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Ecological effects of multi-species, ant–hemipteran mutualisms in citrus

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Abstract. 1. Protection mutualisms between ants and honeydew-producing insects influence arthropod food webs by altering the behaviour, activity and local abundance of ants on plants. Ants often tend multiple species of honeydew-producing insects; however, studies that examine such effects typically consider only pairwise mutualisms. This study investigates how multi-species mutualisms between ants and honeydew-producing insects structure arthropod food webs in citrus.

2. In an organic lemon orchard in San Diego County, California, U.S.A., ants or honeydew-producing insects (or neither) were experimentally removed from individual, mature lemon trees and then abundances of the following were estimated over a 2-year period: the Argentine ant, five species of honeydew-producing hemipterans, and California red scale. Red scale produces no honeydew but indirectly benefits from the presence of ants, which disrupt parasitism by *Aphytis* wasps.

3. Mutualism between ants and honeydew-producing hemipterans indirectly and positively affected red scale. Levels of parasitism decreased with increasing ant recruitment, and red scale density increased with both increasing ant recruitment and increasing honeydew-producing hemipteran abundance. Moreover, abundances of each of three honeydew-producing hemipteran species emerged as positive predictors of red scale density in different analyses; this finding suggests that individual hemipteran taxa exhibit distinct and spatially localised effects on ants.

4. Evidence of positive, indirect effects between two focal species of honeydew-producing Hemiptera was also detected. Guilds of honeydew-producing hemipterans may provide ants with honeydew more consistently or for longer than that produced by any single species. These results argue for an appreciation of how mutualist diversity affects the ecological consequences of mutualisms.

Key words. *Coccus hesperidum*, indirect effects, *Linepithema humile*, mutualist guild, *Planococcus citri*.

Introduction

Interactions between ants and honeydew-producing insects represent an ancient, widespread and ecologically important class of mutualism (Way, 1963; Hölldobler & Wilson, 1990; Davidson *et al.*, 2003; Grimaldi & Engel, 2005; Stadler & Dixon, 2005; Styrsky & Eubanks, 2007; Wilder *et al.*, 2011). These interactions have contributed substantively to a general understanding of mutualisms (Bronstein, 1994; Stadler & Dixon, 2005; Ness *et al.*, 2010) and are increasingly recognised

to influence the food webs in which they are embedded (Wimp & Whitham, 2001; Kaplan & Eubanks, 2005; Lach, 2007). By altering the behaviour, activity and local abundance of ants on plants, for example, mutualisms between ants and honeydew-producing insects can influence the structure of arthropod assemblages and indirectly benefit host plants when ants negatively interact with herbivores other than those they tend (Styrsky & Eubanks, 2007).

As with the study of mutualisms in general, most studies that have examined the ecological effects of mutualisms between ants and honeydew-producing insects consider only pairs of mutualist taxa (Styrsky & Eubanks, 2007). Multi-species mutualisms involving ants and honeydew-producing insects have received scant attention in this light,

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despite being a common phenomenon. Ants, for example, have long been known to interact with multiple species of honeydew-producing insects, often simultaneously and on the same host plant (Addicott, 1979; Bristow, 1984; Sakata, 1995; Fischer *et al.*, 2001; Kaminski *et al.*, 2010; Smith *et al.*, 2008). In cases where species of honeydew-producing insects differ—either qualitatively or quantitatively—in their ability to provide honeydew, inferior mutualists can become abandoned by their ant mutualists, leading to greater predation and parasitism risk, or even suffer predation by ants to a greater extent when they occur in proximity to superior mutualists than when they occur alone (Addicott, 1978; Sakata, 1995; Fischer *et al.*, 2001). By contrast, mutualists that exhibit comparable abilities to provide honeydew, or that are less susceptible to predation by ants, may positively benefit from one another through enhanced levels of ant activity. Positive, indirect effects among honeydew-producing insects may also arise when tending ants maintain large colonies and exhibit strong dietary preferences for honeydew (Kaminski *et al.*, 2010). Irrespective of the exact mechanism involved, positive interactions among co-occurring, honeydew-producing insect taxa could increase honeydew quantity or seasonal availability, enhance or prolong ant presence, and in turn magnify ecological effects of ants on other taxa.

Here we investigate the ecological effects of multi-species mutualisms involving the Argentine ant [*Linepithema humile* (Mayr)] and five species of honeydew-producing hemipterans (HPHs) in a citrus agroecosystem. The Argentine ant often tends HPHs for their honeydew (Way, 1963), and this resource is important for colony growth (Menke *et al.*, 2010), as are carbohydrates more generally (Grover *et al.*, 2007; Kay *et al.*, 2010). Multi-species mutualisms involving ants and HPHs are pervasive in citrus, even at fine spatial scales (e.g. individual flushes of vegetative growth) (Flanders, 1945; DeBach *et al.*, 1951; Bartlett & Lloyd, 1958; Markin, 1970). Strong positive associations among ants and HPHs may partly underlie positive, indirect interactions between ants and a leading citrus pest, the California red scale [*Aonidiella aurantii* (Maskell)], which produces no honeydew but appears to benefit indirectly from inadvertent enemy protection by ants (Samways, 1983; Murdoch *et al.*, 1995; Pekas *et al.*, 2010). Parasitoid wasps regulate populations of California red scale, and experiments show that ant presence reduces levels of red scale parasitism (Murdoch *et al.*, 1995).

To examine the extent to which biological control of red scale via parasitism by wasps is compromised by the presence of a multi-species, ant–HPH mutualism, we carried out a 2-year experiment in an organic lemon orchard where we conducted whole-tree removals of ants and whole-tree removals of HPHs. We tested two hypotheses: (i) ants engaging in mutualistic associations with honeydew-producing insects in the canopy disrupt biological control of red scale; and (ii) densities of red scale therefore increase in the presence of mutualisms between ants and multiple species of HPHs. To support the first hypothesis, we first demonstrate that ant presence in the canopy is strongly linked to HPH presence. In addition to these two hypotheses, we also examine the potential for HPH to exhibit positive, indirect effects on one

another. Our experimental approach represents a unique effort to quantify the functional significance of multiple HPH taxa in accentuating the broader ecological effects of a common mutualism. This approach, more generally, reflects a growing appreciation of the importance of multi-species mutualisms (Stanton, 2003; Holland *et al.*, 2005; Mooney & Mandal, 2009; Kaminski *et al.*, 2010).

Materials and methods

We conducted the experiments described in this study in the late summer of 2009 (year 1) and from early spring to autumn of 2010 (year 2) in a 400-tree, organic lemon grove in Valley Center, San Diego County, California, U.S.A. (33.2940°N, 116.9507°W). This grove supports established populations of the Argentine ant, California red scale, and five common HPH species: brown soft scale (*Coccus hesperidum* L.), citrus mealybug [*Planococcus citri* (Risso)], cottony cushion scale (*Icerya purchasi* Maskell), black scale [*Saissetia oleae* (Olivier)], and woolly whitefly [*Aleurothrixus floccosus* (Maskell)]. For our experiments, we selected 30 trees ranging in height from 2.7 to 4.0 m. We placed sets of three trees into 10 blocks; block identity was determined primarily by tree proximity and secondarily by similarity in canopy size. Within each block, trees were randomly assigned to an ant-removal treatment, an HPH-removal treatment, or an unmanipulated control. Densities of focal insect taxa did not differ among treatment groups prior to the initiation of removal treatments (Appendix S1 and Table S1).

We performed ant and HPH removals as follows. Ant-removal trees were outfitted with sticky barriers to exclude ants from the canopy. Barriers consisted of two layers of high-density foam (in a band 0.2–0.4 m wide) wrapped around the base of each tree trunk. We fixed the foam in place with cable ties, tightly wrapped the foam in plastic wrap, and then applied a 3-cm band of Tanglefoot® (Tanglefoot Company, Grand Rapids, MI) to the plastic wrap. The sticky barrier prevents ants and other crawling insects such as earwigs from ascending the trunk, but does not prevent flying insects, including key natural enemies of red scale and HPHs, from entering the canopy. To maintain the effectiveness of ant barriers, we periodically reapplied Tanglefoot. HPH removals were conducted approximately every 2 weeks to maintain a treatment with consistently low honeydew availability. We carefully searched leaf (including interiors of rolled leaf margins), twig, and bark surfaces for HPH aggregations. All HPHs were manually removed by lightly scrubbing surfaces with a small brush or occasionally by clipping highly infested leaves. Ant- and HPH-removal treatments remained in effect from August through October 2009 and then from January to September 2010. Ant and HPH activities are low in November and December at this site. Removals effectively reduced densities of target insect taxa (Appendix S2 and Table S2).

We sampled ant, HPH and red scale abundances on trees in each experimental group at various frequencies throughout the duration of the experiment (Table S3). For example, we sampled ant abundances approximately every 2 weeks, HPHs

on twigs a total of five times in both years, and red scale infestation on fruit twice in 2010. We started sampling after an initial buffer period (250 degree-days) following initiation of removals to allow time for natural enemies (particularly *Aphytis melinus* DeBach, which is the key enemy of California red scale) to increase in abundance (Murdoch *et al.*, 1995). To estimate ant activity in each tree, we counted either the number of ants ascending (2009) or the mean number of ants ascending and descending (2010) past a fixed point near the base of the trunk in 1 min. All ant counts were obtained between 09.00 and 15.00 hours. We estimated the density of red scale and individual HPH taxa in each tree by counting all individuals found on a specified number of twigs selected at a random height and compass direction within the canopy. In 2009, we sampled three to four twigs per visit for HPHs and red scale, whereas in 2010 we sampled eight twigs per visit for HPHs only. We defined a twig as the three most recent flushes of growth along a randomly selected shoot.

To estimate the density of red scale in the interior of trees, we used a stratified random sampling design modified from Murdoch *et al.* (1995). We first assigned each woody primary (structural) branch emanating from the trunk to a high-density or low-density class (stratum) based on visual inspection of red scale density. We then measured the length of each primary branch starting from the trunk and ending at either 1 m or where the bark began to contain visible chlorophyll (with the shorter of these two distances chosen). Using length measurements and the branch circumference midway along these lengths, we estimated the cylindrical surface area of each branch and thus the proportion of bark surface area in each density class (high or low). We then used a bark corer to extract two, 1-cm² bark samples from the branches in each density class (yielding a total of four samples per tree) in all trees. For logistical reasons in 2010, we sampled bark on control and ant-removal trees only. We also switched to a simple random sampling scheme, where bark cores were taken from the upper surface of four primary branches (random, with replacement) at a random distance up to 0.5 m away from the trunk. After coring, we bagged samples and counted live scale under a dissecting microscope the following day. Because the covers of red scale remain on tree substrates for some time after scale death, we distinguished live individuals from dead ones by lifting and removing scale covers from all surfaces of each twig (Forster *et al.*, 1995). Visibly parasitised individuals were excluded from counts of live red scale.

To estimate the extent of red scale infestations on fruit, we used Integrated Pest Management (IPM) recommendations (Grafton-Cardwell *et al.*, 2009) (<http://www.ipm.ucdavis.edu/PMG/r107301111.html#MANAGEMENT>). In 2010, we counted the number of fruit on each tree that appeared to support more than 10 red scale and the total number of fruit on each tree to determine the proportional infestation level on fruit in all treatments. In control and ant-removal trees, we then estimated the percentage parasitism of red scale on one highly infested fruit per tree and also in bark samples. The most common parasitoid of California red scale in coastal southern California citrus is *A. melinus*, followed

by the endoparasitoids *Encarsia perniciosi* Tower and *Comperiella bifasciata* Howard (Forster *et al.*, 1995). Parasitism of red scale by *A. melinus* is density-independent both spatially (Reeve & Murdoch, 1985) and temporally (Reeve & Murdoch, 1986), although it appears to be greater in the exterior (twigs, leaves, fruit) than in the interior (trunk) (Murdoch *et al.*, 1989) of the tree. All live red scale in fruit and bark samples were staged and then examined for parasitoid eggs, larvae, or pupae found on or in the scale host tissue (Forster *et al.*, 1995). Instar 1, instar 2, and virgin instar 3 developmental stages of red scale are vulnerable to parasitism. Parasitism in instar 1, however, is difficult to assess accurately. Parasitism level per tree was therefore calculated as the number of parasitised individuals in late vulnerable stages (instar 2 and virgin instar 3)/total number of individuals in late vulnerable stages.

Statistical analysis

We focused analyses on determining: (i) reciprocal effects of ant and HPH removals on one another; and (ii) effects of both ants and multi-species HPH assemblages on red scale densities and levels of parasitism. To test for effects of removals on interacting mutualists (e.g. effect of ant removals on HPH abundance), we initially analysed the data with paired *t*-tests. There was, however, high, unexplained spatial variability in ant and HPH abundance (particularly in 2010) that resulted in low ambient insect abundances in some parts of the orchard. Although removal treatments were always effective in keeping densities of focal taxa at low levels (Appendix S2 and Table S2), densities of these same species in some of the control trees also remained as low as those in the removal trees throughout the 2010 sampling period. Rather than drop information from a portion of the experimental blocks, we performed regressions, where continuous abundance estimates of the manipulated species (e.g. ant recruitment level in control and ant-removal trees) were used as explanatory variables in place of the categorical treatment variables (e.g. controls versus ant removals) (see Cottingham *et al.*, 2005). This approach capitalises on the effectiveness of our whole-tree experimental removals and the naturally low abundances of certain focal species in some of the control trees. For abundance and density responses, we applied linear regression analysis (PROC REG; SAS Institute, 2009), and for proportional data, we used logistic regression (PROC LOGISTIC; SAS Institute, 2009) with a binomial error distribution and logit link function. The results of regression analyses for these data were more robust than those of the paired *t*-tests, and are reported herein.

For all regression analyses with multiple HPH explanatory variables (i.e. abundances of citrus mealybug, brown soft scale, and woolly whitefly), we report results of global hypothesis tests (*F*-tests for linear regressions and likelihood ratio χ^2 tests for logistic regressions) for each complete model. For parameter estimation, however, we employed model selection by comparing second-order Akaike information criterion (AIC_c) values for the complete model and all possible subsets (see Burnham & Anderson, 2002; Johnson & Omland, 2004). The candidate model in this set having the lowest AIC_c value is

considered to have the least information loss in explaining the data and is thus the 'best' model if its AIC_c is much lower than the others. In each case, however, multiple candidate models had ΔAIC_c values (AIC_c for each model–minimum AIC_c among all models under comparison) < 10 , indicating model selection uncertainty (Table S4). We therefore used normalised Akaike weights [$\exp(-0.5 \times \Delta AIC_c)$] to determine the 95% confidence set of models for our data. We report model-averaged parameter estimates and unconditional standard errors for the confidence set of models in each analysis (Burnham & Anderson, 2002; Symonds & Moussali, 2011).

We used this hybrid approach, of hypothesis testing along with an information-theoretic approach to model selection and averaging, partly because global hypothesis testing is a valid analysis tool for our formal experimental design (i.e. controls versus HPH removals) and is useful for reporting whether the HPH removals effectively impacted other species' abundances. Additionally, model selection and parameter averaging allowed us to determine which HPH species were most likely driving any global effects on ants or red scale. Because we did not manipulate HPH species independently of one another, the information-theoretic approach to model selection seemed most appropriate for exploring relationships in the data. Additionally, for these regressions, there was little evidence of multicollinearity (variance inflation factors < 4), and therefore no modifications were made on the explanatory variables.

In all models, each observation represents time-averaged data per tree year⁻¹ for date ranges appropriate for the analysis. For example, for analyses with a categorical explanatory variable (i.e. treatment), observations are represented by the weighted means of samples taken across all sampling dates during each year. For regressions, on the other hand, the response variables represent time-averaged data only for dates concurrent with and subsequent to dates when the explanatory variables were measured. Analyses were performed for 2009 and 2010 separately.

Indirect effect of citrus mealybugs on brown soft scale

In addition to the experimental work described in the preceding sections, we also investigated the potential for pairs of HPH taxa to exhibit positive, indirect effects on one another. We conducted a survey of brown soft scale aggregations on unmanipulated lemon trees to determine: (i) whether or not the abundance of neighbouring citrus mealybugs has a significant effect on ant attendance at brown soft scale aggregations; and (ii) if so, whether the sign of this effect is positive or negative. We focused on the effect of citrus mealybug abundance on

brown soft scale mediated through the shared ant partner because citrus mealybug tends to be the most abundant HPH species in lemon, and hence the most likely HPH species to have a significant effect on the population growth of brown soft scale in orchards. In July 2010, we located and tagged up to four discrete aggregations of brown soft scale per tree. Aggregations ($n = 36$) were located on leaf or twig substrates, and on one of the three most recent flushes of vegetative growth per shoot. On three subsequent visits (6, 20, and 34 days after tagging), we recorded the number of settled individuals (i.e. adults and nymphs, but not crawlers) of brown soft scale in each aggregation, the number of ants appearing to tend individuals in these aggregations, and the abundance of settled citrus mealybugs on the same flush as the focal aggregation. Mean abundances were calculated across all three survey dates for each aggregation. We then used multiple linear regression to determine whether or not the size of brown soft scale aggregations or the abundance of citrus mealybugs inhabiting the same flush as the focal aggregations were significant predictors of mean ant attendance per aggregation of brown soft scale.

Results

Relative abundance of HPH species

The abundance of HPHs per twig was 2.8 times greater during the sampling periods in 2009 than in 2010. The citrus mealybug was the most abundant HPH species in both years, whereas black scale and cottony cushion scale were consistently rare by comparison (Table 1). Within each year, differences in the abundance of individual HPH species appeared to influence ant foraging. By observation, for example, the majority of Argentine ant workers in any given tree tended aggregations of citrus mealybug or brown soft scale, while relatively few workers visited aggregations of black scale or cottony cushion scale. Black scale and cottony cushion scale were too rare in samples for statistical treatment in most cases. In all the following analyses, we therefore focused on interactions involving ants and the citrus mealybug, brown soft scale, and woolly whitefly.

Reciprocal effects of removals on mutualists

Removal treatments confirmed the existence of a reciprocally positive relationship between ants and HPHs. Abundances of individual HPH species dropped to low levels in the

Table 1. Proportional abundance of honeydew-producing hemipteran (HPH) species in unmanipulated control trees.

Year	Sampling interval	No. of samples	Total HPH	% CMB	% BSS	% WWF	% WWFa*	% BS	% CCS
2009	18 August to 1 September	110	1271	83.4	9.9	5.6	–	0.1	1.0
2010	9 April to 8 September	240	997	71.2	20.2	–	6.3	1.4	0.9

*In 2010 WWFa were treated as equivalent to individuals for proportional calculations.

CMB, citrus mealybug; BSS, brown soft scale; WWF, woolly whitefly; WWFa, woolly whitefly aggregations; BS, black scale; CCS, cottony cushion scale.

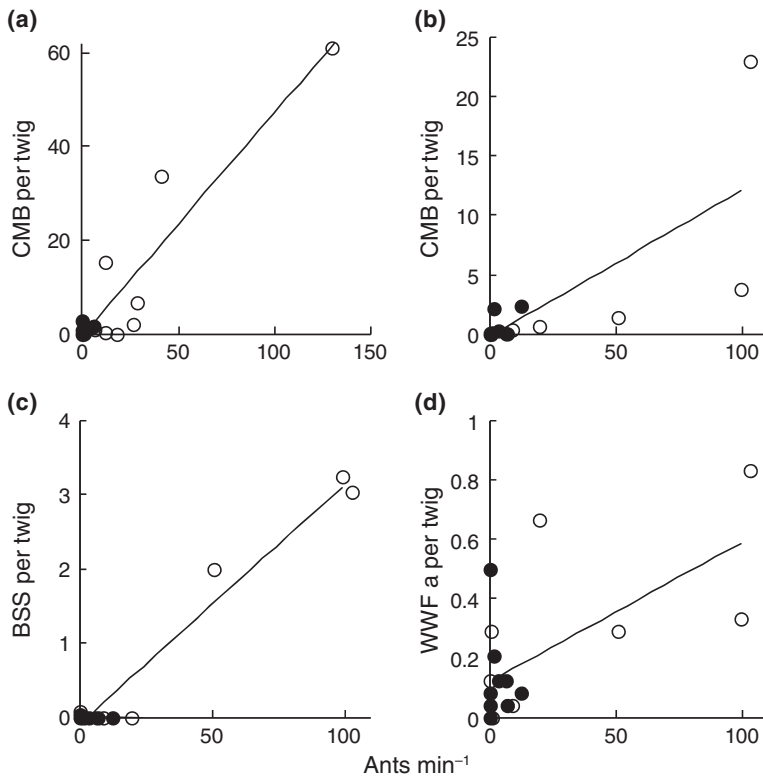


Fig. 1. Effect of Argentine ant recruitment on the abundances of individual species of honeydew-producing hemipterans on twigs in 2009 (a) and 2010 (b–d). Statistical results are reported in Table 2. Open circles, control trees; solid circles, ant-removal trees. CMB, citrus mealybug, BSS, brown soft scale, WWFa, woolly whitefly aggregations.

absence of tending ants and generally increased with increasing levels of ant recruitment (Fig. 1). The significance of linear regressions between the abundances of individual HPH species and ant recruitment exhibited interannual differences. In 2009 only the number of citrus mealybugs per twig in control and ant-removal trees increased [coefficient estimate (SE)=0.48 (0.04)] with increasing ant recruitment, whereas in 2010 the number of citrus mealybugs, brown soft scale, and woolly

whitefly aggregations per twig in control and ant-removal trees increased [coefficient estimates (SE) of 0.12 (0.02), 0.03 (0.00), and 0.00 (0.00), respectively] with increasing ant recruitment (Table 2, Fig. 1).

Honeydew-producing hemipteran removal also reduced ant recruitment in individual trees. In the HPH-removal treatment in 2009, for example, we observed a 63.6% reduction in mean ant recruitment per tree compared with that measured in control

Table 2. Results of global hypothesis tests, by linear regression, for effects of ant recruitment and honeydew-producing hemipteran abundance on species-specific insect abundances.

Year	Response variable	Explanatory variable(s)	R ²	F	d.f.	P	Table for estimates
2009	CMB per twig	Ants min ⁻¹	0.87	120.01	1.18	<0.001	See text
2009	BSS per twig	Ants min ⁻¹	0.01	0.24	1.18	0.632	–
2009	WWF per twig	Ants min ⁻¹	0.01	0.17	1.18	0.686	–
2010	CMB per twig	Ants min ⁻¹	0.58	24.66	1.18	<0.001	See text
2010	BSS per twig	Ants min ⁻¹	0.96	426.80	1.18	<0.001	See text
2010	WWFa per twig	Ants min ⁻¹	0.41	12.47	1.18	0.002	See text
2009	Ants min ⁻¹	CMB, BSS, WWF	0.82	23.98	3.16	<0.001	S5
2010	Ants min ⁻¹	CMB, BSS, WWFa	0.94	72.85	3.15	<0.001	S5
2009	CRS per twig	Ants min ⁻¹	0.39	11.46	1.18	0.003	S6
2009	CRS cm ⁻² bark	Ants min ⁻¹	0.32	8.47	1.18	0.009	S6
2010	CRS cm ⁻² bark	Ants min ⁻¹	0.26	6.35	1.18	0.021	S6
2009	CRS per twig	CMB, BSS, WWF	0.47	4.68	3.16	0.016	S6
2009	CRS cm ⁻² bark	CMB, BSS, WWF	–	1.82	3.16	0.183	S6

For each analysis, data for control trees are pooled with those for the removal trees matching the explanatory variable (i.e. ant-removal trees included in models with ants min⁻¹ as the explanatory variable).

CMB, citrus mealybug; BSS, brown soft scale; WWF, woolly whitefly; WWFa, woolly whitefly aggregations; BS, black scale; CCS, cottony cushion scale; CRS, California red scale.

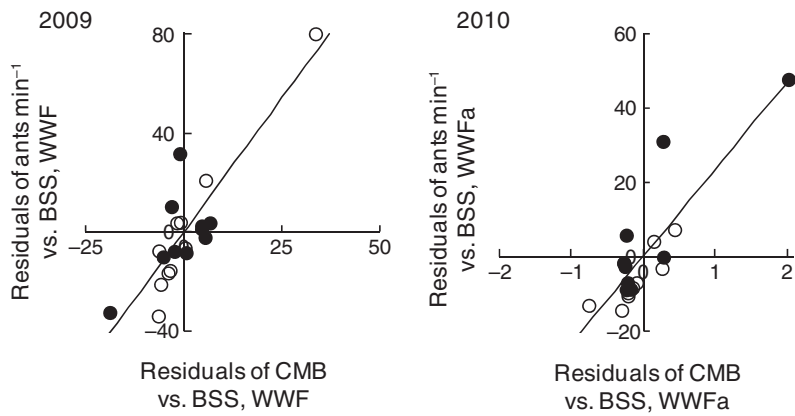


Fig. 2. Partial regression plots showing the effect of citrus mealybug abundance per twig on Argentine ant recruitment rate after controlling for the effects of brown soft scale and woolly whitefly in the multiple regression model (least-squares fit, 2009: $y = 2.18x$, $r = 0.86$; 2010: $y = 23.46x$, $r = 0.86$). Statistical results of full multiple regression models are reported in Table 2. Open circles, control trees; solid circles, honeydew-producing hemipteran (HPH)-removal trees. CMB, citrus mealybug; BSS, brown soft scale; WWF, woolly whitefly; WWFa, woolly whitefly aggregations.

trees (paired $t = 3.21$, $df = 9$, $P = 0.0106$). By observation, the majority of ants foraging in HPH-removal trees visited flowers, presumably to obtain nectar. Levels of ant recruitment appeared primarily driven by the abundance of the citrus mealybug. In multiple regressions using data from control and HPH-removal trees, for example, the relationship between ant recruitment and the abundance of citrus mealybug was significantly positive in 2009 and 2010 after taking into account the effects of other HPH species (Table 2, Fig. 2; see Table S5 for model-averaged parameter and precision estimates).

Indirect effects of mutualist removals on California red scale

The multi-species, ant-HPH mutualism influenced red scale density on multiple substrates. In both 2009 and 2010, red scale densities on bark increased with increasing ant

recruitment (Table 2, Fig. 3a,b). In 2009, red scale densities on twigs increased with increasing ant recruitment (Table 2, Fig. 3c; red scale density was not measured on twigs in 2010). Moreover, the multiple regression of HPH abundances (citrus mealybug, brown soft scale, and woolly whitefly) explained 47% of the variance in red scale density on twigs in 2009 (Table 2), with the strongest individual contribution from brown soft scale per twig (Fig. 3d; see Table S6 for model-averaged parameter and precision estimates). In that year, however, HPH abundance did not significantly affect red scale density on bark (Table 2; Table S6).

In addition to the significant positive relationship between red scale density on twigs and the combined presence of the three focal HPH species, we also observed positive relationships between red scale on fruit and the ant-HPH mutualism. For example, the proportion of fruit infested with red scale increased and the extent of parasitism of red scale on fruit

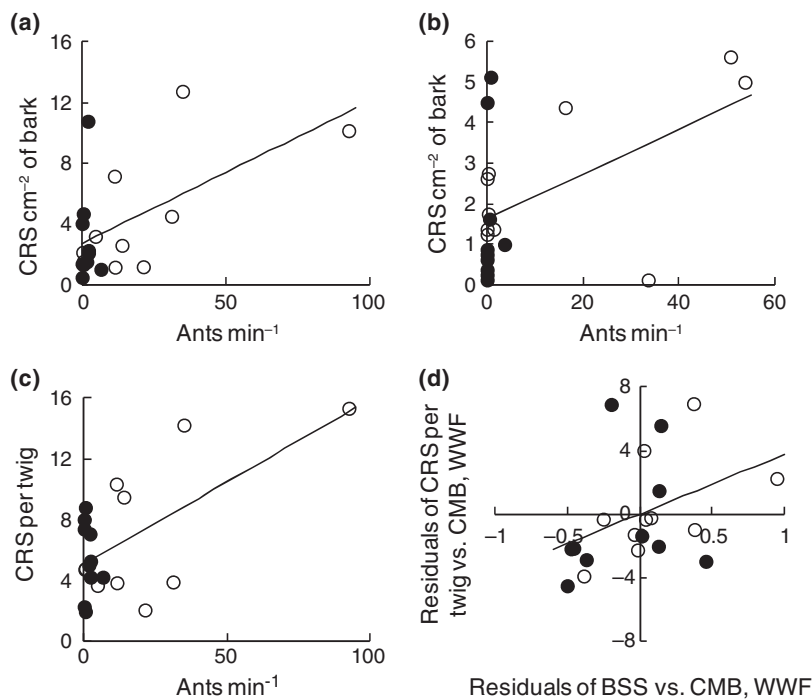


Fig. 3. Effects of Argentine ant recruitment on California red scale (CRS) density on bark substrates in 2009 (a) and 2010 (b) and on twig substrates in 2009 (c). (d) Partial regression plot showing the effect of brown soft scale (BSS) abundance on CRS abundance per twig after controlling for the effects of citrus mealybug and woolly whitefly (least-squares fit, $y = 3.73x$, $r = 0.40$). Statistical results of full linear regression models are reported in Table 2. Open circles, control trees; solid circles, removal trees. CMB, citrus mealybug; BSS, brown soft scale; WWF, woolly whitefly.

Table 3. Results of global hypothesis tests, by logistic regression analysis, for effects of ant and honeydew-producing insect densities on California red scale (CRS) in control and removal trees.

Year	Response variable	Explanatory variable(s)	χ^2	d.f.	<i>P</i>	Table for estimates
2010	Proportion fruit infested	Ants min^{-1}	103.71	1	<0.001	S7
2010	CRS parasitism on fruit	Ants min^{-1}	4.85	1	0.028	S7
2010	Proportion fruit infested	CMB, BSS, WWFa	135.29	3	<0.001	S7
2010	CRS parasitism on bark	Ants min^{-1}	0.26	1	0.613	S7

The regressions of CRS parasitism (on fruit and bark) on honeydew-producing hemipteran (HPH) abundances are not included because CRS parasitism was not sampled in HPH-removal trees.

CMB, citrus mealybug; BSS, brown soft scale; WWFa, woolly whitefly aggregations.

decreased with increasing ant recruitment (Table 3, Fig. 4a,b, Table S7). The proportion of infested fruit also increased with the abundances of citrus mealybug and woolly whitefly, but not brown soft scale (Table 3, Fig. 4c,d; see Table S7 for model-averaged parameter and precision estimates). These effects appear localised, however, because the proportion of parasitised red scale on bark (measured on the trunk) was independent of the level of ant recruitment (Tables 3 and S7).

Indirect effect of citrus mealybug on brown soft scale

Ant attendance at aggregations of brown soft scale was significantly influenced by both aggregation size and the abundance of citrus mealybug on the same flush [model: attendance = f (aggregation size, citrus mealybug abundance); $r^2 = 0.41$, $F = 11.65$, $df = 2, 33$, $P < 0.001$]. In particular, the effect of neighbouring citrus mealybug abundance on ant

attendance of brown soft scale aggregations was significantly positive [$t = 4.23$, $P < 0.001$, estimate (SE) = 0.110 (0.026); Fig. 5], even after accounting for the effect of aggregation size on ant attendance [$t = 2.49$, $P = 0.018$, estimate (SE) = 0.097 (0.039)].

Discussion

In this study we examined whether ants engaging in mutualisms with HPHs disrupt biological control of red scale, and, if so, whether densities of red scale therefore increase in the presence of multiple species of HPHs. Multiple lines of experimental evidence support the first hypothesis: red scale density increased and levels of parasitism decreased with increasing levels of ant activity. Ant activity, in turn, was positively associated with HPH abundance in the canopy. We also received support for our second hypothesis. Different HPH

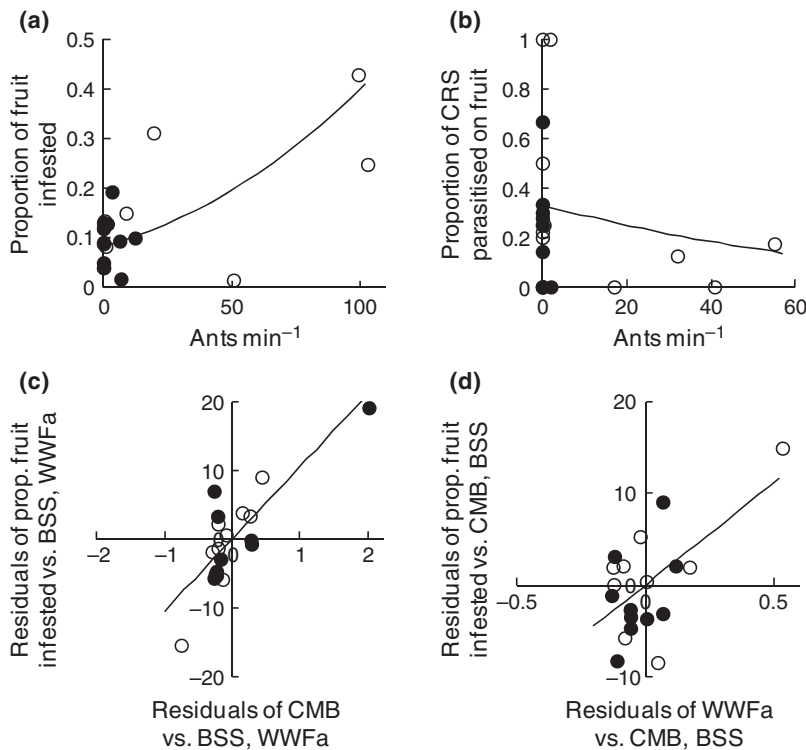


Fig. 4. Effects of Argentine ant and honeydew-producing hemipteran (HPH) abundances on California red scale (CRS) abundance and parasitism on fruit. (a) Proportion of CRS-infested fruit as a function of ant recruitment. (b) Proportion of parasitised CRS (vulnerable instar 2 and virgin instar 3) on fruit as a function of ant recruitment. Partial regression plots showing the individual effects of citrus mealybug (c) and woolly whitefly (d) abundance per twig on the proportion of fruit per tree infested with CRS after controlling for the effects of all other HPH species in the multiple logistic regression model. The curved lines in (a) and (b) are logistic regression models fitted by maximum likelihood estimation. The least-squares fitted models for the partial regression plots are: (c) $y = 10.47x$, $r = 0.82$, and (d) $y = 22.29x$, $r = 0.60$. Statistical results for all logistic regression models are reported in Table 3. Open circles, control trees; solid circles, removal trees. CMB, citrus mealybug; BSS, brown soft scale; WWFa, woolly whitefly aggregations.

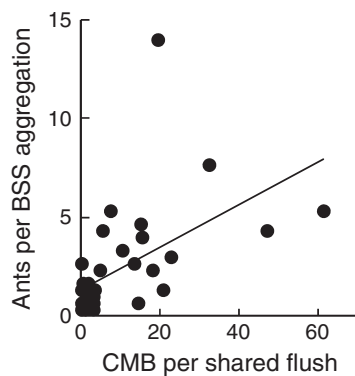


Fig. 5. Positive indirect effect of local citrus mealybug (CMB) abundance (x) on brown soft scale (BSS) aggregations, mediated through ant attendance (y). $y = 0.11x + 1.34$; $P < 0.001$, $r^2 = 0.30$.

taxa emerged as significant predictors of red scale density in different parts of the tree. This finding implies that individual species of HPH give rise to unique and localised effects on tending ants. Lastly, we obtained evidence suggestive of positive, indirect interactions between the two most prevalent HPH taxa. Taken together, these results illustrate the potential for mutualist diversity to influence ecological effects external to the mutualism, presumably through changes in the behaviour or local abundance of the shared mutualist partner. Such effects seem most likely in cases where members of the mutualist guild exhibit positive, indirect effects on one another.

Indirect benefits received by red scale stem from strong, reciprocally positive ant–HPH interactions (Figs 1 and 2, Table 2). Although prior ant-removal experiments document positive, indirect effects of the Argentine ant on red scale (Murdoch *et al.*, 1995), our study is the first to pinpoint the ant–HPH mutualism itself as a mechanism underlying this indirect effect. Importantly, red scale density on twigs and the proportion of fruit infested by red scale both increased in the presence of HPH (Figs 3 and 4). These results further demonstrate the potential for interactions between ants and HPH to influence the structure of arthropod food webs (Wimp & Whitham, 2001; Styrsky & Eubanks, 2007). Interference with parasitoids, coupled with the fact that ants appear to ignore red scale, helps to explain the existence of positive, indirect effects between the ant–HPH mutualism and this abundant herbivore. More common are cases in which ants negatively and directly interact with untended herbivores (Styrsky & Eubanks, 2007).

Although the presence of both ants and HPHs positively influenced the density of red scale on twigs and the proportion of infested fruit, we additionally detected effects of ant recruitment (but not HPHs) on increased density of red scale on bark (Table 2, Figs 3 and 4, Table S6). There are several possible causes of the apparently more pervasive effects of ants on red scale. First, ants forage in lemon trees primarily to tend HPHs, but also to visit flowers. Disruption of biological control of red scale may thus be operating, to some extent, even on trees without HPH. Secondly, ants access trees by forming recruitment trails on the trunk and primary branches, which are locations in the canopy that typically lack HPHs but where

red scale is common. Lastly, enemies of red scale are deterred not by relatively sedentary HPHs, but by highly mobile ants, which wander throughout the tree canopy, including portions of the tree where HPHs are absent.

The effect of ants on red scale might be expected to be strongest in the interior of trees, where ant trails are largest, but we failed to detect effects of ant recruitment (or HPH abundance) on red scale parasitism on the bark. Three possible reasons for this result include the patchy distribution of red scale on bark (i.e. this factor increases the sampling error in estimates of parasitism rate), the naturally low levels of parasitism by *Aphytis* on bark [e.g. *A. melinus* parasitises at higher rates on red scale in the exterior of the tree (Murdoch *et al.*, 1989)], and the tendency for ants travelling along recruitment trails to remain on (or close to) the trails themselves. By contrast, indirect effects of ant recruitment on the density of red scale on twigs and on the proportion of infested fruit were strong, suggesting that the interference of parasitism may be at its highest in areas of the tree preferred by *A. melinus*, the key parasitoid in the system.

A second aim of this study was to evaluate the hypothesis that red scale densities increase in the presence of multiple HPH species. Abundances of each of the focal HPH species emerged as positive predictors of red scale density in different analyses. Red scale density on twigs was positively related to the abundance of brown soft scale (Fig. 3d), whereas the proportion of fruit infested by red scale was positively related to the abundances of citrus mealybug and woolly whitefly (Fig. 4c,d). These findings imply that individual HPH taxa may exhibit unique and spatially localised effects on tending ants. Exclusive focus on a single HPH species could thus yield an incomplete understanding of the ecological effects of ant–HPH mutualisms.

Evidence that the multi-species, ant–HPH mutualism examined here affects the density of a key herbivore illustrates the importance of considering ecological effects of mutualistic interactions that are more inclusive than simple pairwise interactions (see also Kaminski *et al.*, 2010). HPH guilds, for example, may provide ants with honeydew more consistently or for a longer period during the season than that produced by any single HPH species. The two most common HPHs in the present study, for example, exhibit distinctly different seasonal peaks in abundance; brown soft scale reaches its peak abundance in early summer, whereas citrus mealybug reaches its peak in early fall. Assuming these offset phenologies prolong periods of ant activity in lemon trees, negative effects of ant foraging on the parasitoids of red scale would manifest over a longer period of time. Mutualist diversity may also alter honeydew availability across a variety of spatial scales. Within a tree, the presence of multiple HPH species could cause ants to traverse a larger fraction of the tree canopy than they would if tending only a single HPH species. At larger spatial scales, variation in the species diversity and composition of the mutualist guild could play a key role in determining the prevalence and strength of the indirect effect between red scale and the ant–HPH mutualism.

Multi-species, ant–HPH assemblages seem especially likely to give rise to effects external to the mutualism when mutualists

interact positively with one another (Kaminski *et al.*, 2010). Although the present study did not investigate such effects in detail, evidence points to the existence of positive, indirect effects between the citrus mealybug and brown soft scale, which were the most abundant HPH taxa (Table 1). The density of brown soft scale populations depended on the level of ant recruitment into trees (Fig. 1c), and ant visitation to brown soft scale aggregations was in turn influenced by the local abundance of the citrus mealybug (Fig. 5). Given that the Argentine ant forms populous and expansive colonies that depend on carbohydrates for colony growth (Grover *et al.*, 2007; Kay *et al.*, 2010; Menke *et al.*, 2010), it seems unlikely that HPH taxa compete for tending services in this system. Positive indirect effects among HPH taxa that result from ant tending might thus be expected in other situations where ants form large colonies and exhibit dietary preferences for carbohydrates.

Although research on mutualisms increasingly considers the importance of multi-species mutualisms (Stanton, 2003; Holland *et al.*, 2005; Palmer *et al.*, 2010), few published studies consider how the outcome of a mutualism may be contingent upon the presence of multiple, co-occurring mutualists (Kaminski *et al.*, 2010). Our results argue for a greater appreciation of how mutualist diversity may contribute to the broader ecological consequences of mutualisms. In cases where members of a mutualist guild interact positively with one another, one might expect that the behaviour of the mutually shared partner could be altered in ways that differ fundamentally from its behaviour or numerical response to a single mutualist species. Such changes could give rise to trait-mediated or density-mediated effects on other members of the food web; these effects could be critical to understanding the potential ecological importance of mutualisms.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12042

Appendix S1. Pre-treatment insect densities.

Appendix S2. Treatment effectiveness.

Table S1. Results of two-way ANOVAs, with treatment and block as explanatory variables, on log-transformed, pre-treatment densities of focal insect taxa.

Table S2. Effectiveness of removal treatments at reducing target insect densities. One-tailed paired t-tests are reported for planned comparisons between controls and each removal treatment.

Table S3. Sampling schedule.

Table S4. Akaike Information Criterion corrected for finite sample size (AICc) and Akaike weights used for model selection where explanatory variables are the abundances of individual HPH species.

Table S5. Effects of honeydew-producing hemipteran abundance on ant recruitment, by multiple linear regression analysis.

Table S6. Effects of ant and honeydew-producing insect densities on California red scale (CRS) density in control and removal trees.

Table S7. Effects of mutualist abundances on proportion of fruit infested and parasitism of California red scale (CRS) in control and removal trees in 2010, by logistic regression analysis.

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