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Protection against herbivory in the mutualism between *Pseudomyrmex dendroicus* (Formicidae) and *Triplaris americana* (Polygonaceae)

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Abstract

Herbivory significantly impacts the growth and reproduction of plants. Many plants have developed ways to defend against herbivores and one common strategy is to associate with ants. In many ant-plant interactions, ants are known to protect their host. However, in the Neotropical ant-plant genus *Triplaris,* the benefits provided by associated ants have never been tested. Many *Pseudomyrmex* spp. ants are obligate inhabitants of *Triplaris* spp. trees. In this study, *Triplaris americana* was studied in association with *Pseudomyrmex dendroicus*, an ant highly specific to its host (it has not been collected from any other species of *Triplaris*). Ant exclusion experiments were carried out to assess the protective effect of ants. In addition, ant behavior was monitored in control plants to study the mechanisms by which ants might confer protection against herbivory. Ant removal led to a more than 15-fold increase in herbivory. *Pseudomyrmex dendroicus* are active at all times of day and night and aggressively and efficiently remove insect herbivores from their host.

Keywords

Ant-plant interaction, defense, herbivory, mutualism, myrmecophyte, Peru

Introduction

Herbivory can have significant negative effects on plant fitness. Both the incidence and the impact of damage can vary with leaf ontogeny and the specific tissue being attacked, and herbivory can result in decreased survivorship and reproductive output (Marquis 1984, Spotswood et al. 2002). Since herbivory confers often high costs to plant fitness, and its extent is largely dependent on the plant's defense investment (Coley and Kursar 1996, Strauss and Agrawal 1999, Strauss et al. 2002), plants have evolved several ways to protect themselves or to compensate for the effects of attack. The protective strategies plants use include structural defenses, toxins, digestibility-reducing compounds, and mutualistic relationships with natural enemies of herbivores, such as ants (Gong and Zhang 2014).

Myrmecophytes (i.e., plants sheltering the colonies of a limited number of 'plant-ant' species in hollow structures called domatia and sometimes also providing them with food in the form of extrafloral nectar and food bodies) are pervasive and very diverse in the Tropics (Chomicki and Renner 2015). This is a mutualistic association as, in return for being housed and sometimes fed, plant-ants protect their host myrmecophyte from encroaching vegetation, herbivores and pathogens, and/or provide them with nutrients (i.e., myrmecotrophy) (Rico-Gray and Oliveira 2007, Mayer et al. 2014).

Several studies have shown that herbivory increases when ants are excluded from their host (e.g., Janzen 1967, 1972, Agrawal 1998, Michelangeli 2003). However, the degree of protection may vary with the identity of the ant partner (Bruna et al. 2004, Djiéto-Lordon et al. 2004, Frederickson 2005, Dejean et al. 2006), and in some cases the ants provided no protection against herbivory (Fowler 1993, Frederickson and Gordon 2007).

Myrmecophytic-plant genera that have received much attention in the literature include *Acacia, Cecropia, Macaranga, Duroia,* and *Cordia*. However, several other genera remain to be studied. One such case is the myrmecophyte *Triplaris.* Some aspects of the ecological interaction between *Triplaris* and its associate ants have been addressed, but they have been limited to understanding the pruning behavior of *Pseudomyrmex* (Davidson et al. 1988, Larrea-Alcazar and Simonetti 2007), the taxonomy of the *Azteca* that inhabit *Triplaris melaenodendron* (Bertol.) Standl. & Steyerm. (Longino 1996), the foraging behavior of some of the ants associated with *Triplaris surinamensis* (= *T. weigeltiana* (Rchb.) Kuntze; Brandbyge 1986) in Brazil (Oliveira et al. 1987), and host discrimination following chemical cues (Weir et al. 2012).

Questions regarding the effectiveness with which ants associated with *Triplaris* protect the plant against herbivores remain unexplored. The association between *T. americana* L. (Polygonaceae) and *P. dendroicus* Forel (Formicidae) is particular in that it displays high levels of specificity. After examining more than 200 collection records of *Triplaris* and its associated ants (A. Sanchez unpublished data), it was clear that *P. dendroicus* only colonizes individuals of *T. americana*, even when other species of *Triplaris* occur in sympatry. Therefore, in this study we expected a dramatic reduction in herbivory afforded by *Pseudomyrmex* ants compared to an ant-exclusion experiment and high and effective levels of protection.

Methods

Study site

This study was carried out at Los Amigos Biological Station (12°34'9"S, 70°6'0.40"W; ca 250 m) in the department of Madre de Dios in southeastern Peru. Los Amigos conservation area is a private conservation concession established in 2000 by the Peruvian government in conjunction with the Amazon Conservation Association (ACA) and the Asociación para la Conservación de la Cuenca Amazónica (ACCA). The station comprises more than 145.000 ha of lowland Amazonian forest between 250-320 m in altitude, at the confluence of the Madre de Dios and Los Amigos rivers, and protects pristine ecosystems including wetlands, seasonally inundated and terra firme forests, and palm swamps. The climate is characterized by a single dry and wet season each year. The area receives most of its estimated 2000 mm of annual rainfall during the wet season, which typically lasts from November to May (Pitman et al. 1999). During the dry season, from May to November, temperatures are usually at their lowest point, and can be as low as 10 °C. This study was conducted during 42 days of the dry season, in August-September 2008. During our study, the precipitation was sporadic, with only a few days with rainfall amounts greater than 10 mm. The minimum temperature recorded for that period was 13.3 °C, the maximum 35.3 °C, and the average temperature was 23.4 °C (http://atrium.andesamazon.org/).

Study system

The species chosen for this study were *Triplaris americana* and *Pseudomyrmex dendroicus*. *Triplaris americana* is the most common and widespread species in the genus. It is found from Panama to Bolivia and Brazil, usually in lowlands and disturbed areas close to water (Brandbyge 1986). The stems of *T. americana* are hollow (Schremmer 1984, A. Sanchez pers. obs.) and harbor an entire colony of ants. Although these plants produce no food bodies or extrafloral nectaries, rewards to the ants are provided by a third symbiont – scale insects (Coccoidea, Hemiptera) in the form of honeydew (Schremmer 1984, Davidson and McKey 1993, Ward 1999). It has also been suggested that another symbiont – fungi is involved and may also provide food (Schremmer 1984, Defossez et al. 2009, Blatrix et al. 2013). *Pseudomyrmex* (Formicidae, Pseudomyrmecinae) is a genus that comprises ca. 200 species and is distributed in the New World (Ward and Downie 2005). The ants are characterized by large conspicuous eyes, a well-developed post-petiole and a well-developed sting (Ward 1990) with potent venoms (Pan and Hinks 2000, Touchard et al. 2014). Several different clades within *Pseudomyrmex* are associated with distantly related myrmecophytes such as *Acacia*, *Cordia*, *Tachigali*, and *Triplaris* (Ward 1999). Among other characteristics, *Pseudomyrmex dendroicus* is recognized by the coloration of its workers, with a light brown body that contrasts with a dark brown head (Ward 1999). *Pseudomyrmex dendroicus* is an obligate symbiont of the myrmecophyte *Triplaris* and nests exclusively on *T. americana*. However, *T. americana* can be found in association with other species of *Pseudomyrmex* and with ants of other genera such as *Crematogaster* and *Azteca* (Longino 1996, Ward 1999; A. Sanchez pers. obs.). A single *T. americana* plant nearly always hosts only one ant colony (A. Sanchez pers. obs.).

Ant removal and its effect on herbivory

Prior to conducting the ant-exclusion experiments we explored two methods of exclusion, in order to determine which was the most effective. Following previous ant-exclusion experiments (e.g., Stanton and Palmer 2011), we applied a sticky resin in two separate branches per individual (Tangle-trap, Tanglefoot Company, Grand Rapids, MI). We applied the resin directly on the branch and over duct tape. However, the resin was not effective because of the formation of ant bridges (ants connected to each other in order to get over the barrier), and additionally bees ate the resin. Therefore, two to three days after the application of the resin, ants were already moving freely across the barrier and patrolling the leaves from which we intended to exclude them. We then used 0.5% Permethrin (Vidagro, Lima) to kill the entire colony. The use of insecticides has proven to be effective in previous ant-exclusion experiments (Stanton and Palmer 2011, Frederickson et al. 2012). The Permethrin was carefully injected through the prostoma of each internode avoiding contact with other parts of the plants. The prostoma are small unlignified zones between 6 to 10 mm long by which queens gain entry to make nesting sites (Schremmer 1984, Sanchez in review). Prostoma are present on twigs, branches, and the main stem (except at the base of the stem; A. Sanchez pers. obs.). Since adult plants of *Triplaris* can grow more than 15 m high, we chose saplings of less than 2 m tall for this study to ensure appropriate monitoring of herbivory and ant behavior. A total of 22 plants of similar sizes were used, with 11 replicates for each treatment (control and ant-exclusion). The Permethrin had no apparent effects on the plants' viability and growth during the study. The insecticide was used at low concentration (0.5%), and was only applied to the interior of the stem through the prostoma to minimize effects on the plants and on the potential herbivores.

To quantify the effects of ants on herbivory, two fully expanded leaves per sapling were monitored, always choosing the third leaf from the apical meristem from two adjacent branches. Prior to ant exclusion, photographs of every leaf were taken using a digital camera. A transparent sheet subdivided in grids of 1 square cm, each with 25 equidistant points within, was placed on top of the leaf, and photographs were always taken from the same distance. Percentage of herbivory was calculated by counting all the points that fell on areas where there was herbivory and divided by the total number of points that covered the leaf area. Photographs of each leaf were taken every two weeks for a total of six weeks.

We conducted a non-parametric Mann-Whitney *U* test for two independent samples to compare percentage of herbivory between control and experimental plants, using SigmaPlot version 12.5 (Systat Software Inc, San Jose, CA). We compared average herbivory after two, four and six weeks. The two leaves per sapling were used to calculate an average percentage of herbivory for each individual. A non-parametric ANOVA for repeated measures (Friedman ANOVA) was also conducted, to test if there was an increase of herbivory through time in the control and the ant-excluded plants.

Observations on ant behavior

Prior to taking the photographs, we conducted observations on the ants' behavior, recording their patrolling activities and their interactions with potential herbivores and with other ants that occasionally visit *Triplaris*. Plants were monitored for approximately 5 to 10 min every visit. Since ants seem to have patrolling activities that span the 24-hour cycle, observations were also recorded for some plants before dusk (between 1600 h and 1700 h) and at night (between 2100 h and 2200 h).

Results

Ant exclusion

Removal of ant colonies resulted in an increase in the percentage of herbivory. In each time interval (after 2, 4, and 6 weeks) there was a significant increase in herbivory compared to the control (U = 14, P < 0.05; U = 21; P < 0.05; U = 14.5, P < 0.01 respectively; Fig. 1). There was also a higher percentage of herbivory in the ant-excluded plants through time (ANOVA $\chi^2_{N=11,\ df=2}$ = 15.8, P < 0.001; Fig. 1), but not in the control plants (ANOVA $\chi^2_{N=11,\ df=2}$ = 4, P > 0.05).

Seven out of eleven control plants had zero percent herbivory during the six weeks of the experiment. The four other control plants suffered some herbivory by weeks 4 and 6 (~ 1%). In contrast, of the ant-excluded plants, six plants had more than 3% herbivory, having as high as 16% leaf damage (outlier not shown; Fig. 1). By the end of the experiment, the average percent of leaf damage was 3.2 in ant-excluded plants and 0.19 in controls. Plants with ants excluded had, on average, more than 15 times more herbivory than plants with resident ants. It was clear that the effect of herbivory was more pronounced as time progressed, especially in the experimental group (Fig. 1).

Ant behavior

Pseudomyrmex dendroicus actively patrolled their hosts, at all times of day and night, even when the temperature was as low as 13 °C. Whenever the plant was disturbed, they efficiently recruited other workers, and were very aggressive against any intruder. During their patrolling activities they removed any debris found on top of the leaves.

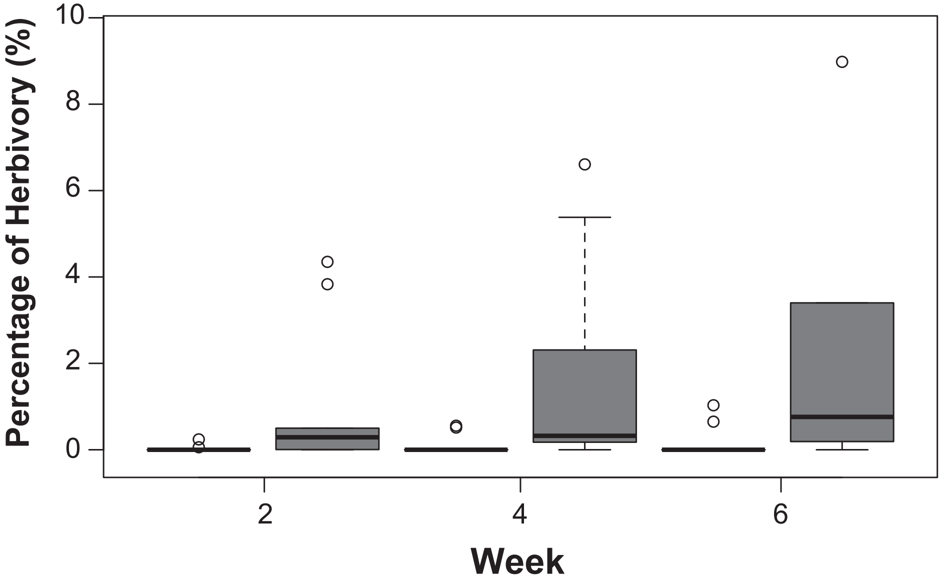

Figure 1. Percentage of herbivory with time for the control and ant-excluded plants (grey). Removing the ant colonies resulted in a significant increase in the percentage of herbivory after 2, 4, and 6 weeks (U = 14, $P < 0.05$; U = 21; $P < 0.05$; U = 14.5, $P < 0.01$ respectively). There was also a significant increase in herbivory through time in the ant-excluded plants (ANOVA $\chi^2_{N=11, df=2} = 15.8$, $P < 0.001$), but not in the control (ANOVA $\chi^2_{N=11, df=2} = 4$, $P > 0.05$).

Figure 2. Interaction between ant workers of *Pseudomyrmex dendroicus* and caterpillars visiting *Triplaris americana*.

They repeated this cleaning process constantly, on all the leaves of their host. In all saplings studied, the leaves had no signs of mosses, fungi or lichens growing them, and no sign of accumulated debris.

From our observations, the most common herbivores were grasshoppers (unidentified; Orthoptera) and caterpillars of the lepidopteran genera *Lophocampa* (Arctiidae, subfamily Arctiinae) and *Hylesia* (Saturniidae). When an ant encountered a caterpillar, a worker approached and detected it with its antennae, and then recruited more workers (Fig. 2). Typically more than 10 workers recruited around the intruder in less than five minutes. Several workers harassed the herbivore by stinging or biting, until it dropped off the plant. The caterpillars usually hung by a silk thread and attempted to move back onto the plant. However, individuals of *Pseudomyrmex* continued to chase them until they dropped again. This cycle was repeated several times. Other herbivores found in *Triplaris* included some Coleoptera. Most of the visitors frequented the plants at night or dawn. In all instances, *Pseudomyrmex* attacked the herbivores aggressively by biting and stinging.

Discussion

Herbivory

This is the first study to report that *Pseudomyrmex* provides benefits to *Triplaris* by reducing herbivory. Our results indicated a significantly higher percentage of herbivory on the plants where ants were excluded (more than 15 times more; Fig. 1), suggesting that the ants play an important protective role against herbivores. This has also been demonstrated in numerous other myrmecophytes, proving that there is a benefit from the effectiveness of the ant defense against herbivores (e.g., Chamberlain and Holland 2009, Rosumek et al. 2009, Trager et al. 2010). Frequent observations of the experimental plants revealed that herbivory tends to occur in a few concentrated events. As discussed by Michelangeli (2003), plants usually remain unharmed for a few weeks or days, but once they are discovered by herbivores, the damage occurs in a short time, often only a few hours.

According to the optimal defense theory (McKey 1974, 1979, Sagers and Coley 1995), defense should be concentrated on young shoots and leaves, since they constitute the most valuable and vulnerable parts of the plant (McKey 1974, Coley and Kursar 1996). In many plant species the nitrogen content in young leaves is very high, due to active growth, making them highly appealing to herbivores (Coley 1982, Kursar and Coley 1991). Vulnerability in young leaves is usually a result of being less tough, fibrous, and the lack of a protective cuticle (Coley and Kursar 1996). Since most of the herbivory damage a tropical plant will suffer throughout its lifetime is accumulated in juvenile leaves (Coley and Kursar 1996), having protection on these vulnerable areas constitute a great advantage. As expected, in several myrmecophytes, patrolling ants concentrate on these younger parts (Janzen 1972, McKey 1984, Fiala et al. 1994, Heil et al. 2001), though they also provide protection for mature leaves. In the association between *T. americana* and *P. dendroicus*, the ants actively guard young leaves and stipules, as well as mature leaves (A. Sanchez pers. obs.).

It has also been suggested that the protective role of ants extends to protecting the host against pathogenic fungi (Heil et al. 2001). Although we did not quantify fungal colonization directly, *Pseudomyrmex dendroicus* constantly removes debris, which could result in protection against fungi and other pathogens (García-Guzmán et al. 2001).

Ants constitute a rapid and direct line of defense, which can mobilize where they are required (McKey 1984, Fiala and Maschwitz 1990, Gaume et al. 1997, Agrawal 1998, Itioka et al. 2000, Michelangeli 2003). *Pseudomyrmex* behaves aggressively against other insects (Janzen 1967, Fonseca 1994, This study), viciously attacking any herbivore on the host, until it leaves the tree. This ant genus is also characterized by possessing potent venoms (Pan and Hink 2000, Touchard et al. 2014), which may allow them to effectively attack herbivores. We observed that *P. dendroicus* workers patrol their host at all times of day and night, even when temperatures are low. Workers do not forage outside the plant and do not eat the insect herbivores they attack (Fonseca 1994; A. Sanchez and E. Bellota pers. obs.). Although in some cases *P. triplarinus* Weddell and *P. dendroicus* take termite bait and/or tuna into the colony (Oliveira et al. 1987; E. Bellota pers. obs.), based on our observations, other larger insects such as caterpillars or Orthoptera do not seem to be preyed upon (as seen in other ant-plant *Pseudomyrmex* by Dejean et al. 2014).

The effects of herbivory may also extend beyond growth, ultimately affecting reproductive success and fitness of the host. Decreased energy spent on reparative growth could translate into increased energy allocation towards reproduction (Maron and Crone 2006). In addition, by protecting young individuals, survivorship would increase enhancing a plant's chances of living into adulthood, even if the production of domatia is costly (Brouat and McKey 2000, 2001). In *T. americana*, queens colonize plants as little as 30 cm tall and the first brood of workers was seen in seedlings ranging between 40 to 50 cm (Schremmer 1984, Sanchez in review). Therefore ants could play a fundamental role in the establishment and success of *Triplaris*.

Conclusion

Ant-exclusion experiments revealed that the myrmecophyte *Triplaris americana* is significantly affected by herbivory in the absence of its symbiotic associate *Pseudomyrmex dendroicus*. Ants actively patrol their host at all times of day and night, and rapidly recruit when an herbivore is encountered. Even though caulinary domatia are costly to produce (Brouat and McKey 2000, 2001), *T. americana* hosts queens of *P. dendroicus* as young seedlings. The specificity of *P. dendroicus* gives a clear difference in the potential survival of these plants. Therefore, following the development of seedlings through time and measuring the impact of herbivory before an ant colony establishes, would be fundamental to understanding how significant ant protection is during plant establishment. Short-term experiments give an idea of herbivore damage, but long-term experiments have revealed how vital ants can be for the plant's survival and the importance of ants on plant vitality, growth, and reproductive success (Heil et al. 2001). In addition, since our experiment was conducted during the dry season, experiments comparing herbivory in the wet versus dry seasons would be beneficial, since higher herbivory rates can occur during the rainy season (Coley 1982).

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