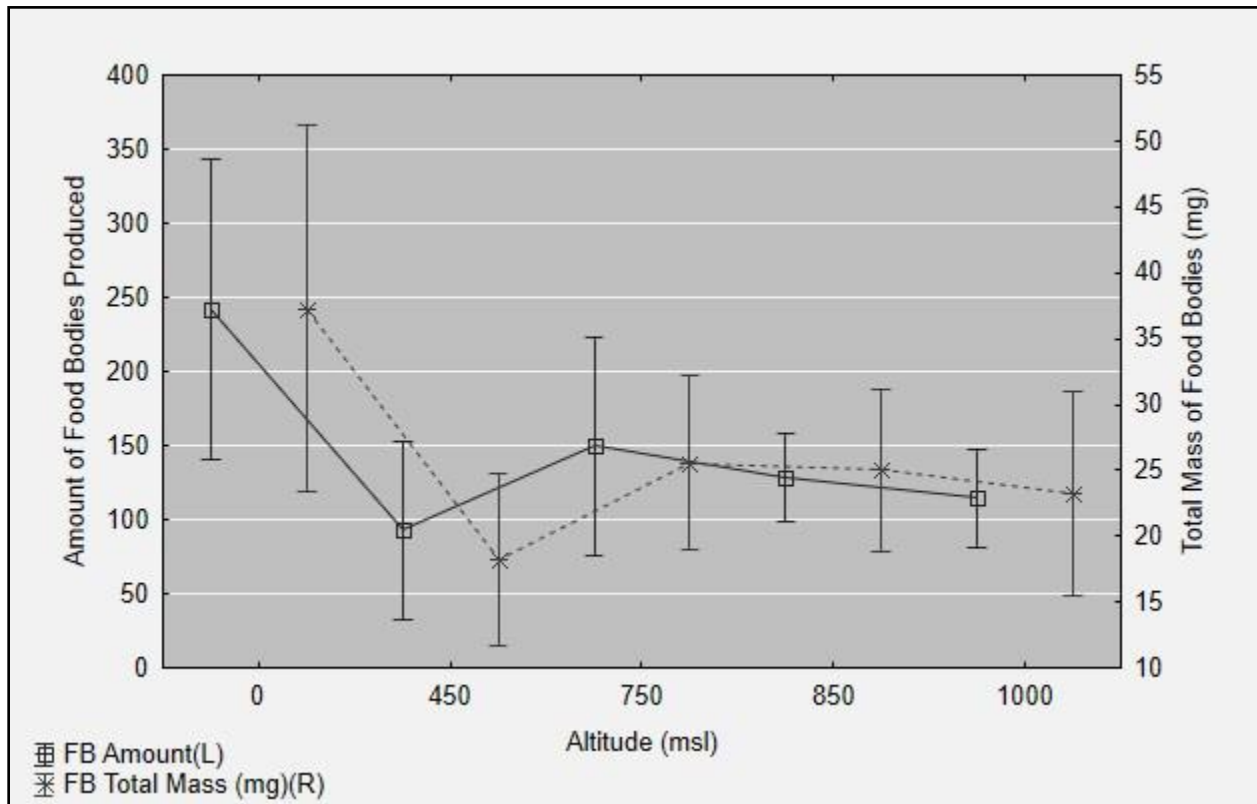


Figure 9. Food body production by *A. hindsii* was not significantly correlated with altitude. Squares represent the mean number of FBs produced per plant (left axis), asterisks represent mean food body total dry mass in milligrams produced per plant (right axis) over 2.5 weeks during May 2015 ($N=21$ total plants in both cases). Altitude is given in meters above mean sea level. Whiskers correspond to standard error.

After the wet season, food body production showed no discernable pattern between study sites and no significant correlation could be detected between the mass ($p = 0.618$, according to Spearman's rank correlation, $N=15$ total sampled plants) or number ($p = 0.762$, according to Spearman's rank correlation, $N=15$ total sampled plants) of the FBs

and the altitude. Again, very high variation within the sampled sites can be observed (Figure 10).

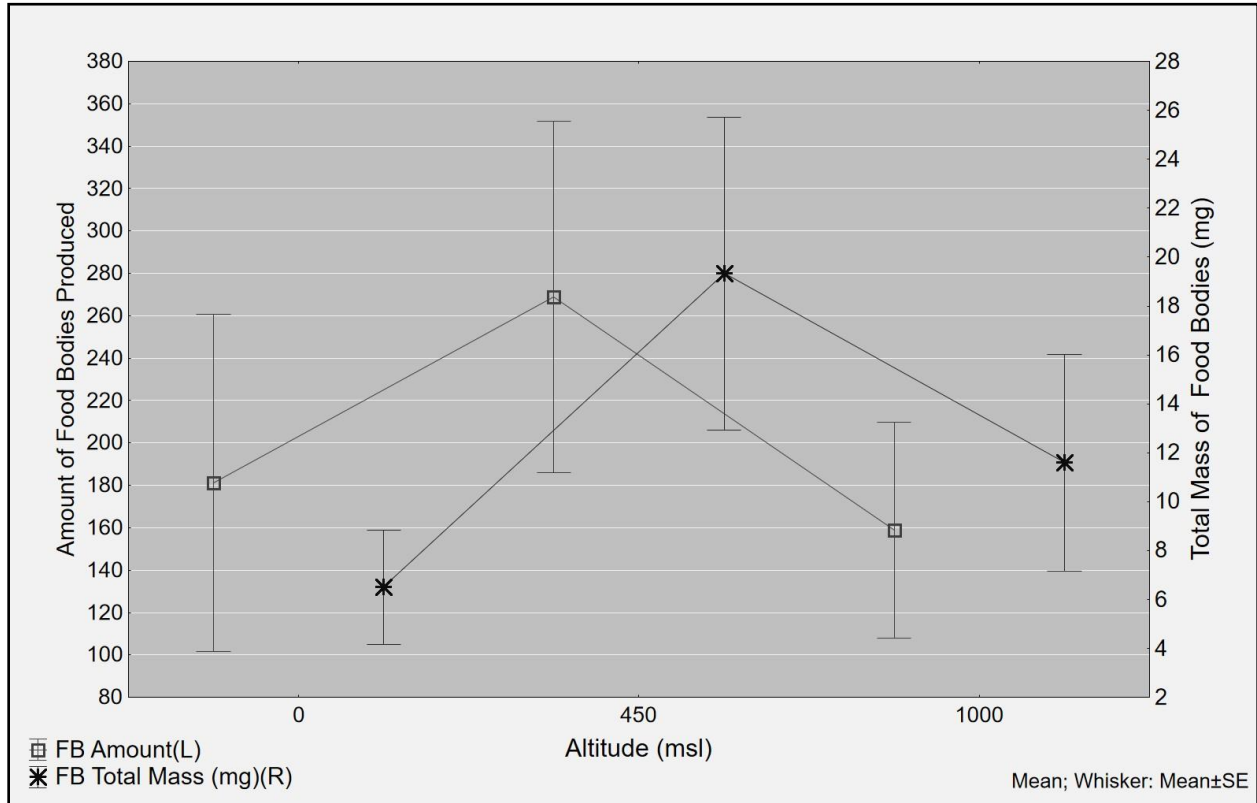


Figure 10. Food body production by *A. hindsii* was not significantly correlated with altitude. Squares represent the mean number of FBs produced per plant (left axis), asterisks represent mean food body total dry mass in milligrams produced per plant (right axis) over 2.5 weeks during December 2015 (N=15 total plants in both cases). Altitude is given in meters above mean sea level. Whiskers correspond to standard error.

5.4. Food Body Protein Quantification

The mean content of total proteins varied among altitude levels with highest values occurring at sea level. However, no significant difference among sampling sites ($p = 0.895$, according to Kruskal-Wallis ANOVA, $n=3$ samples for 0 msl and 450 msl, $n=2$ samples for 1000 msl) or significant correlation with altitude could be detected during the

dry season ($p = 0.95$, according to Spearman's ranks correlation, $N=8$ total samples, Figure 11).

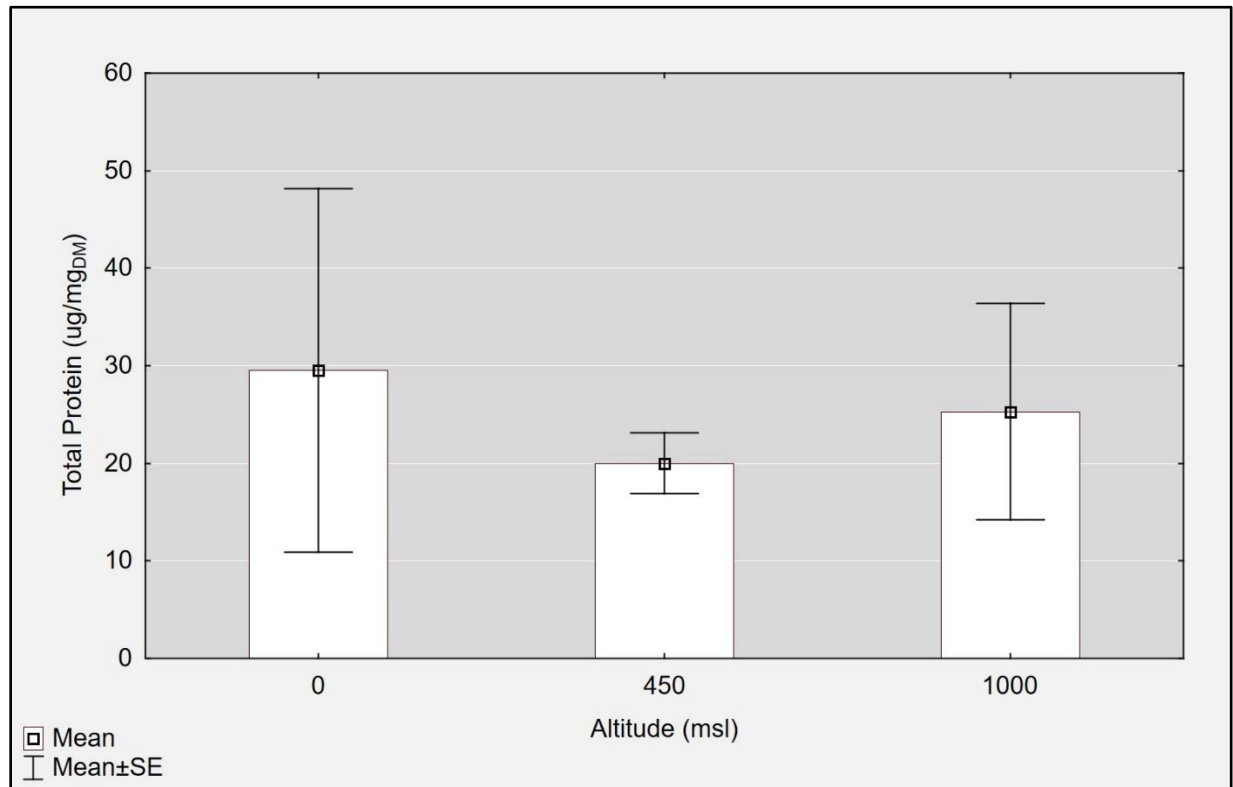


Figure 11. Food body mean total protein content was not significantly affected by altitude during May 2015 (n=3 samples for 0msl and 450msl, n=2 samples for 1000msl). Values in μg of total protein per mg of sample dry mass, altitude in meters above mean sea level. Whiskers correspond to standard error.

In general, the total protein content in the FBs that had been collected after the rainy season was much higher (means from 30 – 125 mg per gram dry mass) than observed in the FBs collected during the dry season (20 – 30 mg per gram dry mass). However, no significant differences could be detected among the protein contents of FBs from plants from different altitudes after the rainy season ($p = 0.2$, according to Kruskal-Wallis ANOVA, n=3 samples for all groups), correlation was not observed either ($p = 0.407$, according to Spearman's ranks correlation, $N=9$ total samples). However, during this

season, the maximum mean protein content was found at 1000 msl, the highest site sampled (Figure 12).

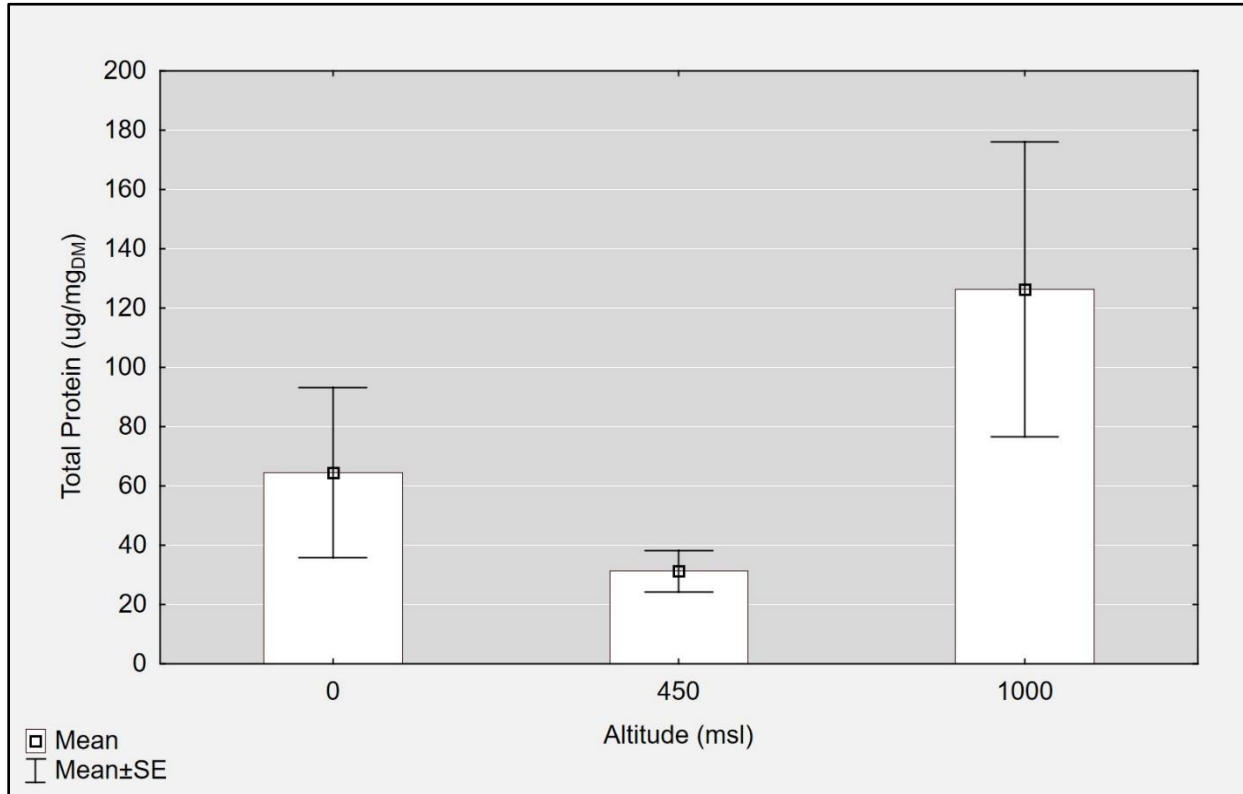


Figure 12. Food body mean total protein content was not significantly affected by altitude during December 2015 (n=3 samples per site). Values in μg of total protein per mg of sample dry mass, altitude in meters above mean sea level. Whiskers correspond to standard error.

5.5. EFN Soluble Solids

The secretion of EFN in terms of soluble solids followed an irregular decreasing pattern along the altitudinal gradient during the dry season. The maximum concentration was found at sea level, while the minimum was observed at 850 msl. There was no significant correlation between soluble solids concentration and altitude ($p = 0.098$, according to Spearman's ranks correlation, $N=40$ total plants), and no significant difference among

sites was observed ($p=0.054$, according to Kruskal-Wallis ANOVA, $n=8$ plants for all groups, Figure 13).

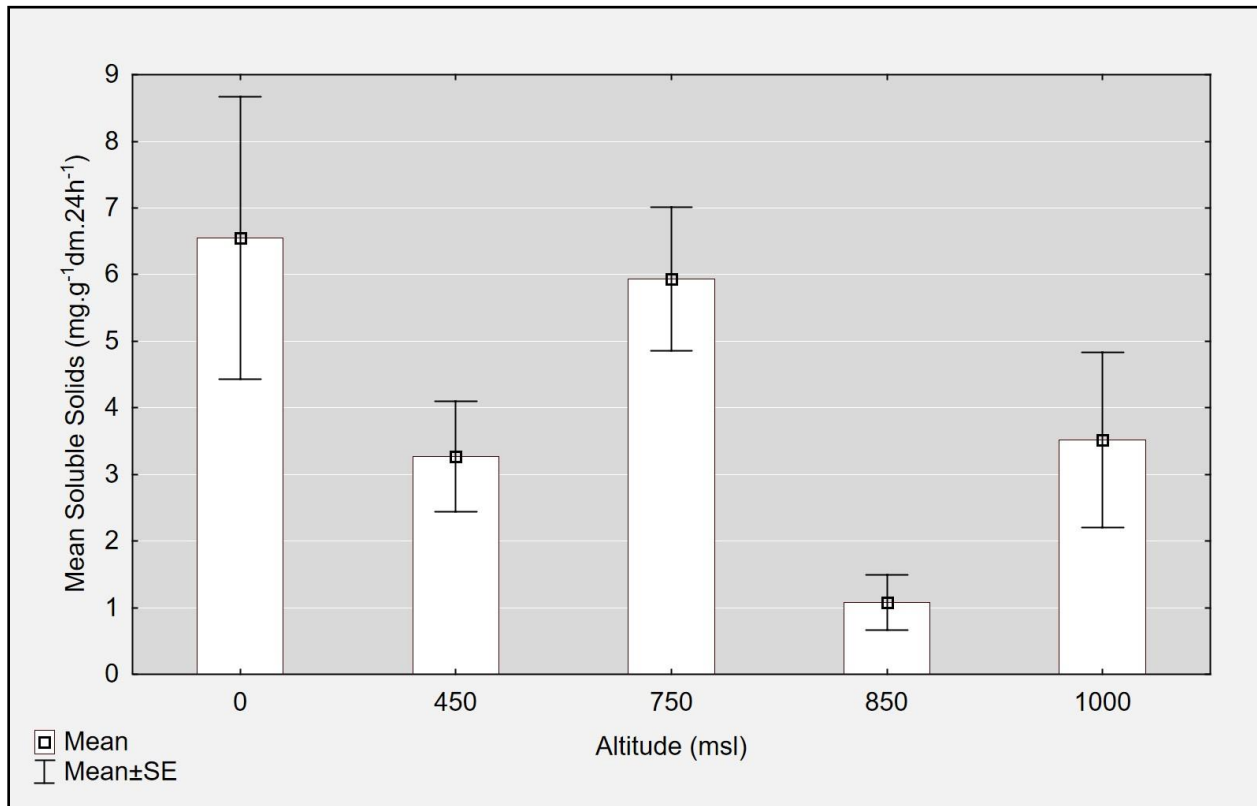


Figure 13. During dry season (May 2015), mean soluble solids in EFN were not correlated with altitude ($N=40$ total plants); additionally, they showed no statistic difference among sites ($n=8$ plants per site). Values in mg of soluble solids per g of leaf dry mass and day, altitude in meters above mean sea level. Whiskers correspond to standard error.

After the wet season, a much clearer pattern could be observed (Figure 14). At this time, the concentration of soluble solids in the EFN was significantly and positively correlated altitude ($p < 0.01$, according to Spearman Ranks correlation, $N=27$ total plants). Nectar concentration at 1000msl was also significantly higher than at sea level ($p = 0.04$, according to Kruskal-Wallis ANOVA, $n=9$ plants per site).

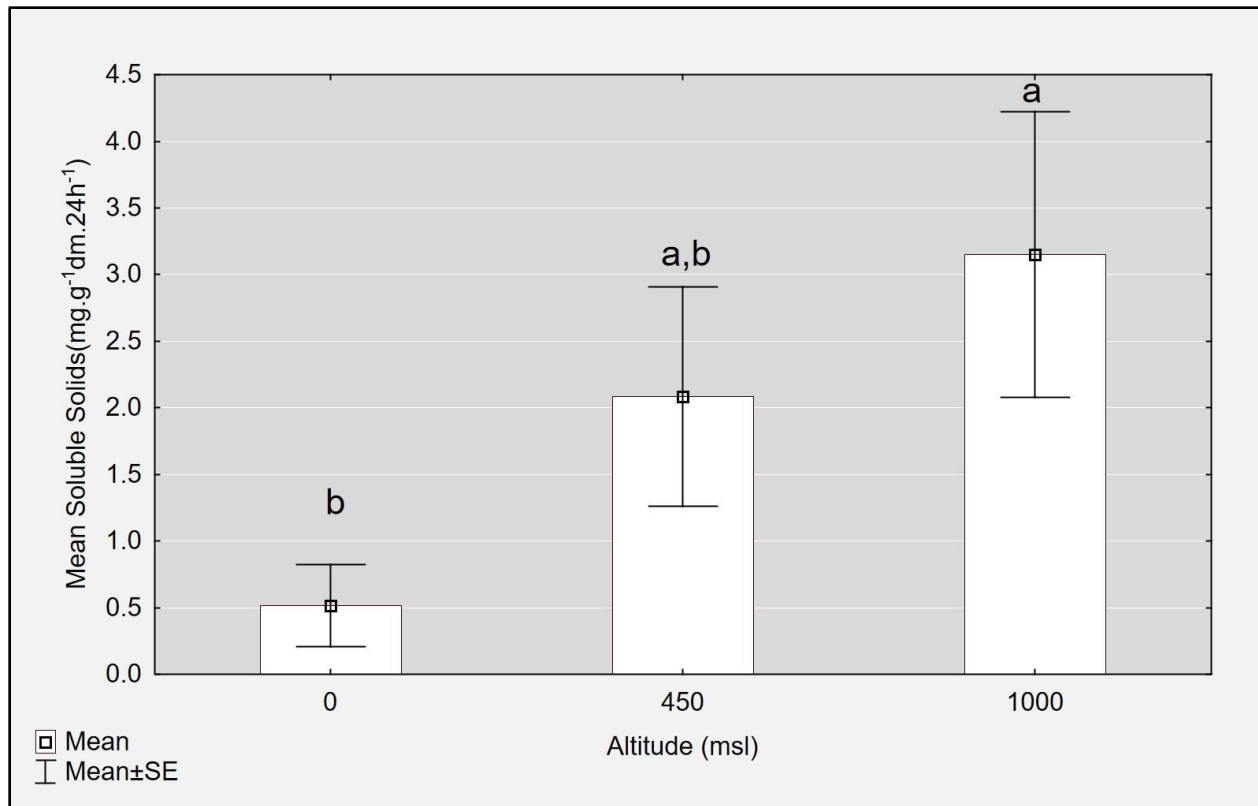


Figure 14. After the rainy season (December 2015), mean soluble solids in EFN were positively correlated with altitude (N=27 total plants); plants at the highest elevation produced in average more soluble solid mass per leaf dry mass than plants at sea level (n=9 plants per site). Values in mg of soluble solids per g of leaf dry mass and day, altitude in meters above mean sea level. Whiskers correspond to standard error; letters indicate significant differences between sites.

5.6. Plant Direct Chemical Defenses

In the feeding experiment, *S. frugiperda* caterpillars that fed on food with leaves from different altitudes showed similar values in mass gain after three days. On days 6 and 10, larvae from the control group had gained significantly more mass than caterpillars feeding on food with *Acacia* leaves ($p < 0.01$, according to Kruskal-Wallis ANOVA, n=15 for all groups). However, this effect was partly reversed over the last 4 days of treatment, during which larvae fed with leaves from the highest elevation reached a mass that showed no

statistical difference ($p = 1.0$, post-hoc Dunn's test, $n=6$ for control group, $n=15$ for treatment group 3) to that of the control group (Figure 15).

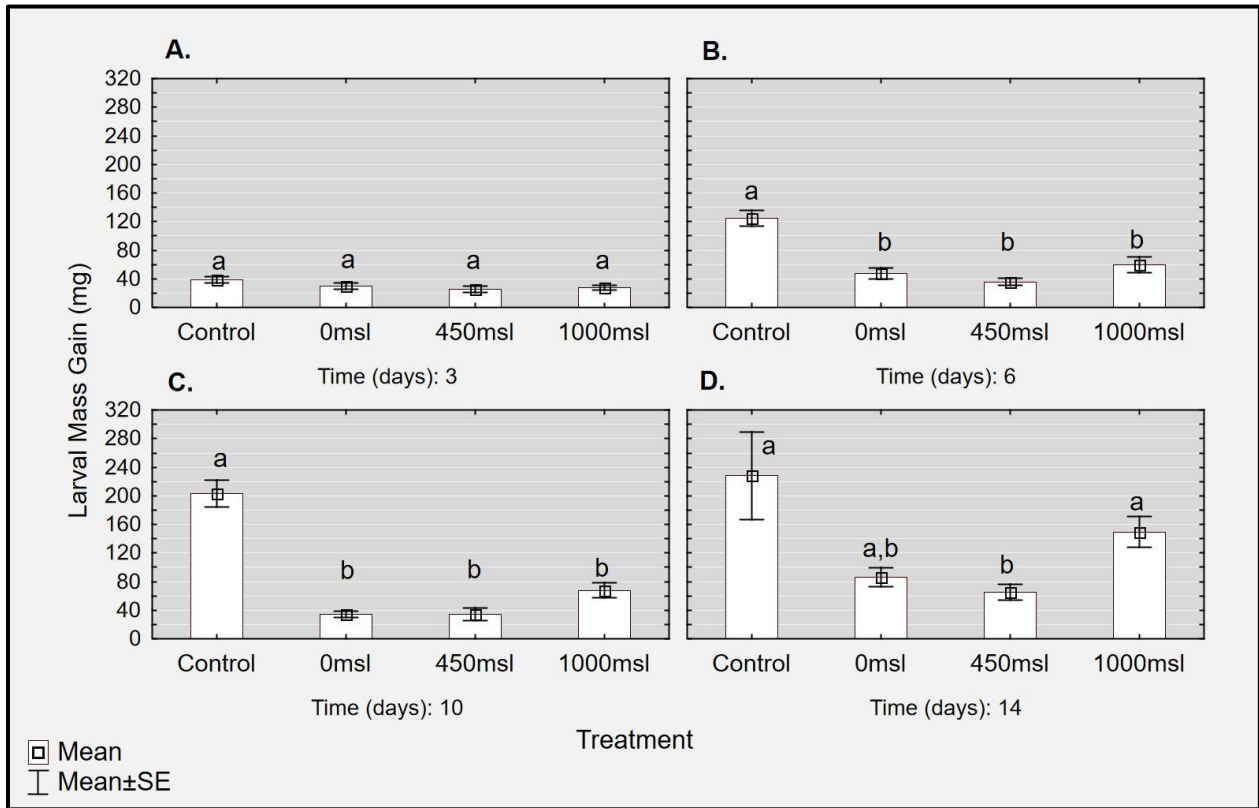


Figure 15. *Spodoptera frugiperda* larvae treated with nutrient rich food mixed with ground dry *A. hindsii* leaves collected at 1000msl gained mass (in mg) statistically similar to the control group after 14 days of treatment ($n=6$ larvae for the control group, $n=15$ larvae treated with 1000msl leaves). Whiskers correspond to standard error; letters indicate significant differences among groups.

As for food intake, larvae subjected to the different treatments responded in a similar way (Figure 16): reduced food intake during the first 10 days of the experiment ($p < 0.05$, post-hoc Dunn's test, $n=15$ larvae per group). Larvae fed with leaves collected at 1000msl showed an increase in food consumption over the last 4 days, attaining a food consumption level that was not statistically different from that of the control group ($p = 0.826$, according to post-hoc Dunn's test, $n=15$ larvae in each group). It is worth

mentioning that only the control group achieved pupation and survival to adult stage, all larvae subjected to treatment failed to pupate and perished.

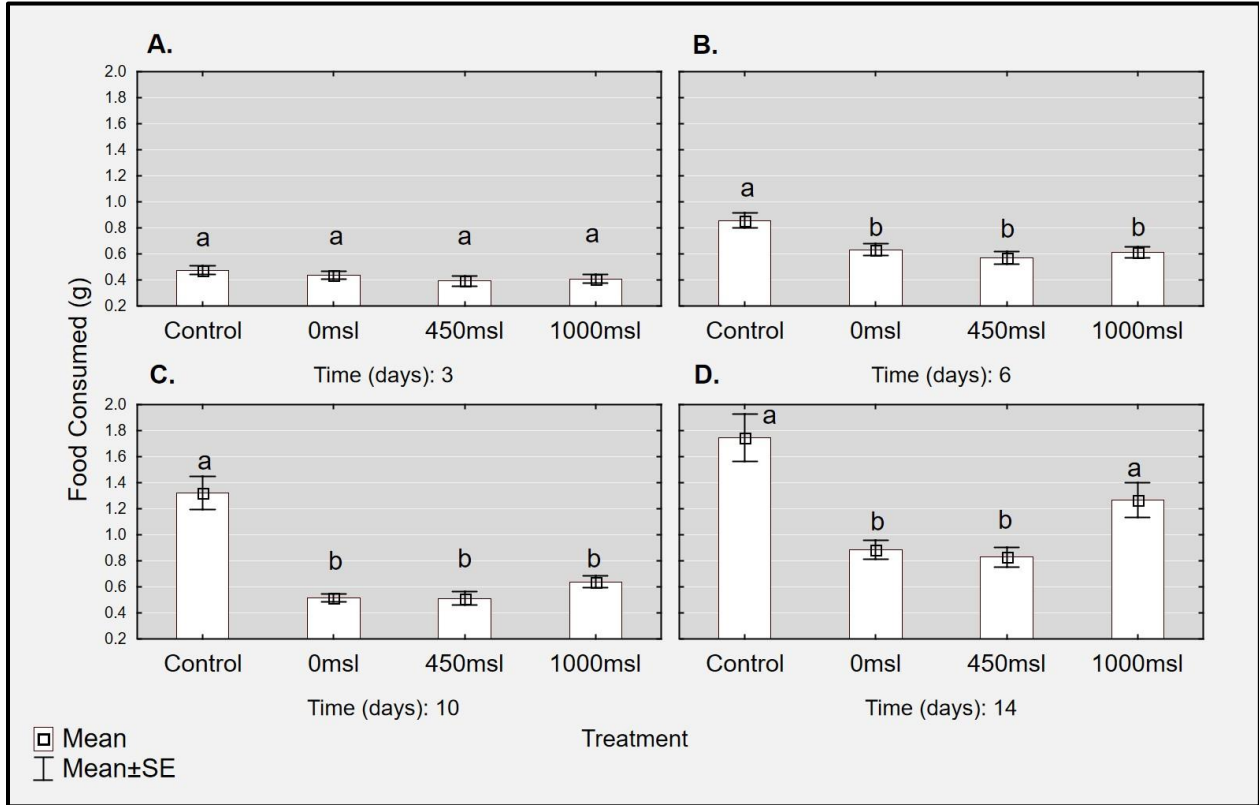


Figure 16. *Spodoptera frugiperda* larvae treated with nutrient rich food mixed with ground dry *A. hindsii* leaves collected at 1000msl consumed food (in g) in statistically similar amounts to the control group after 14 days of treatment (n=15 larvae in each group). Whiskers correspond to standard error; letters indicate significant differences among groups.

6. DISCUSSION

The environmental condition in the tropics are favorable for a great number of species to share the same space, benefitting the evolution of interactions that take advantage of this diversity. This phenomenon has been proposed as the reason mutualisms are centered in the tropics (Boucher *et al.* 1982; Schemske *et al.* 2009). The evolution of defensive ant-plant mutualisms is thought to be a response to the high herbivory rates in warm and humid habitats and to the increased competition in dense tropical forests for resources such as sunlight. In contrast to the four seasons that characterize the climate at more temperate latitudes, tropical climates are characterized by dry and wet seasons and generally lower differences in temperature between these seasons. According to several authors, this long-term environmental stability caused organisms to evolve a low tolerance to changes in their surroundings, a scenario that would predict the dissolution of mutualisms when changes occur (Stevens 1989, 1992; Kiers *et al.* 2010). In the context of the present study, changing environmental conditions might make the production of food rewards by ants-plants costlier for the plant or less stable over the course of the year and thereby negatively affect the functioning of the mutualism.

In practice, it is difficult to follow latitudinal changes in the traits of organisms due to the large distances that must be covered in this type of study. However, similar changes in environment, species diversity and richness as well as distribution can be observed along altitudinal gradients (Janzen *et al.* 1976; Stevens 1992; Sanders 2002). In this study, we made use of this fact and tested over an altitudinal gradient our hypothesis that the limitation of the mutualism between *A. hindsii* and *P. ferrugineus* to lower altitudes is caused by negative effects of the environmental changes at higher altitudes that should

be reflected by differential FB and nectar production at different altitudes and corresponding changes in the behavior of the ant partner.

Thus, the study is focused on the efficiency of the ants' defensive services and the food provisioning by the plant and tested whether the mutualism may be suffering from changes in nectar soluble solids concentration or the number, mass or quality of the FBs produced. However, in summary, the results do not support the mutualism breakdown hypothesis: all plants growing at higher altitudes were occupied by the most efficient mutualistic ant (*P. ferrugineus*), even at the *Acacia*'s elevation limits, we detected no tendency towards changes in the defense provided by ants. Interestingly, the presence of efficient chemical defenses in the *Acacia* leaves at all levels became obvious in terms of reduced growth and survival rate of caterpillars in the feeding experiment. A tendency towards reduced chemical defenses can be observed in terms of significantly higher food consumption at the highest altitude, statistically similar to the control group. However, the difference between the lowest and the highest site was not significant for the final weight gained, which makes this pattern difficult to interpret; and the amount, mass or quality of FBs was not affected by altitudinal changes, despite strong differences between the dry and the wet season. The only significant correlation with altitude was detected for the nectar secretion measured after the wet season.

6.1. Effects of Altitude on *Pseudomyrmex* spp. Ants

Mutualistic *Pseudomyrmex* ants were found throughout the studied altitudinal gradient up to 1000 msl while *Acacia* plants were available.

All the predicted species were present at lower altitudes as well as other non-mutualistic species during the studied timeframes. More than half of censused *A. hindsii* were inhabited by *P. ferrugineus* at all altitudes, with *P. mixtecus* and *P. peperii* living in few plants at low and intermediate altitudes, being entirely absent at high altitudes (Figure 5, Figure 6).

P. mixtecus were more often found at sea level and on certain plants that had their leaves covered in dust from the road. Since the road is highly transited, lack of attendance by *P. mixtecus* is not likely the source of dust accumulation. A possible explanation for only this species to be found on these plants is that dust-covered *A. hindsii* are selected against by other mutualistic ants. Dust most likely renders photosynthesis more difficult, as it blocks out sunlight for the leaves, this would cause lower levels of EFN since the sugary secretion is dependent on the plant's photosynthetic products. Another explanation is that dust covers the nectaries making nectar consumption by the ants difficult. In any of these proposed scenarios, *P. mixtecus* would be more tolerant to low-nectar conditions than other *Pseudomyrmex* mutualists.

P. peperii was found only at intermediate altitudes inhabiting plants that were all adjacent to one another, forming a continuous strip of land filled with only *A. hindsii* inhabited exclusively by *P. peperii*. During dry season, there was only one such area found in transect 2. After rainy season, one was observed in transect 2 and another in transect 3. For each transect, no more *P. peperii* were found on other *A. hindsii* plants. This is the same phenomenon observed by Kautz *et al.* (2009b), where clusters of host plants bearing *P. peperii* were found. The study found that these clusters were supercolonies of closely related ants. The authors concluded that intranidal mating was occurring between

males and daughter-queens derived of the original colony. The establishment of supercolonies would compensate the species' inferior capacity for colony establishment when competing with more successful mutualists as *P. ferrugineus*. This strategy appears to be efficient at intermediate altitudes since *P. peperii* clusters were composed by a higher proportion of plants than those inhabited by *P. mixtecus*, even when the latter has been shown to be a better colony founder (Kautz *et al.* 2009b).

P. ferrugineus stands out amongst the other species since it always controls more than 50% of the *A. hindsii* plants at all altitudes, occupying 100% of them during dry season at the highest altitude. Furthermore, they exhibit an efficient defense response regardless of the plant's location on the mountain range as shown by the significant increase in patrolling workers after damage was made to the plant (Figure 7). This indicates that the ants' response to threats, one of its main roles on the mutualism, is not being impeded by living in high altitudes.

Mean worker size in *P. ferrugineus* remained constant between lowest and highest elevation (Figure 8). Adult size in most insects, and particularly in ants, is directly related to the food quality that was received in larval state (Ezhikov 1934; Smith 1942, 1944). Since chitin exoskeletons are rigid and ants do not molt, their adult form is fixed after emerging from pupation. Usually very young colonies produce smaller workers (Janzen 1967). However, in a mature colony, adult worker size is a reflection of how well larvae are being fed. In the *A. hindsii*-*P. ferrugineus* system, this translates to how good the FBs nutritional content is. The lack of significant variation in worker size thus demonstrates that the plants produce FBs that are able to feed the larvae at *A. hindsii*'s 1000 msl altitudinal limit with the same nutritional quality as those at sea level.

Since these ant species all colonize the same host plants, and they generally do not share hosts, competition for nesting sites is very high (Clement *et al.* 2008; Heil *et al.* 2009; Kautz *et al.* 2012). In altitude, the only host plant species found is *A. hindsii* and its numbers are reduced when approaching its range limit of 1000 msl (Table 2), this would most likely further increase intra-specific competition. Pertaining to the studied system, the only reported ant species living between 900 msl and 1200 msl are *P. peperi* and *P. ferrugineus*. *P. peperi* can only compete with other species through the establishment of supercolonies, as stated before. These clusters are dependent on a queen to found and sustain a colony before branching out to other nearby plants (Kautz *et al.* 2009b). Considering *P. ferrugineus* aggressiveness and success when colonizing new plants, as well as the low resources offered by *A. hindsii* in comparison to High-Reward hosts like *A. cornigera* and the low amount of host plant availability at high elevations, it is highly possible that the high occupancy of *P. ferrugineus* at high altitude is caused by it outcompeting *P. peperi* in response to an environment with less host plants available for colonization. Additionally, the low proportion of unoccupied plants also indicates that the *P. ferrugineus* population at that altitude is enough to colonize almost every plant and defend them against opportunist ants and parasites of the mutualism.

Table 2. Number of *A. hindsii* censused on 3km transects at different altitudes (a) during dry season, and (b) after rainy season.

a)	Mean Transect Altitude (msl)	Number of <i>A.</i> <i>hindsii</i>	b)	Mean Transect Altitude (msl)	Number of <i>A.</i> <i>hindsii</i>
	0	63		0	55
	440	90		320	49
	740	50		550	52
	900	18		790	52
	920	14		920	35

There are reports of ant-bearing *A. hindsii* surpassing 1200 msl in certain urban locations in Central America (Ward 1993). However, there were no *A. hindsii* growing past 1000 msl at the studied site. The few *A. hindsii* found between 920 msl and 1000 msl live in the ecotone between tropical dry forest and the pine-oak forest. It is a known phenomenon that pine needles inhibit seedling growth (Nektarios *et al.* 2005). This, as well as the ant occupancy data (Figure 5, Figure 6), suggest that the plant availability is the limiting factor in expanding the mutualism's range on the Sierra Madre Sur, Oaxaca.

As opposed to what was hypothesized, the mutualism does not break down, and remains very stable throughout the elevational gradient. Myrmecophytic *Acacia* were once restricted to the disturbed lands adjacent to rivers, they became widely distributed with man-made deforestation and habitat disturbance (Janzen 1974). It is possible that this same type of disturbance is what has allowed *A. hindsii*-*Pseudomyrmex* spp. mutualism to colonize higher altitudinal levels in other locations.

6.2. Food Body production

The number and dry mass of FB produced by the plant in two and a half weeks show no significant correlation with the increase in altitude during dry and after wet season. However, it is worth mentioning that during dry season there is a visible trend for the FB production to decrease in higher locations, in mass and number of FB (Figure 9) as well as in protein content (Figure 11). After wet season, there is no discernable pattern in the amount produced or their mass (Figure 10), however there appears to be higher protein

contents in higher locations (Figure 12). It is possible that intraspecific variations between sampled individuals manage to mask a seasonal phenomenon that may be observable with repeated sampling in consecutive years. Since FB are mainly composed of proteins (Heil *et al.* 2004), which are rich in nitrogen, and *Acacia hindsii* flower during dry season (Janzen 1974), this may suggest plants divert resources to reproduction when in altitude at this time of the year, while after rainy season the plant invests more resources promoting ant colony growth.

Food body production often reflects the status and structure of the hosted ant colony. In *Macaranga*, plants producing more FB bear bigger *Crematogaster* colonies (Heil *et al.* 1998, 2001a, 2001b). For *Cecropia* plants growing in secluded or remote locations deprived of herbivores, FB production decreases or stops altogether (Janzen 1973; Johnson *et al.* 1992; Fáveri & Vasconcelos 2004) making the establishment of an *Azteca* colony more difficult. Additionally, in both these cases where the plants can define their partners' colony size, the ants are not entirely dependent on such reward as it is the case for the *Pseudomyrmex ferrugineus* group in *Acacia hindsii* (Janzen 1967). Furthermore, in these other systems FBs are used to feed the adult ants as opposed to *Acacia* FB being used to exclusively feed the larvae (Janzen 1966; Janzen 1967; Heil *et al.* 2004), suggesting that a significant decrease in FB production not only would limit colony growth more severely than in other similar mutualistic systems, but could potentially impede colony establishment. As the production of FBs is statistically without change throughout the altitudinal gradient during dry and wet seasons, it is clear that the *A. hindsii* plants do not need to limit the ant colonies' size even in high altitudes and that the plant keeps relying on the effectiveness of mutualism even in harsher conditions.

6.3. Soluble Solids in Nectar

In a similar fashion, soluble solids in nectar do not significantly correlate with altitude during the dry season, even when there appears to be a trend of their amount irregularly decreasing with the study sites' height (Figure 13). The irregular pattern is explained by the proximity of agricultural settlements to the 450 msl and 850 msl fields and the possible incidence of pesticides on the *A. hindsii* plants during their development.

The decreasing trend itself may be related to the plants' flowering just as suggested for the FBs. Since nectar is primarily composed of carbohydrates (Heil *et al.* 2005, Kautz *et al.* 2009a), it requires an important carbon investment that the plant could be using for growth or the development of reproductive structures. In this case, it may be that plants in greater altitude divert carbon resources towards reproduction instead of nectar secretions when needed. However, further observations are necessary to ascertain if the trend is seasonal or just a random occurrence.

More interestingly, soluble solid concentrations in nectar are significantly correlated with altitude after the wet season (Figure 14). Higher EFN secretion in the *Acacia-Pseudomyrmex* system has been found to increase the ants' activity and defensive response (González-Teuber *et al.* 2012). The soluble solids concentrations are lower after rainy season than those found during the dry season, which is consistent with previous reports (González-Teuber *et al.* 2012), but the increase with altitude suggests that plants higher up on the mountain are prompting their ants to increase their defense. This could

be as a way to avoid being overrun with vines or other competing plants as their growth rate increases after rainy season.

6.4. Plant Direct Chemical Defenses

Spodoptera frugiperda is a nocturnal lepidopteran originating in the tropics and subtropics. Its larval form, called “fall army worm”, is a generalist caterpillar considered a pest for several important crops, corn among them. Larvae undergo 6 instars before pupating underground, this usually takes approximately 4 weeks. Fall army worms and *Acacia* myrmecophytes do not co-exist in nature, which means the caterpillar would not have natural ways to bypass possible chemical defenses and, as a generalist, it is not limited to a specific host plant.

The artificial diet used to feed *Spodoptera frugiperda* larvae is widely used in pest control laboratories in order to nutritionally sustain the caterpillars for research purposes (Greene *et al.* 1976).

Adding ground *A. hindsii* leaves to this mixture ensures not only that the larvae are still receiving the optimum amount of nutrients but also that the only observed effect comes from the chemical contents of the leaves. Any physical defense that could interfere with larval development, like a specialized cuticle or a particular texture is lost when the samples are ground (Heil *et al.* 2002).

Control group larvae consumed increasing amounts of food during their 14-day development and gained mass in a similar manner. Larvae that were treated with *A. hindsii* leaves started showing significantly reduced mass gain compared to controls after

six days of treatment, the trend continued after 10 days; during this same span of time, the treated larvae consumed significantly less food (Figure 15).

This phenomenon makes evident the existence of a compound inside *A. hindsii* leaves that make the food uneatable for *S. frugiperda*. Previous experiments show that *A. hindsii* phenolic compounds such as tannins and flavonoids while present in the leaves, do not show significant differences with the quantities found in other myrmecophytic and non-myrmecophytic Mimosoideae plants. Essays with the caterpillar *S. littoralis* also showed reduced larval growth when fed with leaf extract from different *Acacia* plants, however it was concluded that the biological effect on the caterpillars was likely not due to the phenolic compounds quantified during that experiment, suggesting other substances present on the leaves are responsible for growth inhibition (Heil *et al.* 2002).

According to Heil *et al.* (2000b), myrmecophytic *Acacia* have low levels of chitinases, which are hydrolytic enzymes that break down chitin, the main polysaccharide compound in insect's exoskeletons, so its influence on larval behavior is unlikely.

However, it has been shown that proteinase inhibitors are present in *A. hindsii* FBs as a defense mechanism to keep herbivores and non-mutualist organisms from stealing the mutualists' nourishment (Orona-Tamayo *et al.* 2013). The FBs are modified leaf tissue, it is possible the same proteinase inhibitors present in the FB also exist in the leaves. Certain proteinase inhibitors have also been found in leaves of related non-myrmecophytic *A. nilotica* (Babu *et al.* 2012). Furthermore, the digestive disruption observed in Lepidopteran *Helicoverpa armigera* treated with proteinase inhibitors is consistent with the effects observed during the feeding experiment with *S. frugiperda*

larvae (Babu *et al.*, 2012, Gadge *et al.*, 2015), suggesting this could be causing the growth and feeding decrease in treated caterpillars.

Additionally, there are reports of cyanogenic glycosides present in a wide variety of plants that serve as deterrents against herbivores, making leaves uneatable by releasing HCN which is toxic to most generalists (Vetter 2000; Møller 2010). In a study that tested several myrmecophytic *Acacia* species for cyanogenic glycosides, the authors found that three out of twelve species had cyanogenic activity. Among these, *A. hindsii* specimens native to Mexico had the compound present in its leaves (Seigler & Ebinger 1987).

It is then proposed that the lower food consumption by *S. frugiperda* fed with *A. hindsii* leaves could be related to the presence of deterrent chemical compounds in the nutritional preparation. Taking this into account, it is important to notice that food consumption increased significantly for larvae being fed leaves from 1000 msl to levels statistically similar to controls in the last four days of treatment (Figure 16). For the control group, this high food consumption coincides with the days prior to pupation. This leaves few possibilities for explaining that larvae treated with 1000 msl leaves were able to feed as much as the control larvae: either treated caterpillars could tolerate consuming the deterrent compound or the chemical's concentration was lower than in other treatment groups. Since the other treated larvae continued the trend of having low levels of food intake, it is unlikely that this group spontaneously developed tolerance to the compound present in leaves.

Despite these observations, the chemical defenses present in the leaves remain extremely efficient: none of the treated larvae survived. Furthermore, there was no significant difference in growth between larvae feeding on leaves from sea level and those

feeding on leaves from the highest altitude. This makes it difficult to interpret the pattern, since on the one hand there seems to be a reduction in the deterrent effect allowing the larvae to feed in higher quantities, but on the other hand, there is still the same growth inhibition and lethal effect in all altitude levels.

Thus, even though these results are not conclusive enough to demonstrate a change in the chemical composition of the leaves, there is a trend that suggests leaves in high altitude may contain less deterrent chemicals than in lower or intermediate altitudes, and previous studies point towards these compounds being cyanogenic glycosides or proteinase inhibitors.

While it is widely accepted that myrmecophytic plants possess little to no chemical defenses against herbivores owing to the ant's efficient defense (Rehr *et al.* 1973; Seigler & Ebinger 1987; Heil *et al.* 2000a, 2000b), *A. hindsii* is considered to be a Low-Reward myrmecophyte. This means its investment on mutualistic food rewards is inferior to High-Reward plants such as *A. cornigera* (Heil *et al.* 2009). It is then possible for the studied species to balance chemical and biotic defenses, even if maintaining both may appear redundant. In this case, a possible reduction in herbivore deterrent effect at the highest elevation may suggest reduced investment in chemical direct defenses by a Low-Reward mutualist, potentially in favor of indirect biotic defenses when growing in a harsher environment. In order to fully understand this phenomenon, future studies focusing on altitude influence on leaf chemical composition are needed.

7. CONCLUSIONS

As mutualisms are considered to be unstable interactions and hypothesized to break when faced with environmental change, the study focused on finding evidence of this mutualism breakdown by comparing the production of food rewards at different altitudes and observing the resident ants' behavior. It is now evident that the mutualism breakdown hypothesis had to be rejected in this case in light that the EFN and FBs that constitute food rewards from *Acacia hindsii* are not affected by altitude, nor is the *Pseudomyrmex ferrugineus* ants' defensive service or colonization capabilities.

Even though results on altitude influence on leaves' chemical defenses are inconclusive, there is an observable trend towards diminished chemical defenses at high altitudes. Future studies focusing on the analysis of the leaves' chemical composition and its changes, likely by making use of specialized techniques such as Gas Chromatography and Mass Spectrometry, are required to further understand the *A. hindsii*-*P. ferrugineus* group's mutualism and its adaptation to harsh environments.

Contrary to the initial hypothesis, the mutualism between *A. hindsii* and the *P. ferrugineus* group did not dissolve. Unexpectedly, the mutualism was maintained in the altitudinal survival limit of the plant with virtually no change in the interaction. Thus, mutualisms often thought to be fragile or unstable, reveal to be much more resilient to environmental change.

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