

**Micro-geographic variation in the *Cecropia-Azteca* pair-wise association as shown
through herbivory levels, light environment, and stable isotope analysis**

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Table of Contents

Acknowledgements 2
Abstract..... 4
Introduction..... 5
Materials and Methods..... 8
Results 12
Discussion 13
Literature Cited 18
Tables and Figures..... 22

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Abstract

Mutualisms, biological interactions in which both involved species benefit, have been widely studied. It is known that the quality of mutualisms may vary spatially and temporally, and that environmental variation can affect the ecological benefits and costs of species interactions. Since environmental properties may influence the evolutionary course of these highly conditional pair-wise interactions, how the environment affects these interactions has become an important question in the study of mutualisms. This project looked at the effects of certain environmental variations using a common tropical ant-plant association, the interaction between *Cecropia* trees and *Azteca* ants. *Azteca* ants live in *Cecropia*'s hollow stems. Plants provide food and domatia and, in turn, ants defend their host against insects that might damage them. In this project, the amount to which *Azteca* rely on *Cecropia* for food in different light environments was assessed using stable isotope analysis in the University of Arkansas Stable Isotope Laboratory. By assessing the diet of *Azteca* in different light environments, we can better understand the conditional nature of mutualisms and explore their evolutionary and ecological histories. Leaf tissues of *Cecropia* spp. (Urticaceae) and four species of symbiotic ants were collected at the Organization for Tropical Studies' La Selva Biological Station, Heredia Province, Costa Rica, in May 2009. ImageJ® software was used to analyze the relative amount of herbivory on each host plant. Each species of *Azteca* associated with a *Cecropia* tree was identified. The amount of light available to each tree was qualitatively assessed, and measured the height of each sample. The study found that *Cecropia* plants

with active ant colonies had lower levels of herbivory than did plants without ants, though light environment had no effect on herbivory levels. The species of ant present on *Cecropia* had no effect on herbivory levels. Different species of ant were not uniquely present at different heights. Stable isotope analysis showed that while ants did not contribute carbon or nitrogen to their hosts, overall carbon and nitrogen concentrations were higher in sunny environments. Ants were relying on sources besides the trees for their nutrients and that light environment influenced plant-water relations. These results contribute to a greater understanding of insect-plant mutualisms regarding micro-geographic variance.

Introduction

Mutualisms are interspecific consumer-resource interactions in which one or both individuals experience a net benefit. In the case of ant-plant associations, plants provide food and often nesting sites to the ants; the associated ants in turn provide protection from both herbivores and encroaching vegetation. Defense by symbiotic ants is an effective form of protection from herbivores; ants are highly mobile and tend to defend younger, more vulnerable leaves, thereby fulfilling a plant function usually achieved through chemical defenses (Heil et al. 2000). Historically, there were two well-formulated views regarding plants' necessity of their associated ants. Over a century ago, Belt (1874) and Müller (1874, 1876) hypothesized that the saplings of many tree species benefit from protective ant interactions. The suggestion of a mutualistic relationship between ants and plants provoked a flurry of debate. Indeed, many prominent naturalists such as W.M. Wheeler described these relationships as neutral or even parasitic (Wheeler

1942). Detailed field studies and experimental work was lacking, however, until the pioneering work of Janzen (1966, 1967, 1975) reignited interest in ant-plant associations and established that plants may benefit from associations with ants. It is now understood that plants may benefit in numerous ways from ant associations: ants protect plants from herbivores and encroaching vegetation, they help disperse seeds and fruits, they can pollinate flowers, and they can feed plants essential nutrients (Beattie 1985).

Mutualistic ant-plant interactions range from species-specific, co-evolved obligate relationships to generalized, facultative associations in which one or both partners can live successfully without the other (Bronstein 1994). In some cases, such as the obligate *Acacia-Pseudomyrmex* mutualism, neither the ants nor the *Acacia* can survive without the other. The *Pseudomyrmex* aggressively defend their host trees against herbivores and epiphytes in exchange for nesting sites in the tree's specialized, hollow thorns and specialized food bodies containing protein, carbohydrate and fats (Janzen 1966). Ant-*Acacia* species have even reduced their investment in chemical defenses, implying that they rely heavily, if not exclusively, on their resident ants (Janzen 1966, Gonzalez-Sulser and Thompson 2004). In other cases, the association becomes more facultative and ambiguous. Wild cotton (*Gossypium thurberi*), for example, attracts various generalized ant species to its extrafloral nectaries (EFNs) in order to gain protection from herbivores, but when EFN production wanes, so does the aid of the ants (Rudgers 2004). This facultative system also occurs in *Ipomea carnea*; generalized ants are attracted to EFNs in order to gain protection from herbivores, but the ants are not associated with the plant in other ways that are common in obligate systems (Keeler 1977).

The *Cecropia-Azteca* association is one of the most conspicuous ant-plant mutualisms in the neotropics (Longino 1989). The hollow internodes of *Cecropia* provide domatia for *Azteca*, and Müllerian bodies on trichilia at the base of the petioles provide food. *Azteca* are known to protect the *Cecropia* from herbivores and encroaching epiphytes (Janzen 1969, Shupp 1986, Heil et al. 2000). Some species of *Azteca* are more aggressive than others; in many cases, a slight shake will cause the ants to swarm up and down the tree looking for potential threats, whereas other species will not come to tuna baits or react to violent blows to the tree (Longino 1991, pers. obs.). Effects of spatial conditionality on mutualisms can be seen in some *Cecropia* species in Puerto Rico and most other Caribbean islands. These *Cecropia* are not occupied by ants, and the plants no longer produce the trichilia that make the glycogen-rich Müllerian bodies which feed the ants, as is common with *Cecropia* on the Neotropical mainland (Janzen 1973). Habitat-related variables may also influence herbivory levels and the success of *Cecropia-Azteca* interactions once they have been established (Bronstein 1998).

Mutualistic interactions between species can be temporally or spatially variable, creating a gradient in conferred benefits for participants from strong mutualists to antagonists (Bronstein 1994). Abiotic factors such as light availability have been shown to condition the outcome of ant-plant interactions (Kersch and Fonseca 2005), and in *Cecropia*, higher light intensity increases the production of Müllerian bodies and the levels of condensed tannins and total phenolics (Folgarait and Davidson 1994). When a *Cecropia* produces more food bodies, the inhabiting ant colony is able to benefit more from those nutrients and may be less likely to rely on other sources of food to survive. Using stable isotope analysis, we can determine how much of the ants' diets are coming

from the host plant itself. As *Azteca* ants are completely arboreal, they are only able to get their nutrients from the host plant and from insects that land on or climb up the trees. By assessing the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ levels in ants inhabiting *Cecropia* in different light environments, we can determine whether variance in this pair-wise association has an effect on the importance of *Cecropia* to their inhabiting ants, and vice-versa. Carbon and nitrogen exist in two stable (non-radioactive) forms: ^{12}C , ^{13}C , ^{14}N , and ^{15}N . Stable isotope analysis assesses the ratio of the amounts of the relatively heavier isotopes to the amounts of the lighter isotopes, and can be used to determine individual diet sources and the extent of the interdependence of the association.

This project was designed to explore the following questions in order to better understand the *Cecropia-Azteca* mutualism:

- What are the benefits of ant association?
- Do costs and benefits differ between light environments?

The results of this study will ultimately contribute to a greater understanding of insect-plant mutualisms as regards to micro-geographic variance, and will shed light on the evolution of cooperation as a whole.

Materials and Methods

From April 27-30, 2009, leaves from *Cecropia* trees and their inhabiting ants, if present, were collected at the Organization for Tropical Studies' La Selva Biological Station, Heredia Province, Costa Rica (10° 26' N, 83° 59' W). Juvenile *Cecropia* trees and their affiliated ants were sampled from secondary forest in and around the abandoned Huertos project plots along the Sendero Tres Rios, Sendero Occidental, and Sendero Las

Vegas trails of the La Selva station. The Huertos project was a long-term agroforestry project in which chemical supplements, particularly nitrogen, were added in different concentrations to each plot (Tillberg 2004). The Huertos project ended in 2002-2003, and since then *Cecropia* and other pioneer species have colonized the plots. *Cecropias* in these plots were oftentimes clustered in groups of 2-4, and were usually located between two and thirty meters from the cleared trails.

To determine whether the presence or absence of ants has fitness consequences for *Cecropia*, 34 juvenile *Cecropia obtusifolia* and 7 *Cecropia insignis* 1 m to 6 m tall were sampled. For each individual, the height to the top of the youngest node was calculated using a Suunto® clinometer and a meter tape. The potential sunlight availability in the immediate area above the individual tree (sunny, shady) was qualitatively assessed based on the amount of canopy over the trees.

The presence or absence of ant colonies on each tree was determined by extensive shaking and tapping of the tree and was recorded. If ants were present on a tree, samples were placed in Eppendorf tubes containing 95% ethanol for later identification by R. Vargas at La Selva. Four randomly selected juvenile *C. obtusifolias* that were determined to not have ants were cut open to make sure there was no colony present.

To assess herbivory, the second and third leaves from the top of each tree were collected and taken back to the La Selva laboratory. The first, and therefore newest, leaves were not collected because they would not have had adequate time to experience herbivory; similarly, older leaves were not collected because they would have displayed older, non-contemporary herbivory. Leaves were photographed against a white background using a Nikon D200 DSLR camera, with manual settings of 1/125, f/3.5,

ISO-320, and on-board flash, under fluorescent lighting from a distance of approximately 1.75 m and saved as JPEG files. ImageJ 1.41o software (National Institutes of Health 2009) was used to trace the leaves at 4x magnification to measure the total area of each leaf as well as the total leaf area eaten. For each tree, measurements from leaves two and three were combined, and used to calculate percent herbivory for the tree. Leaf and ant samples were then placed in drier ovens at 50°C for 48 hours and then sealed in plastic bags in preparation to be transported to the University of Arkansas by C. Sagers.

Stable isotope analysis was conducted in the University of Arkansas Stable Isotope Lab (UASIL) from March 2010 to June 2010. Leaf samples were freeze-dried, homogenized by grinding, and weighed in tin capsules. Ant samples were leached in 99% ethanol to remove lipids (see DeNiro and Epstein 1978 and Post et al. 2007), freeze dried, and then weighed into tin capsules. Samples were analyzed at UASIL with a Finnigan Delta+ isotope ratio mass spectrometer. Isotopic analysis requires very precise analytical techniques. It has been found that measuring absolute isotopic composition is not as reliable or convenient as measuring the isotopic differences between a given standard and the sample in question. Assessing the differences between samples and defined laboratory standards over a shorter period of time provides higher precision and repeatability than assessing absolute values alone due to drift over time in mass spectrometry instruments (Ehleringer et al. 1986, Ehleringer and Osmond 1989). Isotopic ratios are therefore expressed as “per mil” (‰) deviations from an arbitrary standard, such that

$$\delta = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \times 1000\text{‰}$$

where R is the absolute isotopic ratio, and R_{sample} and R_{standard} are the molar ratios of the heavier to the lighter isotope of the sample and standard, (Sagers et al. 2000, Trimble and Sagers 2004). The $\delta^{13}\text{C}$ of whole plant tissues depends on both the $\delta^{13}\text{C}$ of the source CO_2 and discrimination during carbon fixation. The carbon isotopic composition of whole animal tissues closely reflects the isotopic ratio of food eaten, with a slight (0.5-1‰) enrichment at each trophic level. The $\delta^{15}\text{N}$ of whole plant tissues typically reflects the $\delta^{15}\text{N}$ of the nitrogen source. The nitrogen isotopic composition of whole animal tissues reflects the isotopic ratio of food eaten with a substantial ^{15}N enrichment (3-5‰) at each trophic level.

A Stepwise Multiple Regression in the forward direction with a probability to enter of 0.25 and a probability to leave of 0.1 was used to evaluate the presence/absence of ants, tree height, the type of herbivory, and the relative light levels as potential determinates of percent herbivory for *C. obtusifolia* individuals. As a result, a Mixed Model ANOVA was constructed with ant presence/absence and tree height as explanatory variables. A post-hoc Wilcoxon Sign-Rank test and a simple linear regression were used to evaluate the effect of each variable separately. A Wilcoxon Sign-Rank test was used to determine whether the species of ants inhabiting the trees had an effect on the percentage of herbivory on the *C. obtusifolia* leaves. A Oneway ANOVA was run to compare: *Cecropia* leaf $\delta^{13}\text{C}/^{12}\text{C}$ to light environment; *Cecropia* leaf $\delta^{15}\text{N}/^{14}\text{N}$ to light environment; ant $\delta^{13}\text{C}/^{12}\text{C}$ by ant species; ant $\delta^{15}\text{N}/^{14}\text{N}$ by ant species; *Cecropia* leaf %C and %N by the presence and absence of ants; *Cecropia* leaf $\delta^{13}\text{C}/^{12}\text{C}$ and $\delta^{15}\text{N}/^{14}\text{N}$ by presence and absence of ants. A contingency table analysis was run to determine if *Cecropia* growing in high light environments are more likely to have ants

than *Cecropia* in shade. All statistical analyses were conducted in JMP 8.0.2 software (SAS Institute 2009).

Results

Of 34 *C. obtusifolia*, thirteen had active ant colonies: four had *Crematogaster spp.*, four had *Azteca xanthochroa*, four had *A. alfari*, and one had *A. constructor*. Of seven *C. insignis*, one was colonized by *A. constructor* (Table 1). Two of the four juvenile *C. obtusifolias* that were cut open were each found to have one *A. constructor* queen, one tree had one *A. xanthochroa* queen, and one tree had two *A. xanthochroa* queens. None of these trees had workers present and were excluded from further analysis.

Only ant presence/absence ($p = 0.16$) and tree height ($p = 0.09$) entered the ANOVA model generated by the Stepwise Multiple Regression to assess the factors that influence herbivory. Type of herbivory and light environment did not. When all *Cecropia* trees were included in the analysis, both ant presence/absence and tree height were significant determinants of percent herbivory (ANOVA: $F = 3.74$, $df = 2$, $p = 0.04$; $R^2 = 0.21$; Effects test: ant presence/absence $p = 0.05$, height $p = 0.03$). A post-hoc Wilcoxon Sign-Rank test showed that ant presence/absence was having an effect on herbivory levels ($Z = -3.1$, $p = 0.002$). A post-hoc linear regression on percent herbivory versus height showed that height was having a minor effect on herbivory levels ($F = 3.13$, $df = 1$, $p = 0.09$; $R^2 = 0.1$).

After one outlier, a tall tree with high herbivory, was excluded, tree height ceased to be a predictor of percent herbivory ($p = 0.35$; Figure 1), whereas ant presence continued to be associated with reduced levels of herbivory ($p = 0.001$; Whole Model: F

= 6.85, $df = 2$, $p = 0.004$; $R^2 = 0.33$). Height did not affect the level of herbivory but the herbivory levels were significantly lower when ant colonies were present (Figure 1).

The species of ant inhabiting *Cecropia* trees with active colonies did not have a significant effect on percent herbivory, with or without the outlier (Wilcoxon-Sign-Rank test including outlier: $\chi^2 = 2.72$, $df = 3$, $p = 0.44$. Wilcoxon Sign-Rank test excluding outlier: $\chi^2 = 3.59$, $df = 3$, $p = 0.31$. Table 1).

Light levels were found to influence plant-water relations (ANOVA: $F = 9.19$, $df = 1$, $p = 0.004$, $R^2 = 0.19$). *Cecropia* nitrogen sources were found to differ among light environments (ANOVA: $F = 11.07$, $df = 1$, $p = 0.002$, $R^2 = 0.22$). Ant species were found to rely on different sources for carbon (ANOVA: $F = 3.04$, $df = 3$, $p = 0.07$, $R^2 = 0.45$) and nitrogen (ANOVA: $F = 7.93$, $df = 3$, $p = 0.004$, $R^2 = 0.684$). Carbon (ANOVA: $F = 13.22$, $df = 1$, $p = 0.0008$, $R^2 = 0.25$) and nitrogen (ANOVA: $F = 8.26$, $df = 1$, $p = 0.007$, $R^2 = 0.18$) concentrations in *Cecropia* were found to differ between light environments. Ants were not found to contribute carbon (ANOVA: $F = 2.12$, $df = 1$, $p = 0.15$, $R^2 = 0.05$) or nitrogen (ANOVA: $F = 2.95$, $df = 1$, $p = 0.09$, $R^2 = 0.07$) to their hosts. *Cecropia* in sunny environments were no more likely to have ants than those in shade (Contingency Table Likelihood Ratio: $\chi^2 = 2.33$, $df = 1$, $p = 0.13$, $R^2 = 0.04$).

Discussion

Interspecific mutualisms are interactions in which two species experience higher fitness when they occur together than when they occur independently (Bronstein 1998). Protective ant-plant interactions have been shown to benefit both the myrmecophytic plant and its specialized ants.

As expected, *Cecropia* with active ant colonies had significantly lower levels of herbivory than trees without ants. Protection continues to be one of the benefits conferred by ants on myrmecophytic hosts, and has been shown in numerous studies (Bronstein 1998, Heil and McKey 2003, Janzen 1966, Janzen 1969, Schupp 1986).

Regarding herbivory, however, light environment seemed to have no significant effect. The qualitative assessment of available light—sunny and shady—might not have been accurate enough to show any effect of available light on ant activity in the *Cecropias*, which might explain why the results were non-significant. Most of the *Cecropia* (N = 26) were in shady lighting, whereas only twelve were in sunny areas. Increased light has been shown to increase the production of Müllerian bodies and thus should make the tree more attractive to ants (Folgarait and Davidson 1994, Kersch and Fonseca 2005), but such a trend was not found.

Contrary to predictions, the species of ant present did not have an effect on herbivory levels in *Cecropia* either. However, four trees were found to be inhabited by *Crematogaster spp.*, which are not usually considered an obligate mutualist with *Cecropia* in Costa Rica, although they, along with other genuses (Longino 1989, Yu and Davidson 1997), are found on some *Cecropia* elsewhere such as central Amazonia (Bonato et al. 2003) and the island of Hawaii (Wetterer 1997). This study found that the *Azteca* ants were no more effective against preventing herbivory than the *Crematogaster* ants were. *Crematogaster* has previously been shown to also be aggressive when inhabiting *Cecropia* (Tillberg 2004).

While it has been shown that trees are colonized by ants at a young age, and, therefore, a shorter height (Longino 1989), the leaves being tested were young—the

second and third newest—and thus the herbivory they had experienced had to have been relatively recent compared to that of the older leaves. Taller trees should not have experienced more herbivory by default, since their ants had already been established and should have been protecting the tree since before leaves two and three appeared.

Contrary to Longino's (1989) finding that different ant species should inhabit *Cecropia* of different heights, this study did not come to similar conclusions. That said, the trees sampled in this study were only juveniles between 1m and 6m tall, which could have affected the frequency and nature of ant colonization and dominance. It can also be assumed that the trees sampled in this study were not only of different heights, but of different ages as well. If the trees had started growing at the same time but under different conditions, more significant variation in ant occupants or even antiherbivore defense might have been found (Folgarait and Davidson 1994).

Although it was found that *Cecropia* in sunny environments were no more likely to have ants than were *Cecropia* in shade, this study did conclude, through the stable isotope analysis of the carbon signature of ants, that different ant species rely on different energy and food sources. The carbon signature is often linked to carbohydrate sources for animals (Ottonetti et al. 2008, Tillberg 2004), showing that these species of ants may be obtaining their carbohydrates from different sources. Although the result was not statistically significant ($p = 0.07$, $F = 3.04$), it is more than likely biologically significant due to such a small sample size; there would be no way to detect significance even if the result were in fact significant. It was also found that the $\delta^{15}\text{N}/^{14}\text{N}$ of different ant species was significantly different, giving further evidence that the different species rely on

different sources for nutrients. This shows that different ant species do rely on different sources for energy and food, revealing variation in this ant-plant mutualism.

None of the four species of ant were found to contribute unique sources of carbon or nitrogen to their hosts. Other studies have come to the opposite conclusions, both in *Cecropia* (Sagers et al. 2000, Sagers and Goggin 2007) and with other species (Treseder et al. 1995). This calls into question the benefits other than protection that *Cecropia* obtain from their ant symbionts, at least in the La Selva region of Costa Rica.

However, overall carbon and nitrogen concentrations were found to differ significantly between light environments. Whereas the presence of ants did not differ among light environments, the fact that carbon and nitrogen concentrations increased with the increase in light levels may indicate that the plants are more likely to produce Müllerian and other food bodies and grow more quickly (Folgarait and Davidson 1994) and may affect the mutualism in other ways not measured by this study.

Light environment was found to influence plant-water relations, as $\delta^{13}\text{C}/^{12}\text{C}$ for *Cecropia* was found to be less negative in sun (Figure 2), where they are expected to experience greater water stress compared to in shade (Farquhar and Richards 1984). Water use efficiency has been shown to have an effect on stable isotope signatures of carbon, so this shows that light may have been having an effect on the isotope signatures in *Cecropia* (Martin and Thorstenson 1988). Plant nitrogen sources were also found to differ among light environments, indicating that the micro-climate has an effect on more than just carbon signatures in plants (Ehleringer et al 1986) and showing variation in the ways that plants feed ants (Fischer et al. 2002).

Leaf carbon and nitrogen concentrations were significantly different between light environments. As the trees do not seem to be acquiring carbon or nitrogen from the ants, this can be attributed directly to the amount of sunlight the trees were receiving. While many studies have shown geographic and micro-climactic variation in the extent of insect-plant mutualisms (Alonso 1998, Ehleringer et al 1986, Longino 1989, Rico-Gray et al. 1998), some even go as far as to show that high light intensity can enhance both levels of condensed tannins and phenolics—which are comprised mostly of carbon compounds, rather than nitrogen—and the production of Müllerian bodies (Folgarait and Davidson 1994).

Since environmental properties may influence the evolutionary course of these highly conditional pair-wise interactions, how the local environment affects these interactions has become an important question in the study of mutualisms. By assessing the micro-geographic and micro-climactic variation in the *Cecropia-Azteca* mutualism, this study has shown that habitat is driving at least some aspects of this pair-wise association and has added information about the evolutionary balance and development of mutualisms to the literature.

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Tables and Figures

Table 1: The species of ant present on *Cecropia* did not cause a significant difference in percent herbivory on the trees. *Azteca* ants are the genus typically associated with *Cecropia*, especially in Costa Rica, though *Crematogaster* is known to inhabit the trees as well.

Species	Number of Trees	Mean Percent Herbivory	Std Dev
<i>Crematogaster spp.</i>	4	0.0331	0.0206
<i>A. Alfari</i>	4	0.0631	0.1158
<i>A. Constructor</i>	2	0.0355	0.0517
<i>A. Xanthochroa</i>	4	0.0168	0.031

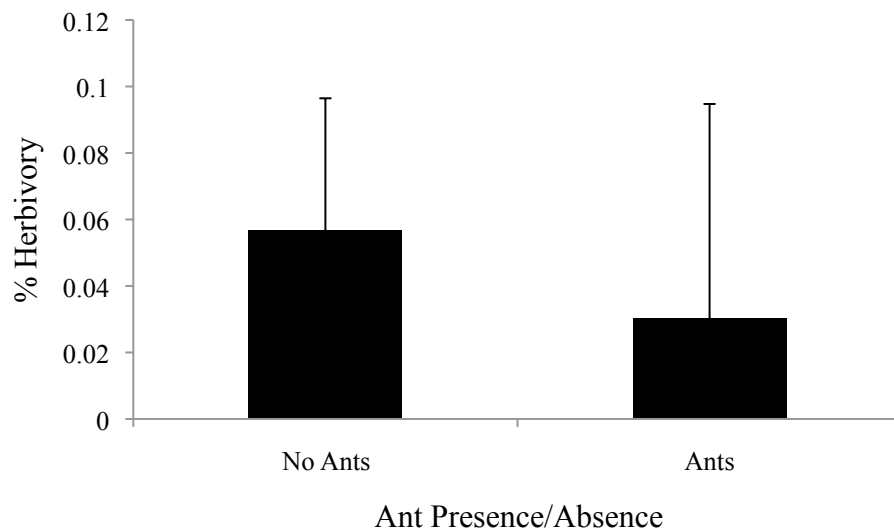


Figure 1: Comparison of herbivory levels in *Cecropia* with and without active ant colonies. Error bars represent \pm SE.

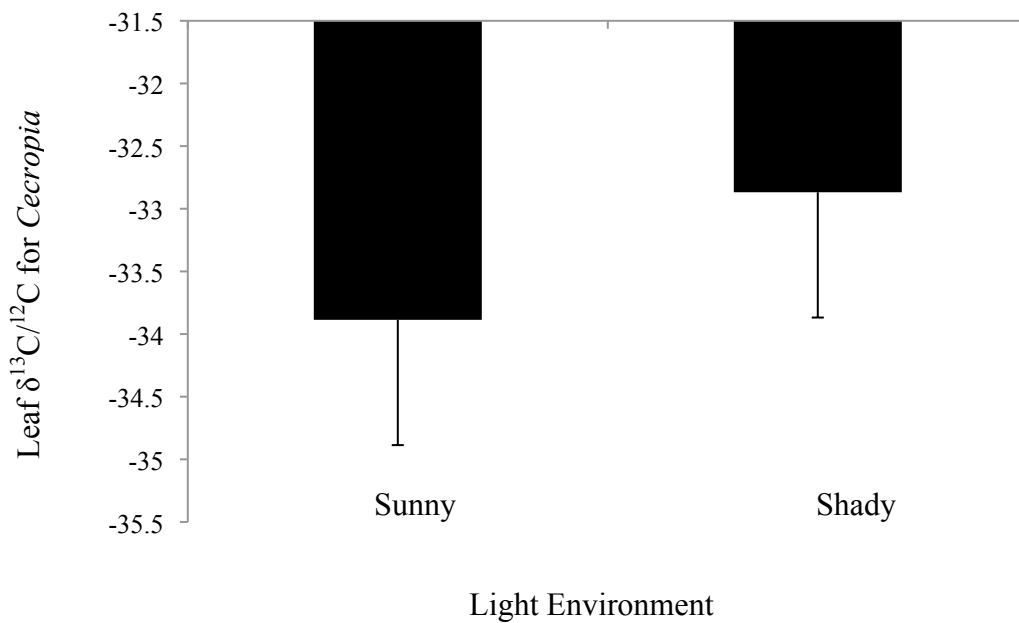


Figure 2: Difference in leaf $\delta^{13}\text{C}/^{12}\text{C}$ for *Cecropia* leaves by light environment is significant. Error bars represent \pm SE.