



Short-term plasticity and variation in acacia ant-rewards under different conditions of ant occupancy and herbivory

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Abstract

In ant–plant defense mutualisms, plants known as myrmecophytes provide food and shelter to ant partners in exchange for defense against herbivores and pathogens. To ensure interaction pay-off, myrmecophytes must regulate their investment in ant-rewards depending on local conditions and herbivore pressure. We investigated how myrmecophyte investment in multiple ant-rewards relates to herbivory, ant defense, and ant occupancy over time. Specifically, we examined the plasticity of ant-rewards produced by swollen-thorn acacias (*Vachellia collinsii*) under different ant occupancy and herbivory conditions. We compared food rewards (number of extrafloral nectaries and pinnules as a proxy for food bodies) and housing rewards (domatia dimensions) of *V. collinsii* for three conditions: (1) occupied (defended by the obligate mutualist *Pseudomyrmex spinicola*) versus unoccupied trees, (2) occupied trees subject to an experimental herbivory manipulation versus control trees, and (3) trees occupied by different ant species varying in their level of defense (*P. spinicola*, *P. simulans*, *Crematogaster crinosa*). We found that food rewards were more likely to vary in time depending on ant occupancy and resident species. Conversely, housing rewards varied with the condition (occupancy or species of partner) and less through time. A one-time herbivory manipulation did not cause any changes to the ant-rewards produced. Our results reveal short-term plasticity in *V. collinsii* ant-rewards and demonstrate that myrmecophytes with constitutive rewards can adjust their investment in ant-rewards depending on the presence and identity of ant partners.

Keywords Ant–plant interaction · Swollen-thorn acacia · Myrmecophyte · *Pseudomyrmex* · *Vachellia*

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Introduction

Since the evolution of the first land plants, herbivores have imposed strong pressures on plants, impacting their diversification, population dynamics, community structure, and outcome in competitive interactions (Labandeira 2007; Myers and Sarfraz 2017). To reduce the effect that herbivory has on their growth, survival and reproduction, plants have evolved multiple herbivore defense strategies. These include direct defenses that impact herbivore feeding performance and preference through physical and chemical barriers such as thorns and trichomes, or secondary metabolites, and indirect defenses that recruit natural enemies of herbivores through the constitutive or induced production of attractive compounds (Aljibory and Chen 2018). One such natural enemy that is recruited through plant indirect defenses are ants.

Myrmecophytes are plants in facultative or obligate mutualistic associations with ants. In exchange for protection against herbivores, pathogens and encroaching vegetation, myrmecophytes provide ants with housing and food rewards

that are essential to their ant partner's colony (Janzen 1966; Mayer et al. 2014; Chomicki and Renner 2015). Housing is provided in the form of specialized structures called domatia (e.g., hollow spines), and food is provided as nectar, and in some cases, as food bodies (Rico-Gray and Oliveira 2007). Maintaining such associations with ants can be costly for plants as resources are allocated to supporting ant colonies through the production of ant-rewards (Stanton and Palmer 2011; Frederickson et al. 2012). In addition, the return on the investment may vary depending on herbivore pressure and ant occupancy. Hence, myrmecophytes must regulate their investment in ant-rewards to maximize their fitness, presumably optimizing the benefits of ant association under different conditions. Adjustments in the production or size of ant-rewards and physical defenses, depending on herbivore leaf damage, have been demonstrated in several myrmecophytic genera (e.g., *Acacia*, *Cecropia*, *Cordia*, *Macaranga*; Heil et al. 2001a; Mayer et al. 2014).

Although the plasticity of ant-rewards is evident in induced rewards, the plasticity or variation of constitutive ant-associated structures is not well documented. Plants in obligate defense mutualisms are known to produce the structures for ants even when a colony is absent (Janzen 1974; Heil et al. 1997; González-Teuber and Heil 2015). In some species, the presence of ants or herbivores triggers the production of ant-rewards (Risch and Rickson 1981; Letourneau 1990). If ant-defense is lacking or herbivory is low, and there is a mechanism by which the plant can detect this, then natural selection would favor the allocation of energy to a different structure or function (e.g., reproduction or growth) thereby reducing the investment in ant-rewards. Alternatively, under high herbivore pressure, the plant may invest relatively more in structures that benefit the ants to enhance the defense they provide.

We investigated the relationship between ant-rewards, ant occupancy, and herbivory in a myrmecophytic Mesoamerican acacia, *Vachellia collinsii* (formerly *Acacia collinsii*, Fabaceae). *V. collinsii* is a species of swollen-thorn acacia that occurs from Mexico to Colombia (Janzen 1974). Within its range, *V. collinsii* is most typically found dry or xerophytic environments and it is occupied by at least eight species of obligate ants throughout its range, whose colonies depend on its swollen-thorn life form and ant-rewards (Janzen 1966, 1974).

Regardless of ant occupancy, *V. collinsii* produces multiple ant-rewards on each node: stipules may swell and fuse to develop a swollen hollow spine (domatia; Fig. 1), a compound leaf is produced with one to five extrafloral nectaries at the base of the petiole and occasionally with an additional nectary at the leaf tip ("apical nectary"; Fig. 1), and protein-rich food bodies, called Beltian bodies, develop on the tips of each leaflet pinna (Fig. 1). Over their lifetimes, *V. collinsii* trees can be inhabited by

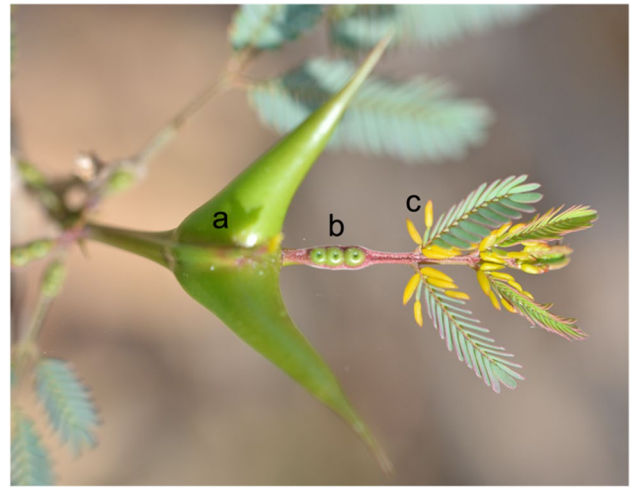


Fig. 1 Ant-rewards produced at the nodes of *V. collinsii*: **a** developed swollen spines ("domatia") that house the ant partners, **b** extrafloral nectaries located at the base of the leaf petiole that secrete nectar, and **c** Beltian bodies produced at the tips of leaf pinnules that are harvested by ants

various colonies of the same or different defending and non-defending ant species, or be left to fend for themselves if they lose their ant colony (Janzen 1966). Swollen-thorn acacia trees can be also inhabited by non-mutualistic ants (so-called parasites of the mutualism) that use ant-rewards but do not defend the tree (Janzen 1975).

In our study, we compared food and housing ant-rewards of *V. collinsii* trees under different ant occupancy conditions, herbivore pressures, and with different species of ant partners at two points in time. We first investigated differences in ant-rewards and herbivory levels between occupied (ant-defended) and unoccupied (not ant-defended) *V. collinsii* trees. Next, we conducted an herbivory experiment where we pruned occupied trees (inhabited by *Pseudomyrmex spinicola*) and examined whether the ant-rewards produced in new nodes were adjusted in response to increased levels of herbivory compared to control plants. Finally, we described how ant-rewards vary in trees occupied by ant species that provide different levels of defense (*P. spinicola*, *P. simulans*, *Crematogaster crinosa*). Given the importance of ant partners for the survival and reproduction of acacia trees (Janzen 1966), we expected trees with higher levels of herbivory to invest more energy in the short-term production of ant-rewards, depending on ant occupancy and the ant species occupying them. We also expected trees that had been unprotected for a long time (unoccupied or inhabited by weak or non-defending ant species) to allocate energy to other structures or functions that provide defense against herbivores (e.g., via direct defenses) and therefore produce fewer or smaller ant-rewards than ant-defended trees.

Methods

Study sites

We described the ant-rewards of *V. collinsii* at two seasonally dry forests in Panama during the wet season (September to December 2019): Parque Natural Metropolitano (PNM) in the Canal Zone (Panama Province, 8°59'36" N, 79°32'46" W) and Bosque Seco de Coronado, Reserva Eugene Eisenmann (hereafter, "Coronado"; Panama West Province, 8°32'04" N, 79°54'07" W). *V. collinsii* trees at PNM are only inhabited by the obligate ant mutualist *Pseudomyrmex spinicola*, whereas trees in Coronado can be occupied by a colony of one of three ant species: *P. spinicola*, *P. simulans*, and *Crematogaster crinosa*. More details on the study sites are provided in Online Resource 1.

Pseudomyrmex spinicola is a well-known obligate mutualist that attacks potential herbivores and removes encroaching and neighboring vegetation (Janzen 1966). Adult *P. spinicola* workers feed on nectar produced by extrafloral nectaries and harvest Beltian bodies from the tips of young leaves to feed larvae (Janzen 1974). *P. simulans* is an obligate acacia-ant that occupies spines, consumes nectar, and harvests Beltian bodies (Ward 1993). The defense it provides to *V. collinsii* is not well understood, but data suggest that it is a weaker defender than *P. spinicola* (Amador-Vargas et al. 2021). In contrast, *C. crinosa* is a facultative acacia inhabitant that occupies spines and consumes nectar but provides little defense against herbivores (Janzen 1967; Longino 2003). *C. crinosa* queens and workers are unable to cut open entrance holes on swollen spines and thus can only inhabit trees that are or were occupied by an obligate *Pseudomyrmex* species. *C. crinosa* is also visually, taxonomically, and behaviorally difficult to distinguish from two other *Crematogaster* species with similar acacia interactions (*C. rochai*, *C. torosa*; Longino personal communication). Hence, although we identified ant specimens from trees in our study using the key to *Crematogaster* (Longino 2003), it is possible that some trees were occupied by *C. torosa* or *C. rochai*.

Ant-reward samples and measurements

We conducted three sets of ant-reward comparisons for *V. collinsii* trees under different conditions. At PNM, we compared ant-rewards for (1) occupied trees (*P. spinicola*-inhabited) versus unoccupied trees (without any resident colony), and (2) occupied trees (*P. spinicola*-inhabited) where half received an experimental pruning treatment versus control trees. (3) In Coronado, we compared ant-rewards of trees occupied by different ant species (*P. spinicola*, *P. simulans*, *C. crinosa*).

For all three comparisons, we quantified ant-rewards from and measured herbivory of leaves on new branches (where the stem and spine color were still green) twice over the course of the wet season in Panama. Sampling the new growth in the same trees through time allowed us to distinguish between variation and plasticity by quantifying adjustments in the ant-rewards produced in new tissue under different conditions. We numbered the nodes from the base to the tip of each branch and obtained the following measurements on food ant-rewards produced on new leaves: (1) number of extrafloral nectaries; (2) presence of an apical extrafloral nectary (observed only in trees at Coronado); (3) number of pinnules, used as a proxy for the number of Beltian bodies produced by that leaf; (4) Beltian body dry mass.

To estimate the number of Beltian bodies produced on new leaves, we used photographs of the newest bipinnate compound leaves in each selected branch and counted the number of pinnules per leaf. Beltian bodies develop on the tip of each pinnule, are produced only once in a pinnule's lifetime, and are harvested by ants to feed larvae; hence, counting the number of pinnules on a leaf serves as a good estimate for the number of Beltian bodies produced by that leaf, even after Beltian bodies have been harvested by ants. To measure Beltian body dry mass, we collected a minimum of 15 Beltian bodies from newly developed leaves and dried all Beltian bodies from a given leaf for 25 min at 50 °C. We weighed Beltian bodies for each leaf in bulk, due to their small size and limitations of our measurement apparatus, using an Accuris balance (Model: W3100A-120, ± 0.1 mg). We did not measure the mass of Beltian bodies from trees occupied by different ant species in Coronado due to limited sample size. Measurements taken from photographs or in the laboratory (e.g., Beltian body mass) were done by tree ID code. Therefore, investigators were blind to ant occupancy, ant partner, or pruning treatment conditions.

For housing ant-rewards, we recorded whether stipules had developed as a pair of swollen spines at each new node of a branch. As swollen spines represent the nesting space available to ants and determine the colony size that a tree can host, their number and size in a tree indicate its investment in ant-mediated indirect defense against herbivores (Fonseca 1993; Orivel et al. 2011). For each pair of developed spines, we measured the base diameter (d) and length of each side (L_1 , L_2) using calipers (± 0.1 mm). With these values, we calculated the "spine asymmetry," used as an indicator of plant environmental stress (Díaz et al. 2004; Alves-Silva and Del-Claro 2013, 2016), as the difference in length between the left and right side of a pair of spines ($L_1 - L_2$). We then estimated the total spine volume (i.e., ant housing space) using the formula for the volume of two cones added together, following Amador-Vargas et al. (2020).

Estimates of herbivory associated with ant-rewards

For each selected branch, we estimated the amount of herbivory associated with the ant-rewards produced on new nodes using photographs of the newest bipinnate compound leaves. This herbivory measure represented an estimate of the baseline herbivory levels that new leaves were subjected to in the field by insects prior to our sampling efforts. For each leaf, we counted the total number of (1) leaflets, (2) missing leaflets, (3) intact pinnules (i.e., a full pinnule with no evidence of herbivory), and (4) damaged pinnules (with any evidence of herbivory). If a leaflet was missing or subject to extreme herbivory (missing > 70% of pinnules), it was assigned the same number of pinnules as the corresponding pair. If both leaflets in a pair were missing, no estimates for the number of intact and missing pinnules for each pair could be made, but a 100% herbivory measure was assigned to the pair of leaflets. We then calculated the average herbivory proportion of a leaf across its leaflet pairs (hereafter referred to simply as herbivory) and described herbivory levels across the three sets of comparisons.

Ant-reward comparisons

Ant-rewards in occupied and unoccupied trees at PNM

To examine differences in ant-rewards produced by occupied and unoccupied trees, we surveyed acacia trees along four trails at PNM, taking measurements on trunk diameter (5 cm above ground) using a caliper (± 0.1 mm). We noted whether trees were solitary or growing in patches with clonal shoots and if they hosted *P. spinicola*. Because unoccupied trees at PNM were scarce ($N = 12$), we randomly selected trees occupied by *P. spinicola* ($N = 12$) within the trunk diameter range of unoccupied trees (1.8–37.2 mm) for a total sample size of 24 trees. We selected occupied trees within this diameter range to account for potential size-dependent differences in ant-rewards between occupied and unoccupied trees. For trees located in clonal shoot patches, we only included the tree with the largest diameter in each patch to ensure that each occupied tree hosted an independent *P. spinicola* colony.

We described food and housing ant-rewards in five new-growth branches (i.e., where branches were still light green) and took photographs of the three newest bipinnate compound leaves in each branch (total of 15 photographs per plant) to estimate leaf herbivory levels and number of Beltian bodies. We marked the measured branches with wires at the points of new growth, to facilitate the identification of selected branches in our second sampling date when new nodes were produced (4 to 7 weeks later).

Ant-rewards of occupied control and pruned trees at PNM

Using the remaining occupied trees in our survey at PNM ($N = 36$), we conducted an herbivory manipulation experiment that investigated ant-reward adjustments in response to a one-time, severe herbivory treatment. We randomly assigned treatments (control vs. pruning) to the remaining solitary trees in our survey ($N = 19$) and then supplemented group samples with individual trees within shoot patches ($N = 17$).

For trees receiving the herbivory manipulation treatment (pruning), we counted the total number of new-growth nodes up to a height of 2 m from the ground. We used scissors to systematically remove half of all the new leaves produced by trees, starting with those located at the base of trees and making our way up until we reached our target 50% herbivory treatment. To simulate a leaf being eaten, we made five to eight cuts to each leaf that was removed and cut leaflets from the apex to the base of the leaf until all leaflets in a leaf were fully removed. We left nodes of branches from which we measured the ant-rewards untouched. Trees in the control group were left intact (i.e., no leaves were pruned on trees in this group).

We measured ant-rewards in newly produced nodes 4 weeks after the pruning treatment, following the sampling methods explained above, sampling ant-rewards in three new-growth branches per tree and taking photographs of the two newest bipinnate compound leaves in each branch (total of six leaf photographs per plant).

Ant-rewards in trees with different species of ant partners in Coronado

To examine differences in ant-rewards produced by trees with different species of ant partners (*C. crinosa*, *P. spinicola*, and *P. simulans*), we surveyed trees in Coronado and randomly selected a sample of 36 trees ($N = 12$ per species). We measured ant-rewards on nodes in three new-growth branches per tree, taking photographs of the two newest bipinnate compound leaves in each branch (total of six leaf photographs per plant). Ant-rewards from new nodes were sampled 5 weeks after the first sampling date.

Statistical analyses

In general, to compare ant-rewards and herbivory of (1) occupied and unoccupied trees, (2) pruned and control trees, and (3) trees with different species of ant partners, we ran linear mixed-effect models (LMMs) or generalized linear mixed-effect models (GLMMs) depending on the response variable. We ran all models (unless stated) in R (R Core Team 2016) using the “lme4” package (Bates et al. 2015), with individual acacia tree as a random effect, tree diameter

as a covariate (log transformed), and the predictor variable (ant occupancy, herbivory treatment, or ant partner) and sampling time (first vs. second sample) as fixed effects with their interaction. For significant predictors, we ran pairwise post hoc comparisons using the “emmeans” R package, specifying Holm adjustment (Russell 2020). We graphed all results using the ggplot2 package (Wickham 2016). More detailed descriptions of our methods for statistical analyses are provided in Online Resource 1.

Results

Ant-rewards in occupied and unoccupied trees at PNM

Leaves in occupied and unoccupied trees at PNM had more extrafloral nectaries and pinnules (and therefore also more Beltian bodies per leaf) in the first than in the second sample, and this difference was more pronounced for both ant-rewards in unoccupied trees (LMM, interaction term (extrafloral nectaries): $F_{1,364}=7.63$, $P=0.006$; interaction term (pinnules): $F_{1,299}=14.19$, $P<0.0001$; Fig. 2, 2). We did not compare average Beltian body dry mass in trees with different ant-occupancy, but Beltian bodies in occupied trees were marginally lighter in the first than in the second sample ($F_{1,23}=4.07$, $P=0.055$; Online Resource 2: Fig. S1).

The proportion of nodes with developed spines did not differ among occupied and unoccupied trees (GLMM, $Z=-1.68$, $P=0.09$) or between samples ($Z=1.40$, $P=0.16$; Online Resource 2: Fig. S2). Due to there being few spines in our second sample, we could only compare the dimensions of spines in the first sample: spines in nodes of ant-occupied and unoccupied trees were similar in length (LMM, $F_{1,17}=0.27$, $P=0.61$; Fig. 3), diameter ($F_{1,19}=0.006$, $P=0.94$; Fig. 3), and volume ($F_{1,18}=0.019$, $P=0.89$; Fig. 3). Spines were marginally more asymmetrical

in unoccupied trees than in occupied trees ($F_{1,18}=3.47$, $P=0.08$; Fig. 3).

Unoccupied trees had herbivory levels almost two times greater than those in occupied trees (LMM, $F_{1,22}=9.79$, $P=0.005$; Online Resource 2: Fig. S3). Herbivory in the first sample was greater than herbivory in the second sample for both groups of trees ($F_{1,262}=66.33$, $P<0.0001$; Fig. S3), and this effect was more pronounced for unoccupied than occupied trees (interaction term: $F_{1,267}=4.41$, $P=0.03$).

Herbivory associated with ant-rewards and ant-occupancy at PNM

We found that herbivory was associated with the number of extrafloral nectaries on a leaf ($F_{3,255}=5.42$, $P=0.001$) and ant occupancy ($F_{1,22}=7.89$, $P=0.01$), but not their interaction ($F_{3,259}=1.50$, $P=0.21$). Leaves with one extrafloral nectary showed less herbivory than leaves with two, three, or four extrafloral nectaries (Online Resource 2: Table S1) and this effect was more pronounced in unoccupied trees (Fig. 4). Also, the number of pinnules per leaf was negatively correlated with herbivory (Fig. 4; $F_{1,262}=11.96$, $P=0.0006$), and the effect was stronger in occupied trees (interaction: $F_{1,265}=4.51$, $P=0.03$). Therefore, the greater the number of pinnules per leaf in an occupied tree, the less herbivory it showed compared to unoccupied trees. We also found that leaves with heavier Beltian bodies showed less herbivory compared to leaves with lighter Beltian bodies ($F_{1,11}=5.84$, $P=0.03$; Fig. S4). Herbivory on a leaf was negatively correlated with the length of spines on that same node ($F_{1,47}=4.55$, $P=0.04$; Fig. S5a), but not with spine diameter ($F_{1,45}=0.49$, $P=0.49$; Fig. S5b), volume ($F_{1,44}=1.23$, $P=0.27$; Fig. S5c), or asymmetry ($F_{1,45}=0.11$, $P=0.75$; Fig. S5d), and this effect was similar for both occupied and unoccupied trees (interaction for length: $F_{1,47}=1.62$, $P=0.21$; diameter: $F_{1,44}=0.13$, $P=0.72$; volume: $F_{1,44}=0.32$, $P=0.58$; asymmetry: $F_{1,44}=2.28$, $P=0.14$).

Fig. 2 Food ant-rewards in occupied and unoccupied *V. collinsii* trees at Parque Natural Metropolitano (mean \pm SE): number of **a** extrafloral nectaries and **b** pinnules per leaf, in the first sample (circle) and after 4 to 7 weeks (triangle)

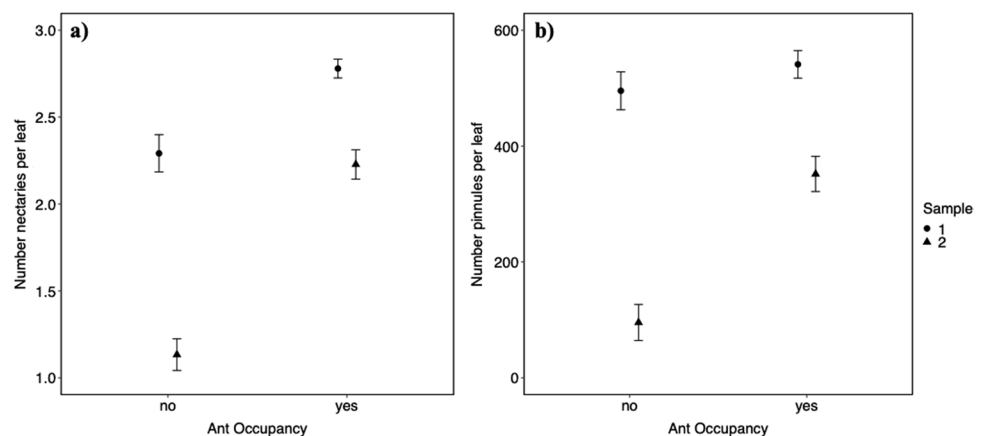


Fig. 3 Dimensions (mean \pm SE) of swollen spines in nodes of occupied and unoccupied *V. collinsii* trees at Parque Natural Metropolitano: spine **a** length (mm), **b** diameter (mm), **c** volume (mm^3), and **d** asymmetry (mm)

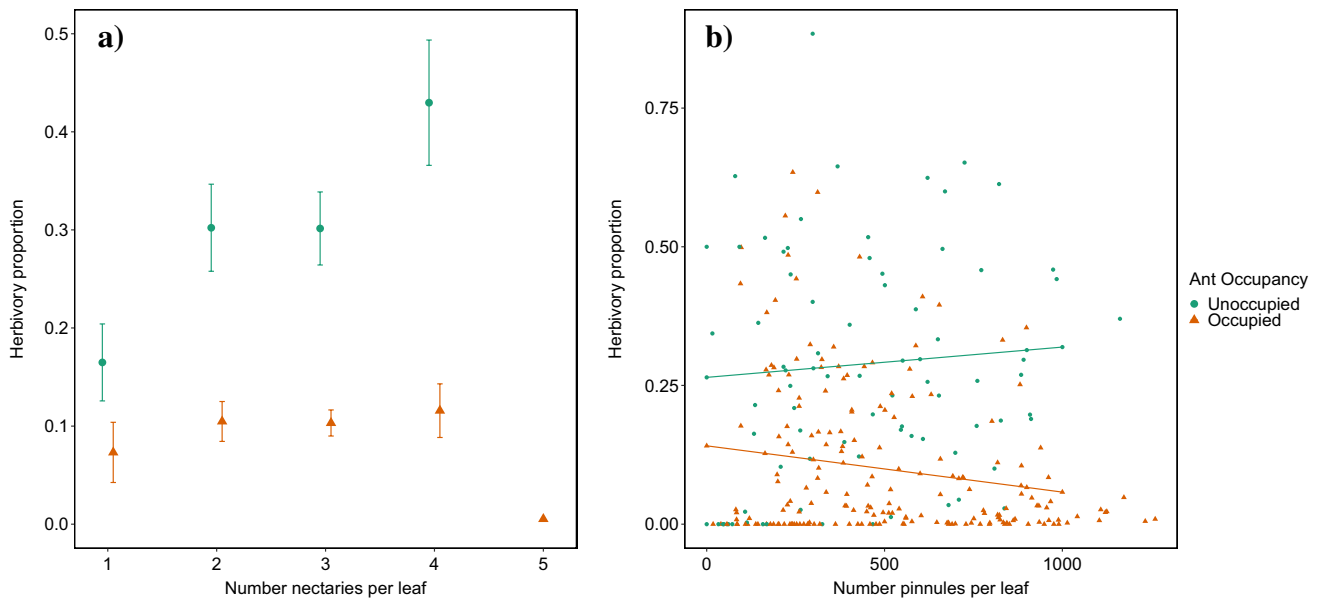
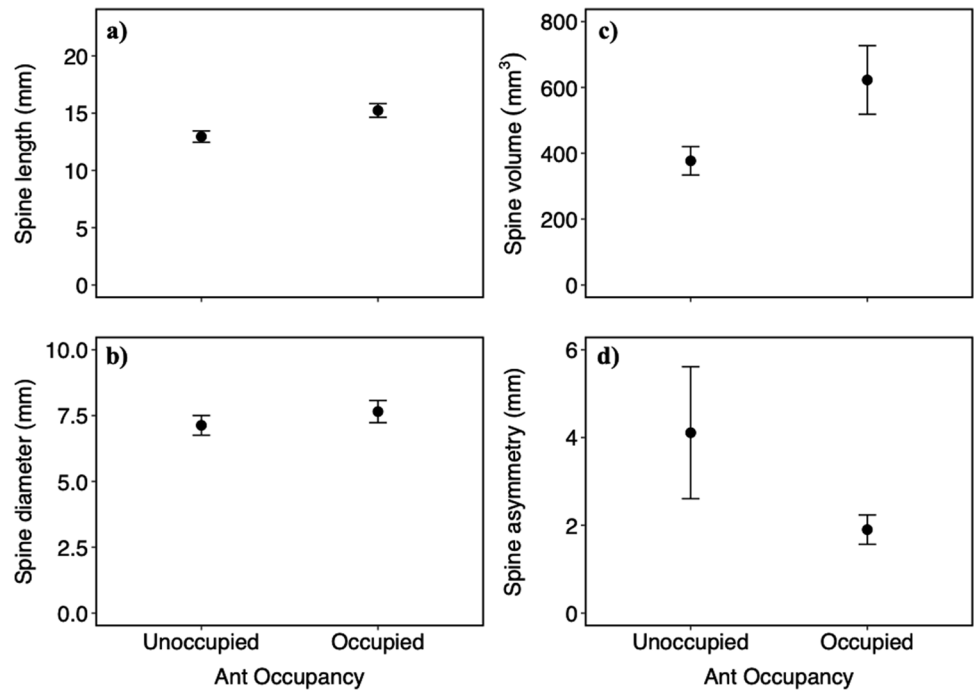


Fig. 4 Leaf herbivory proportion (mean \pm SE) in occupied (triangles) and unoccupied (circles) *V. collinsii* trees at Parque Natural Metropolitano, and the number of **a** extrafloral nectaries per leaf and **b** pinnules per leaf

Ant-rewards of occupied control and pruned trees at PNM

In our herbivory manipulation experiment with occupied trees, all trees had similar initial levels of herbivory per leaf (mean \pm SE; control: 0.20 ± 0.018 ; pruned: 0.18 ± 0.018 ; $F_{1,33} = 0.47$, $P = 0.49$). After the pruning treatment, new

leaves in both control and pruned trees had fewer extrafloral nectaries, and this decrease was marginally greater in pruned than in control trees (LMM, interaction term: $F_{1,380} = 3.46$, $P = 0.06$; Online Resource 2: Fig. S6a). For other ant-rewards, we did not find any effect of treatment: there was no interaction between sampling time and treatment for number of pinnules per leaf ($F_{1,348} = 1.80$, $P = 0.18$; Fig. S6b),

Beltian body mass ($F_{1,24}=0.37, P=0.55$; Fig. S6c), proportion of nodes with developed spines (GLMM, $Z = -0.056, P=0.96$; Fig. S7e, S7f), or spine length ($F_{1,95}=0.10, P=0.75$; Fig. S7a), diameter ($F_{1,101}=0.001, P=0.97$; Fig. S7b), volume ($F_{1,101}=0.055, P=0.82$; Fig. S7c), and asymmetry ($F_{1,72}=0.88, P=0.35$; Fig. S7d). However, ant-rewards differed over time in both control and pruned trees: compared to the first sample, leaves in the second sample had fewer extrafloral nectaries ($F_{1,702}=43.8, P<0.001$; Fig. S6a) and pinnules ($F_{1,352}=46.7, P<0.001$; Fig. S6b), and nodes had narrower spines ($F_{1,100}=4.03, P=0.04$; Fig. S7b). The length ($F_{1,94}=24.21, P<0.001$; Fig. S7a) and asymmetry ($F_{1,65}=3.75, P=0.05$; Fig. S7d) of spines in the second sample were also greater than for spines in the first sample, but spine volume did not change over time ($F_{1,100}=0.62, P=0.43$; Fig. S7c).

Ant-rewards in trees with different species of ant partners in Coronado

Herbivory differed among trees occupied by different ant partners ($F_{2,44}=7.9, P=0.001$; Online Resource 2: Fig. S8): it was greater in *C. crinosa*-inhabited trees (hereafter, “*C. crinosa*-trees”) compared to the two *Pseudomyrmex* species (post hoc, *P. simulans*: t -ratio = 3.74, $P=0.001$, *P. spinicola*: t -ratio = 3.93, $P=0.01$), and was similar between *P. simulans* and *P. spinicola* (post hoc, t -ratio = 0.29, $P=0.77$). Five weeks later, newly produced leaves showed a similar pattern

as those in the first sample ($F_{1,299}=2.51, P=0.11$; Fig. S8). Trees occupied by different ant partners had similar diameters at the beginning of our experiment (LMM, $F_{2,34}=0.19, P=0.82$; Fig. S9).

With respect to the ant-rewards produced by these trees, we found that the number of extrafloral nectaries per leaf did not differ among trees with different ant partners (GLMM, all $P>0.05$; Online Resource 2: Table S2). Conversely, after 5 weeks, new leaves in all trees had marginally fewer extrafloral nectaries ($Z = -1.77, P=0.077$). The number of pinnules per leaf depended on the ant partner: *C. crinosa* trees had leaves with fewer pinnules than *P. simulans* (GLMM, $Z=2.97, P=0.003$) and *P. spinicola* trees ($Z=3.42, P=0.0005$). In contrast, *P. simulans* trees had fewer pinnules than *P. spinicola* trees ($Z = -2.04, P=0.04$). For all ant partners, trees produced leaves with fewer pinnules in our second sampling period (i.e., after 5 weeks; $Z = -11.13, P<0.0001$) and the degree of pinnule reduction depended on the ant partner: *C. crinosa* trees showed the greatest reduction in the number of pinnules (Table S3). We also found that *C. crinosa* trees were more likely to have leaves with an apical extrafloral nectary than *P. simulans* trees ($Z = -2.08, P=0.03$) and marginally more likely than *P. spinicola*-trees ($Z = -1.7, P=0.08$). Leaves in trees with either species of *Pseudomyrmex* did not differ in the likelihood of having an apical extrafloral nectary ($Z=0.20, P=0.83$).

Regarding housing ant-rewards, spine length and asymmetry were similar among trees occupied by different ant species (LMM, $F_{2,33}=1.63, P=0.20$; $F_{2,28}=0.84, P=0.44$;

Fig. 5 Food ant-rewards in *V. collinsii* trees occupied by different species of ant partners in Coronado (mean \pm SE): number of **a** extrafloral nectaries per leaf, **b** pinnules per leaf, and **c** leaves with an apical nectary (blue—present, orange—absent). Dot shape in (a) and (b) denotes sampling time (circle—first; triangle—after 5 weeks)

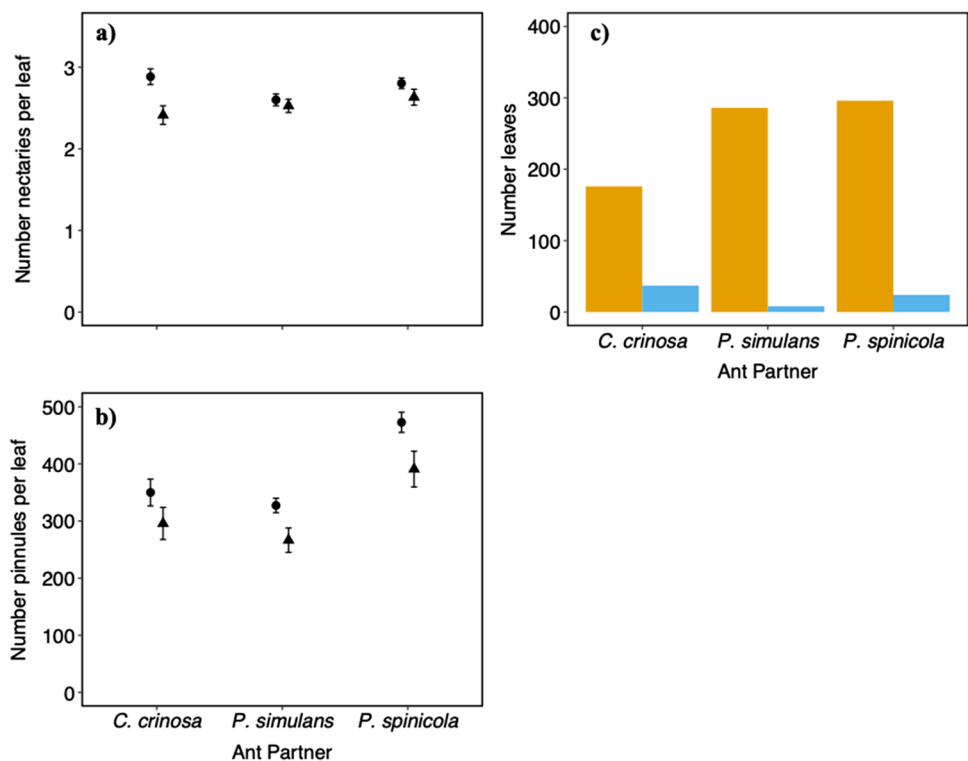


Fig. 6 Dimensions (mean \pm SE) of developed swollen spines in nodes of *V. collinsii* trees with different species of ant partners in Coronado: **a** spine length (mm), **b** diameter (mm), **c** volume (mm^3), and **d** asymmetry. Dot shape denotes sampling time (circle—first; triangle—after 5 weeks)

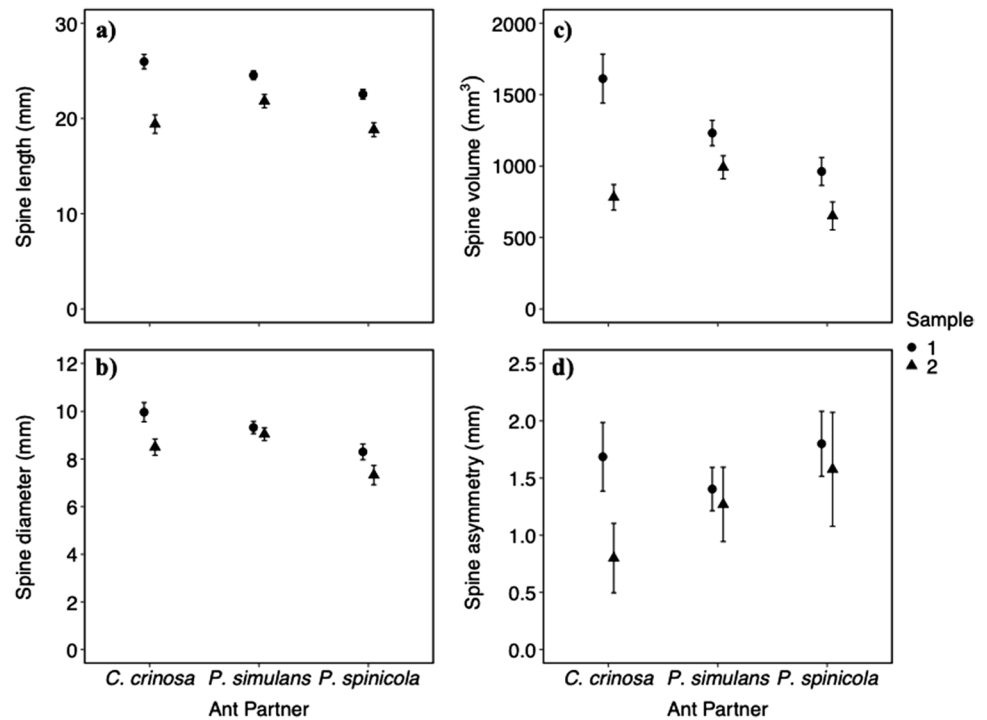


Fig. 5 and 5 respectively). Spines were shorter in our second sample ($F_{1,266} = 47.1$, $P < 0.001$), and this effect depended on the ant partner (interaction term: $F_{1,266} = 4.36$, $P = 0.013$): in *C. crinosa* trees, the reduction in spine length was greater than in *P. spinicola* or *P. simulans* trees (Online Resource 2: Table S4). Spines differed in diameter ($F_{2,35} = 3.72$, $P = 0.03$; Fig. 5) and volume ($F_{2,34} = 3.96$, $P = 0.02$; Fig. 5) depending on the ant partner occupying the tree: *P. simulans* trees had marginally wider spines than *P. spinicola* trees, but similar to *C. crinosa* trees (Table S5). Spines of *P. spinicola* trees had marginally smaller volumes than *P. simulans* trees (post hoc, t -ratio = 2.39, $P = 0.07$). The volume of spines in *C. crinosa* trees was more variable and therefore not different from those in trees with either of the *Pseudomyrmex* partners (Table S5). We also found no difference in the proportion of nodes with developed spines for trees occupied by different the species of ant partners: 62 to 67% of the nodes on a tree did not develop a swollen spine (Table S6, Fig. S10) (Fig. 6).

Discussion

Our results reveal short-term plasticity in some *V. collinsii* ant-rewards, which vary through time and depend on the presence and identity of ant partners. In general, we found that food rewards were better correlated with ant occupancy than housing ant-rewards. Food rewards also

more often showed an interaction of time and ant presence or ant partner identity and were the only ant-rewards that varied after a short-term experimental pruning.

Food rewards

More specifically, we found that the number of extrafloral nectaries per leaf depended on ant occupancy, while the number of pinnules per leaf (our proxy for the number of Beltian bodies) depended on both ant occupancy and the species of ant partner occupying a tree. Leaves in unoccupied trees also had fewer extrafloral nectaries and pinnules per leaf than those in trees with *P. spinicola*. Two hypotheses could explain this pattern: (1) greater herbivory in unoccupied trees causes trees to allocate resources to structures or functions that are not related to ant-rewards; or (2) that these trees are unoccupied in the first place because they had fewer extrafloral nectaries and Beltian bodies, and founding ant queens preferentially nest in high-reward individuals (Heil et al. 2009; Razo-Belman et al. 2018). Our results show that the number of pinnules and extrafloral nectaries in unoccupied trees decreases over time. This result supports the first hypothesis of a plastic response of trees but does not preclude the second hypothesis.

When occupied by different ant species in Coronado, trees had a similar number of extrafloral nectaries per leaf. Also, leaves in *C. crinosa* trees were more likely to have an apical extrafloral nectary (i.e., additional nectary at the

leaf tip). This result is surprising because apical extrafloral nectaries in *V. collinsii* are rarely observed. Nectaries have been demonstrated to indirectly protect trees from herbivory when in obligate or facultative associations with ants (Heil et al. 2001a; Calixto et al. 2018). A nectary at the leaf tip (i.e., apical extrafloral nectary) may thus promote better protection by weak defenders like *C. crinosa* by ensuring that ants are patrolling the entire length of leaves. *C. crinosa* ants take over trees that were formerly occupied by *Pseudomyrmex* obligate inhabitants (Janzen 1967). Hence, it is also possible that *C. crinosa* workers are more likely to colonize trees that produce many nectar rewards (as those are the only food rewards that they consume), or that these trees formerly housed smaller or weaker colonies of mutualistic ants that were easier to displace and also had apical extrafloral nectaries.

Swollen-spine production and dimensions

In contrast, spine dimensions varied more with ant presence and partner identity and less with time: spine diameter and volume differed marginally depending on the resident ant species (*P. spinicola* or *P. simulans*, but not *C. crinosa*). Surprisingly, spines in unoccupied trees did not differ from occupied trees in any of the dimensions, except for spine asymmetry (i.e., the difference in length between the two sides of a pair of swollen spines), which was marginally greater in unoccupied trees compared to trees occupied by *P. spinicola*. Asymmetry in bilateral plant traits and structures is widely considered to be an indicator of developmental instability resulting from environmental stress (Díaz et al. 2004). Plants growing in resource-limited environments or that are subjected to high levels of herbivory are thus expected to produce asymmetrical structures with large deviations from bilateral symmetry (Alves-Silva and Del-Claro 2013, 2016). The greater spine asymmetry in unoccupied trees in our study may indicate herbivory-induced plant stress in the absence of defending ants, although further studies are needed to validate this hypothesis.

We also found no differences in the likelihood of stipules developing as swollen spines in any of our comparisons. This result contrasts with the finding that trees inhabited by parasitic ants in a dry forest in Costa Rica have fewer developed spines than those inhabited by *P. spinicola* (Amador-Vargas et al. 2020). Likewise, in Costa Rica, 82 to 98.15% of nodes in occupied trees developed swollen spines, whereas in Panama this value never exceeded 38% (26% at PNM and 38% in Coronado). Differences in climate between study sites may contribute to these distinctions. Panama (and a small section of Colombia) host the southernmost populations of *V. collinsii* and are perhaps where trees experience the wettest conditions (Janzen 1974). Water stress has been

demonstrated to impact the strength of ant–plant mutualisms (Pringle et al. 2013) and the production of ant-rewards (González-Teuber et al. 2012). As such, it is possible that differences in water availability across sites in Panama and Costa Rica influence whether nodes develop swollen spines or not. There may also be some seasonality in spine production given that in Amador-Vargas et al. (2020), trees from Costa Rica were sampled at the start of the dry season, while we sampled in the middle of the rainy season in Panama.

Ant-rewards through time

Our data also show strong plasticity of ant-rewards as the wet season in Panama progresses. We found that food ant-rewards (number of extrafloral nectaries and pinnules per leaf) decreased over time, both at PNM and Coronado. The mass of Beltian bodies from occupied and unoccupied trees at PNM, on the other hand, increased over time. By contrast, spine length decreased with time in Coronado, but increased at PNM for trees in our herbivory manipulation experiment. Pairs of spines in both control and pruned trees at PNM also became increasingly asymmetrical and narrower over time. Similar changes in ant-rewards throughout a season have been reported for other swollen-thorn acacia species in Oaxaca, Mexico: ant-mediated indirect defense in both *A. cornigera* and *A. hindsii* decreased during the wet season, while plant growth increased and extrafloral nectar production and ant activity decreased (González-Teuber et al. 2012). Our results, in conjunction with previous studies, thus demonstrate that changes in ant-rewards can occur over the course of a season or merely a few weeks and correspond with ant occupancy and ant partner identity. They also suggest that there may be an endogenous seasonal response in Mesoamerican acacias that allocates resources to growth or direct defense during the wet season, when herbivore pressure is low, rather than to indirect defense. This hypothesis, however, remains to be tested and the mechanisms involved are not well understood.

Ant-rewards after experimental pruning

In our herbivory manipulation experiment, we applied a one-time, severe herbivory treatment to *P. spinicola*-occupied trees. Unlike other studies that have found various adjustments in ant-rewards in response to a one-time herbivory manipulation (Heil et al. 2001a, b; Mondor and Addicott 2003; Mondor et al. 2006), we found no significant differences in the ant-rewards produced by control and pruned trees. The only effect that we detected was a marginally greater reduction in the number of extrafloral nectaries per leaf in pruned trees. However, published reports on changes in ant-rewards in response to herbivory manipulations come from myrmecophilic (“ant-loving”) species and report

changes in the number extrafloral nectaries per plant or their nectar. For myrmecophytes, adjustments in ant-rewards have only been reported in long-term experiments in response to changes in herbivore pressure (Frederickson et al. 2012). Specifically, for acacias, adjustments in spine dimensions (and thus physical defense) have been reported in African acacias (*Acacia drepanolobium*) after excluding herbivores for 22 months (Young and Okello 1998), 7 years (Huntzinger et al. 2004), and 10 years (Palmer et al. 2008). The one-time mechanical pruning treatment in our experiment thus perhaps was insufficient to induce adjustments in ant-rewards in the short term. On the other hand, it is possible that the lack of herbivore oral secretions and saliva components in our pruning treatment did not elicit jasmonic acid pathways involved in induced plant defense responses (Arimura 2021). Physical defense traits (such as spine dimensions) are generally inducible (Barton 2016), but it is unclear whether spine production in *V. collinsii*, which is tied to ant housing rewards, is induced by herbivore-released elicitors or mechanical wounding of tissue. Alternatively, it is possible that our herbivory treatment induced changes in other ant-rewards or traits that we did not measure, like extrafloral nectar quality and quantity or Beltian body composition (Calixto et al. 2020). Also, as the association evolved with megafauna (Chomicki et al. 2015), it is possible that herbivory by large animals is what triggers a response in structures for the ants.

Natural herbivory in trees with different defense conditions

With regards to natural herbivory levels of plants in the field, we documented greater herbivory in unoccupied trees than in those occupied by *P. spinicola*—as expected. In addition, we compared herbivory among trees occupied by *P. spinicola*, a facultative parasite (*C. crinosa*), and an obligate associate (*P. simulans*). Herbivory was greatest in *C. crinosa* trees and comparable between *P. simulans* and *P. spinicola* trees. *C. crinosa* is considered a parasitic ant in acacia trees, as it consumes nectar and nests inside the spines but provides little defense against herbivores or encroaching vegetation (Janzen 1966; Longino 2003). Conversely, the ecology and behavior of *P. simulans* have not been studied, and the interaction with the host plant is not well understood (Ward 1993). Our results support general understandings of anti-herbivore defenses provided by *C. crinosa* and *P. spinicola* but indicate that *P. simulans* may defend *V. collinsii* against herbivores as effectively as the obligate ant mutualist, *P. spinicola*. However, our herbivory quantification did not consider damage caused by other types of insect herbivores. In a different tropical dry forest site in Panama, *P. simulans* trees were more heavily infested with Cecidomyiidae-induced stem galls—also present in our site—than

other sympatric plants that were inhabited by *C. crinosa* or *P. spinicola* (Amador-Vargas et al. 2021). As such, it may be that *P. simulans* successfully defends *V. collinsii* against leaf-eating herbivores but not against other herbivores like gall-inducing midges.

Natural herbivory and ant-rewards

Lastly, to capture ant-reward variation in response to natural herbivory, we assessed correlations between ant-rewards and herbivory in occupied and unoccupied trees. In our data, herbivory was positively correlated with the number of extrafloral nectaries on a leaf. This correlation was strong in unoccupied trees but not occupied trees. This result supports the notion that in the *Vachellia–Pseudomyrmex* mutualism, greater investment in extrafloral nectar enhances the recruitment, activity, and aggression of mutualistic ants, resulting in greater protection against herbivores at the scale of individual leaves (González-Teuber et al. 2012) as well as the entire tree (Heil 2013). In contrast, herbivory was negatively correlated with the number of pinnules per leaf (i.e., number of Beltian bodies), particularly in trees with *P. spinicola*. Less is known about the role that Beltian bodies (or food bodies generally) play in regulating ant defense; however, an increase in Beltian body production should translate to greater protection against herbivores by providing nourishment to ant larvae and facilitating the production of workers. Young leaves with Beltian bodies are also regularly patrolled by ant workers, which collect them one by one after a period of guarding them. Finally, as expected, herbivory was also negatively correlated with spine length. Altogether, these results suggest that investment in ant-rewards like Beltian bodies, which are tied to leaf size and facilitate the production of more workers, may favor greater long-term protection from herbivores when occupied by *P. spinicola*. The return in investing in the production of Beltian bodies, however, would be delayed due the intrinsic time-lag involved in the development of larvae into workers. In contrast, high investment in ant-rewards such as extrafloral nectaries may be costly if a tree is unoccupied and resources are not allocated towards other, perhaps more direct forms of defense (e.g., via the production of chemically defended leaves).

Conclusions

In sum, our study examined how food and housing ant-rewards in *V. collinsii* relate to herbivory, ant occupancy, and ant defense over the course of a wet season in Panama. Our data revealed short-term plasticity in the ant-rewards of the same individual, with changes over time depending on ant occupancy and ant partner identity. We also

demonstrated that a one-time pruning event may not be sufficient for ant-defended *V. collinsii* trees to adjust their investment in ant-rewards, although experiments on plants that have sustained constant herbivory without ant defense have yet to be conducted. These results call for mechanistic studies to reveal the factors driving and influencing the differences in ant-rewards that we observed over time. Finally, our data also suggest that the obligate acacia ant, *P. simulans*, deters leaf-eating herbivores as effectively as the well-known mutualist *P. spinicola*—a finding that further contributes to the current understanding of associations between Mesoamerican acacias and obligate ant partners.

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Author contributions SAV, FG and MG conceived ideas and designed methodology; FG, MG, and YG collected the data; SAV and FG analyzed the data; SAV and FG led the writing of the manuscript, and made equal contributions.

Data availability Dataset and code available at Figshare <https://doi.org/10.25573/data.13344455>.

Declarations

Conflicts of interests The authors declare that they have no conflicts of interest.

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