Introduction

Herbivory is a biotic interaction between plants and animals that has a variety of consequences for plants, from partial leaf loss to the death of whole organisms (Herrera & Pellmyr, 2002). Herbivorous insects have diverse relationships with plants, spending most of their lives or whole lifecycles on

Abstract

To reduce herbivory, plants bearing extrafloral nectaries interact with ants and attract them by providing food. As plant bodyguards, ants respond to the resource provision and, using their antennae, detect chemical messages from the host plants that help them to locate herbivores. Ants can also use their vision to explore the environment; however, information is lacking on how interactions between visual signs and the availability of extrafloral nectar affect ant aggressiveness near resources. We addressed the following question in this study: does the ants’ ability to visualize potential herbivores enhance their aggression under a constant provision of a high-quality food source? Using an experimental approach within the semiarid intertropical region of Tehuacan-Cuicatlán (Mexico), we manipulated the availability of food sources by constantly offering artificial nectaries on the shrub *Prosopis laevigata* (Fabaceae). Over two time periods (day and night), we tested how the presence of a high-quality food source affected ant aggressiveness to herbivores. Therefore, we offered dummy caterpillars and counted the number of marks left by enemies. Overall the attack rate was extremely high: 84.25% of the dummy caterpillars were injured. Ants were responsible for 86.22% of the marks left by enemies, and their aggression increased during the day, especially towards caterpillars in trees with high-quality food sources. During the night, ants probably rely mostly on their antennae to detect potential herbivores; therefore, their ability to detect dummy caterpillars was greater during the day. We show that, besides nectar quality and availability, visualizing herbivores may enhance ant aggressiveness.
Extrafloral nectar is rich in carbohydrates, amino acids, lipids, and other organic components, and it attracts a great variety of visitors (Del-Claro et al., 2016; Lange et al., 2017). By providing extrafloral nectar, plants feed ants in exchange for protection against potential herbivores (Koptur, 1984; Campos & Camacho, 2014; Dáttilo et al., 2017; Lange et al., 2017). However, this relationship is subject to environmental constraints (e.g. temperature and humidity) and the specific characteristics of ants, plants and their natural enemies (e.g. period of activity of ants and nectar quality) (Gottsberger et al., 1984; Falcão et al., 2014; Anjos et al., 2017; Lange et al., 2017).

The quality and quantity of resources provided by extrafloral nectaries differ among plant species and individuals and are influenced by factors such as the time of day, nutritional input and changes in the environment, e.g. wind temperature and relative humidity (Gottsberger et al., 1984; Rico-Gray & Oliveira, 2007; Lange et al., 2017). For instance, extrafloral nectaries of the shrub *Prosopis laevigata* (Fabaceae) are more productive at night (when the abundance of herbivores is higher), thus increasing nearby ant activity (Dáttilo et al., 2015). Moreover, the presence of extrafloral nectaries affects the composition and frequency with which ant species forage on plants (Dáttilo et al., 2014; Anjos et al., 2017). It has also been observed that the activity and quality of extrafloral nectaries vary with ant activity and, therefore, with the hour of the day (Lange et al., 2017). Plants can also increase nectar production near leaves attacked by herbivorous insects, which leads to an increase in the local recruitment of ants (Ness, 2003). In this way, the variation in extrafloral nectaries' productivity and the quality of the resource provided affects ant activity, aggressiveness and, consequently, performance as plant defenders (Anjos et al., 2017; Fagundes et al., 2017; Lange et al., 2017; Flores-Flores et al., 2018).

Ants detect chemical signals, such as pheromone and non-pheromone compounds, using their antennae when exploring the environment, to locate their nest or to locate a food source (Knaden & Graham, 2015). When interacting with plants, ants are influenced by volatile and non-volatile compounds in the nectar produced by extrafloral nectaries or produced by plants under attack from herbivorous insects (herbivore-induced plant volatiles) (Nelson et al., 2019). However, ants also use visual stimuli when foraging (Knaden & Graham, 2015) and to identify their position in relation to their nest, including landmarks, panoramic characteristics and other visual cues (Graham & Cheng, 2009; Buehlmann et al., 2018; Fernandes et al., 2018; Freas et al., 2018). The ants’ visual system is related to their habit; nocturnal species have a greater number of photoreceptors (Greiner et al., 2007), which capture more light to the detriment of visual accuracy (Land, 1997; Yilmaz et al., 2014), whereas diurnal ants benefit from higher optic resolution, as light is not limited (Yilmaz et al., 2014). In fact, it has already been demonstrated that ant species can identify and attack potential herbivores, differentiating caterpillar models and dead caterpillars from cubes made of plasticine (Leles et al., 2017). Although it is known that visual and, mainly, chemical signals assist ants when foraging and exploring the environment (Knaden & Graham, 2015), there is still a lack of information on whether, and how, the interactions between chemical (e.g. detection of food with antennae) and visual (e.g. visualization of a herbivorous insect) stimuli influence ants’ performance in protecting plants against herbivores.

In semiarid regions, extrafloral nectaries tend to be more active at night, when environmental conditions are more suitable for herbivores to forage and plants require more protection (Fitzpatrick et al., 2014; Dáttilo et al., 2015). However, ants are constantly foraging on plants during the day, despite the presence of extrafloral nectaries, since microclimatic characteristics are more suitable above than on the ground (Fitzpatrick et al., 2014; Luna et al., 2016). Higher temperatures also affect nectar production — extrafloral nectaries are usually less active during the day when plants suffer from water stress, which may cause loss of nectar due to evaporation (Falcão et al., 2014; Dáttilo et al., 2015).

During the dry season, nutrients, and especially water, are limiting factors for both ants and plants (Rico-Gray et al., 2006). Therefore, under these limiting conditions, plants alter their nectar provisioning; they can increase the nectar quality, enhancing the ants’ protection when the cost of losing tissue is higher (Pringle et al., 2013; Lange et al., 2017) or reduce their investment in indirect defences — nectar provisioning — and increase investment in direct defences, such as trichomes (Yamawo et al., 2012; Calixto et al., 2015). Either way, ants are subject to the plants’ responses to environmental conditions and nectar production; therefore, one may expect ant aggression towards potential herbivores located close to sources of food, such as extrafloral nectaries, to increase in drier environments and/or seasons.

In the semiarid Tehuacan-Cuicatlán Valley (Mexico), it has been shown that there is a turnover of ant species on the same individual of the shrub *P. laevigata* between day and night periods; therefore, each species is associated with a different quality and amount of resource, since *P. laevigata’s* extrafloral nectaries are mostly active at night (Dáttilo et al., 2015). In semiarid regions, nectaries and herbivores are more active at night; therefore, ant species foraging during this period are more effective plant defenders (Flores-Flores et al., 2018). However, considering that ants can also visually detect potential herbivores (Knaden & Graham, 2015; Leles et al., 2017) and the differences in visual accuracy between nocturnal and diurnal species (Yilmaz et al., 2014), how would the constant provision of high-quality food sources over day-night periods affect ant aggressiveness? Would the aggressiveness of ants be enhanced by the interaction between chemical (detection of food with antennae) and visual (visualization of a potential herbivore during the day) stimuli? The aim of our study was to evaluate the effects of a constant source of food during day-night periods on...
ant aggressiveness towards potential herbivores (dummy caterpillars) in a population of *P. laevigata*. We assessed (1) the difference in the frequency of attacks by ants on dummy caterpillars between shrubs with constant sources of high- and low-quality food and (2) the difference in the frequency of attacks between day and night. We predicted that, since the abundance of ants and their aggression increases in plants with higher quality nectar (Anjos et al., 2017; Flores-Flores et al., 2018) and during the day ants can visually detect potential herbivores (Yilmaz et al., 2014; Leles et al., 2017), we hypothesised we would observe the highest frequency of attacks on dummy caterpillars in trees with a higher quality food source during the day.

**Material and Methods**

**Study area**

The study was developed within the limits of the Helia Bravo Hollis botanic garden (97W 27° 29′, 18N 19° 55″), located in the municipality of Zapotitlán Salinas (Puebla, Mexico), part of the Biosphere Reserve of Tehuacán-Cuicatlán (Fig 1A). The area is located within the Mexican xerophytic region with pronounced seasonality, with a long dry season between November and May and a rainy season between June and October. The mean annual temperature is 18–22°C and mean total annual precipitation is 400mm.

**Experimental design**

The study was conducted in April 2019 in an area dominated by the thorny shrub *Prosopis laevigata* (Fabaceae) (locally known as mezquitera) (Fig 1A). We randomly selected 40 trees separated by at least eight meters to avoid the same ant colonies foraging in more than one sampled plant. In each shrub, we fixed 10 artificial nectaries, represented by 1.5 ml microtubes, on ten different branches approximately 1.60 m above the ground and separated from each other by at least 30 cm. *P. laevigata* individuals were divided into two treatment groups in a pair wise design. Twenty shrubs received artificial nectaries containing a high-quality food source: a solution of water and honeybee nectar (similar to Flores-Flores et al., 2018) with 30% sugar, measured in Brix degrees. The artificial nectaries of the remaining 20 shrubs were filled with water, hereafter, a low-quality food source.

The use of dummy caterpillars has the potential to provide insights into the influence of a constant provision of resources on ants’ responses towards potential herbivores. In general, models made of plasticine are distributed over the branches or leaves to resemble Lepidoptera larvae feeding on plants (Low et al., 2014; Roslin et al., 2017). When trying to feed on or attack the dummy caterpillars, different animal groups leave distinct marks, allowing their identity to a coarse, but informative, taxonomic level (e.g. birds, reptiles or chewing insects) (Low et al., 2014) (Fig 1B). These models have already provided useful information about the effects of plant and vegetation structure (Leles et al., 2017; Frey et al., 2018) and caterpillar characteristics (Hossie & Sherratt, 2012) on the relationships between caterpillars and their natural enemies. Ants have been shown to be responsible for most of the attacks on dummy caterpillars (Roslin et al., 2017), evidence that caterpillar models are efficient tools to assess the ants’ response towards herbivorous arthropods (Leles et al., 2017).

![Fig 1](image_url)

**Fig 1.** A. Study area within the Helia Bravo Hollis botanic garden (Zapotitlán Salinas, Puebla, Mexico), highlighting the area dominated by the thorny shrub *Prosopis laevigata* (Fabaceae). B. Dummy caterpillar with a mark left by an ant species. C. Lepidoptera larvae from the Geometridae family observed in the study area.
To evaluate the effects of food resource quality on ant aggressiveness towards potential herbivores, we fixed 10 dummy caterpillars (totaling 400 caterpillars) made of non-toxic green plasticine (Dixon Comercializadora S.A., Tultitlan, Mexico) to each shrub (Fig 1B). We modeled the caterpillars by pressing the plasticine against a metal lemon squeezer, then cutting it while measuring with a scale ruler to ensure that all caterpillars were the same length and circumference (3.5 x 0.5 mm, respectively). Caterpillars resembled Lepidoptera larvae from the Geometridae family (Fig 1C) and were fixed in a looping position (as Roslin et al., 2017) using instant glue (Kola Loka®, DuPont, Wilmington, USA) on the same branches as and 10 cm away from artificial nectaries. Caterpillars were affixed at 7 AM on the first day, and we performed four visits after every 12 hours, at 7 PM (to monitor the attacks made by natural enemies during the day) and 7 AM (to monitor night-time attacks), totaling 48 hours of the experiment. During each visit, we renewed the contents of the artificial nectaries and counted the number of marks left on each dummy caterpillar by potential natural enemies (hereafter, frequency of attacks). We identified them as ants or other organisms using guidelines and available literature (Low et al., 2014; Roslin et al., 2017). Since we wanted to measure differences in the ant’s aggressiveness towards potential invaders between day and night, the caterpillars were not retrieved after being attacked for the first time so we could obtain a cumulative attack rate per caterpillar and per treatment.

Data analysis

We compared the frequency of attacks by ants on dummy caterpillars during the day and night in shrubs with low- and high-quality food sources using a Generalized Linear Mixed-Effects Models with the R software (R Core Team, 2018). We considered the frequency of attacks by ants in each treatment as dependent variables and the periods of the day (day and night) and food source quality treatments (each shrub represented a random block) as fixed factors. We counted the number of marks on each caterpillar, we used a Poisson error distribution with a logarithmic transformation. Furthermore, we measured the explained deviance using Wald $\chi^2$ test.

Results

We observed marks left by natural enemies in 337 (84.25%) dummy caterpillars at least once during the whole experiment (two caterpillars could not be retrieved). Considering the cumulative attack rate, we observed 624 marks left by natural enemies (mean ± SD = 15.60 ± 3.88 marks per shrub), and ants were responsible for 538 (13.45 ± 4.90 marks per shrub) or 86.22% (Fig 2). The remaining marks (86, 13.78%) were left by other animal groups, such as birds, reptiles, mammals and other arthropods (e.g. parasitic wasps) (Fig 2). Considering treatments, we did not observe differences between the frequency of attacks by ants on caterpillars in shrubs with high- (264 marks) and low-quality resources (274 marks) ($\chi^2 = 0.14, df = 1, p = 0.7$) (Table 1). However, the frequency of attacks by ants on caterpillars was higher during the day ($\chi^2 = 25.56, df = 3, p < 0.0001$) (Table 1, Fig 3). In shrubs with low-quality resources, we observed a high number of attack marks during the first 24 hours, followed by a clear decrease (Fig 3). When assessing the interaction between fixed factors, we observed that the frequency of attacks by ants against dummy caterpillars was higher during the day in trees with high-quality nectar ($\chi^2 = 12.01, df = 1, p = 0.007$) (Fig 3).

Table 1. Frequency of attacks by ants on dummy caterpillars near artificial nectaries fixed on the branches of the shrub Prosopis laevigata during day and night and under different food source quality treatments over a 48-hour period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Frequency of attacks by ants during the day</th>
<th>Frequency of attacks by ants during the night</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-quality food source</td>
<td>164</td>
<td>100</td>
<td>264</td>
</tr>
<tr>
<td>Low-quality food source</td>
<td>142</td>
<td>132</td>
<td>274</td>
</tr>
</tbody>
</table>

Discussion

We found that the interactions between detecting a food source and visual signs enhanced ants’ aggressiveness.
towards potential invaders. Our data shows that ants foraging during the day, which are attracted by artificial nectaries with a high-quality food source, might be able to visually detect dummy caterpillars. As expected, the ant’s aggressiveness near these resources was higher compared with ants near the artificial nectaries filled with water. Even with resources *ad libitum*, ants continuously attacked potential herbivores when they were able to visually detect them. However, nocturnal ants probably rely more on chemical signals from their host plant and the invader, picked up using their antennae, to detect herbivores; therefore, they were attracted by our artificial nectaries but did not efficiently detect the dummy caterpillars.

In arid environments, resources are transient, and territorial and competitive animals, such as ants, will protect them against invaders (Rico-Gray, 1989). Therefore, not only were the artificial nectaries containing a high-quality food source considered valuable by the ants, but also those with a low-quality food source, as they were a source of water; in the Tehuacán Cuicatlán valley, the dry season started eight months before our experiment. Furthermore, a turnover of dominant ant species over day-night periods has been demonstrated in *P. laevigata*; the dominant ant species during the day was *Camponotus rubritorax*, while *C. atriceps* was dominant at night (Dáttilo et al., 2015). At the beginning of our experiment, each ant species was attracted by the new source of water in the trees with a low-quality food source, which probably explains the high number of marks during the first 24 hours. However, the high-quality food source contained honeybee nectar (i.e. fructose), rich in carbohydrates and amino acids as well as water, making it a better food resource and increasing its attractiveness to ants and, thus, the aggressiveness of ants against potential invaders (Grover et al., 2007; Anjos et al., 2017; Flores-Flores et al., 2018). Carbohydrate scarcity has been shown to reduce ant activity and aggressiveness, and access to carbohydrates and protein-rich resources contribute to their competitive behavior (Grover et al., 2007; Passos & Leal, 2019). Therefore, the ants initially defended both resources, but only the high-quality food source maintained a higher level of aggression over the entire 48-hour period.

Ant species have different morphological adaptations to the availability of light – diurnal species have higher optical accuracy, whereas the eyes of nocturnal species capture light more efficiently (Land, 1997; Greiner et al., 2007; Yilmaz et al., 2014). The 60% difference in the attack rate between day and night on the shrubs with high-quality nectar is indicative of the ability of *C. rubritorax* to visually detect the dummy caterpillars. It is likely *C. atriceps* detected our caterpillars using their antennae when exploring the environment near the artificial nectaries at night, not having enough visual accuracy to efficiently detect the dummy caterpillars by sight. Possibly, caterpillars were attacked more on the first night because they were first detected then as an unknown strange object. By the following night, they were ignored by the ants, which is evident from the reduced frequency of attacks in the shrubs with both high- and low-quality food sources. Therefore, we have demonstrated that, when the ants were exploiting the high-quality food sources, their ability to visually detect potential herbivores enhanced their aggressiveness, since the frequency of attacks only remained constantly high during the day. More studies are required to confirm if there are morphological and physiological differences in the eyes of the two species since evidence has shown that *C. rubritorax* is adapted to activity in the day and *C. atriceps* at night (Flores-Flores et al., 2018).

The lower frequency of attacks at night in trees with a high-quality food source could also be related to the activity of *P. laevigata*’s extrafloral nectaries. *C. atriceps*, which forages mostly at night, has several small sources of nectar available, as its foraging time coincides with the activity of *P. laevigata*’s extrafloral nectaries (Dáttilo et al., 2015). Consequently, during the day, ants such as *C. rubritorax* do not have the same resources from *P. laevigata* available and probably concentrated more on our artificial nectaries. Therefore, higher numbers of ants near the artificial nectaries and their ability to visually detect the dummy caterpillars enhanced *C. rubritorax*’s aggressiveness against potential herbivores.

We observed an extremely high proportion of attacked caterpillars during the whole experiment: 84.25% in only two days. Studies conducted in different environments, such as tropical savanna, urban area and tropical forest, that left models exposed for over four days observed attack marks on only 11.7, 16.2 and 20.9% of caterpillars, respectively (Moreno & Ferro, 2012; Leles et al., 2017; Frey et al., 2018). Therefore, our study demonstrated that caterpillar models can be used efficiently to explore the relationships between ants, the resources provided by plant species and herbivorous insects. This method can be applied when other resources are available, for example, to assess the relationship between ants
and potential invaders when plants are flowering or fructifying. More studies are needed to explore if this higher predation rate can also be observed in other systems or environments.

We have demonstrated that the interaction between detecting a high-quality food source and visual signs enhances ant aggressiveness towards potential herbivores. In shrubs with high-quality artificial nectaries, the frequency of attacks remained higher during the day, indicating that ants invest more energy defending a nutritious food source than water, and their ability to visualize a potential herbivore may enhance their effectiveness as plant defenders. During the night, ants probably detected our caterpillars using their antennae and had grown accustomed to the strange object by the following night. Therefore, not only resource availability and quality but also visual signs, modulate ant-plant-herbivore interactions. In addition, both visual cues and chemical signals influence the ants’ behaviour.

Acknowledgements

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References


