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Multitasking in a plant-ant interaction: how does *Acacia myrtifolia* manage both ants and pollinators?

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Abstract

Plant associations with protective ants are widespread among angiosperms, but carry the risk that ants will deter pollinators as well as herbivores. Such conflict, and adaptations to ameliorate or prevent the conflict, have been documented in African and neotropical acacias. Ant-acacia associations occur in Australia but little is known of their ecology. Moreover, recent phylogenetic evidence indicates that Australian acacias are only distantly related to African and American acacias, providing an intercontinental natural experiment in management of ant-pollinator conflict. We examined four populations of *Acacia myrtifolia* over a 400 km environmental gradient in southeastern Australia using ant and pollinator exclusion as well as direct observation of ants and pollinators to assess the potential for ant-pollinator conflict to affect seed set. Native bees were the only group of floral visitors whose visitation rates were a significant predictor of fruiting success, although beetles and wasps may play an important role as “insurance” pollinators. We found no increase in pollinator visitation or fruiting success following ant exclosure, even with large sample sizes and effective exclusion. Because ants are facultative visitors to *A. myrtifolia* plants, their presence may be insufficient to interfere greatly with floral visitors. It is also likely that the morphological location of extrafloral nectaries tends to draw ants away from reproductive parts, although we commonly observed ants on inflorescences, so the spatial separation is not strict. *A. myrtifolia* appears to maintain a generalized mutualism over a wide geographic range without the need for elaborate adaptations to resolve ant-pollinator conflict.

Introduction

Mutualisms between plants and ants are geographically widespread and taxonomically diverse (Davidson and McKey 1993; Heil and McKey 2003; Rico-Gray and Oliveira 2007). Some of the most striking examples of such mutualisms involve acacias. On three different continents, acacias attract ants with food or domiciles and receive, to various degrees, protection from herbivory (Janzen 1966; Buckley 1982 and references therein; Heil et al. 2009). However, this attention from ants may harm a host plant's reproductive success by interfering with pollinators or directly damaging reproductive structures. Such ant-pollinator conflict is known to occur among African and American acacias (Willmer and Stone 1997; Nicklen and Wagner 2006), and has been documented in other ant-plant taxa such as *Cordia nodosa* (Yu and Pierce 1998) and myrmecophilous *Ferocactus wislizeni* (Ness 2006). Dual effects such as these pose an adaptive challenge for host plants, which must simultaneously attract pollinators while profiting from the protection ants might offer. No investigation of such conflict among Australian species of *Acacia* has been reported. Our work examines the effect of ants on pollination and fruiting of *A. myrtifolia* in southeast Australia, providing a contrast with acacias elsewhere that can be interpreted in the context of new discoveries about the phylogenetic relationships among the world's acacia lineages (Maslin et al. 2003, Brown et al. 2008, Murphy et al. 2010).

A potentially important distinction for the strength of plant-ant interactions depends on whether host plants house resident ant colonies in domatia or attract ants facultatively from their surroundings (Rico-Gray and Oliveira 2007; Chamberlain and Holland 2009). Many acacias in the Old World and the neotropics produce domatia and have strong interactions with their resident colonies. Elegant strategies to avert conflict between ant guards and pollinators have been demonstrated in some of these species. *Acacia zanzibarica* in Tanzania, *A. collinsii* in Costa

Rica, *A. hindsii* in Mexico, and *A. constricta* in Arizona temporarily separate pollinators and ants by repelling ants from open flowers, probably with volatile chemicals (Willmer and Stone 1997; Ghazoul 2001; Raine et al. 2002; Nicklen and Wagner 2006). The repellent effect occurs only during the single day of anthesis, but this brief segregation of ants and pollinators allows rates of fruit set to be positively correlated with overall ant activity despite the potential for ant-pollinator conflict (Willmer and Stone 1997).

In contrast, acacias in Australia do not seem to form obligate mutualisms with resident ants (Knox et al. 1986), but instead recruit a wide variety of visitors from local ant communities, attracting them with extrafloral nectar (EFN). Extrafloral nectaries are common in Australian *Acacia* (Boughton 1981; Marginson et al. 1985), as they are in the related genus *Inga* and throughout the Fabaceae generally (Weber and Keeler 2013). Strategic placement of extrafloral nectaries may ameliorate the risk of conflict between ants and pollinators, as has been suggested for some North American species. For example, *Acacia constricta* in Arizona appears to use large numbers of extrafloral nectaries with unequal nutritional quality of nectar to distract visiting non-resident ants and reduce their presence on flowers (Wagner and Kay 2002). Similarly, extrafloral nectaries on new vegetative growth in *A. hindsii* lure dwelling ants away from inflorescences produced on older shoots, reducing conflict between the beneficial and inimical activity of ants (Raine et al. 2002).

The quantity and quality of EFN may even play a role in regulating the nature of a mutualism (Heil et al. 2009). A comparison of *A. cornigera* and *A. hindsii* in Mexico, for example, showed that higher concentration and amino acid composition of EFN elicited greater activity and aggressiveness of mutualist *Pseudomyrmex* ants (González-Teuber and Heil 2009; González-Teuber et al. 2012). The mutualism of *A. hindsii* with *Pseudomyrmex* involves sucrose-

free EFN, which *Pseudomyrmex* requires but exploiter species dislike, thus reinforcing the mutualism (Kautz et al. 2009).

The wealth of present evidence concerning acacia-ant interactions comes almost exclusively from African and American species. Very little is known about the ecology of acacia-ant interactions in Australia, including the potential for conflict between ant protection and pollinator service (Stone et al. 2003). This limitation in our knowledge is especially unfortunate as the Australian acacias represent a remarkable radiation of more than 1000 species (Luckow et al. 2003; Murphy 2008). Together with a diverse ant fauna (Fisher 2010), they provide an underutilized opportunity to explore the evolution of plant-ant interactions.

Here we report an experimental study of the effect of ants and floral visitors on reproduction in the Australian species, *Acacia myrtifolia* (Sm.) Willd., which has a widespread geographical distribution, suitable size for experimental manipulations (less than three meters tall), and is visited by several ant species. Our main aim was to determine whether ant attendance on plants poses a net risk to pollination and fruit set. Because the degree of any plant-ant conflict could be context-dependent, we conducted experiments at four geographically separated sites spanning a range of habitat characteristics and background ant faunas. We start with the supposition that facultative ant visitors to *A. myrtifolia* are capable of interfering with pollinator activity on inflorescences, but with no knowledge of whether the degree of interference matters for maternal reproductive success of the plants. However, we hypothesize that the generalized plant-ant relationship found in this species is not sufficiently intense to pose the same conflict with pollinators documented for acacias elsewhere. Specifically, we hypothesize that ant exclusion will have no short-term consequences for reproductive success. We manipulated both ant and pollinator access to our study plants and measured the resulting fruit and seed production.

We also used direct observation of pollinator activity and pollinator exclosures to assess the roles of different kinds of pollinators in effecting fruit set.

Materials and methods

Study species

Acacias around the world were placed in a single genus for two centuries (Miller and Burd 2014), but they are now known to form a paraphyletic group of morphologically and ecologically similar species. The African and American lineages, now assigned to the genera *Vachellia*, *Senegalia*, *Mariosousa* and *Acaciella*, are only distantly related to the crown group of *Acacia sensu stricto*, which occurs predominantly in Australia (Maslin et al. 2003; Brown et al. 2008; Murphy et al. 2010) (Online Resource 1). In this article, we use the genus name *Acacia* to refer to members of this paraphyletic group to maintain consistency with older literature. However, *A. collinsii*, *A. constricta*, *A. cornigera*, *A. hindsii*, *A. nilotica*, *A. tortillis* and *A. zanzibarica* are now placed in *Vachellia*, and *A. senegal* in *Senegalia*.

Acacia myrtifolia is a member of the *Acacia sensu stricto* crown group of acacias. It is an understory shrub usually 0.5 to 3 meters tall, distributed mainly in southern Australia (Australia's Virtual Herbarium 2011) and often locally abundant in sandy soil of forests, woodland, and heath (Flora of Australia 2011). *Acacia myrtifolia* bears a prominent gland on the margin of each phyllode that secretes EFN all year (Bernhardt and Walker 1984; Marginson et al. 1985; pers. obs.), with standing crops usually less than 2 µl (Martínez-Bauer 2014). Thus, EFN secretion in *A. myrtifolia* is constitutive rather than induced in response to herbivory, as occurs in some

Central American acacias (Heil et al. 2004). Flowering usually occurs from August to October, during late austral winter and early spring (Knox et al. 1985). Populations are in flower for about 6 weeks, with a peak about halfway through this period, and inflorescences are visited by a wide variety of insects. Inflorescences are racemes of 3–20 globular heads (capitula) which themselves contain 2–5 yellow, nectarless flowers.

Acacia myrtifolia is visited throughout the year by several ant species. Pitfall trapping revealed that ants from eight of the nine subfamilies in Victoria (Shattuck 1999) occurred in our sites. Ants were abundant at each site, and there appeared to be no shortage of potential ant partners to interact with *A. myrtifolia*.

Study sites

We selected four widely separated sites in southeastern Australia containing *A. myrtifolia* along a general gradient from temperate rainforest in the southeast to dry, sparse *Eucalyptus* woodland in the northwest. The easternmost site, Croajingolong National Park (NP), is the largest and least disturbed of the four sites (Online Resource 2 and 3). The westernmost site, Grampians NP, is second in size to Croajingolong and also relatively undisturbed (Online Resource 2 and 3). Brisbane Ranges NP consists of forested hills in an agricultural landscape, but at 77 km² is relatively small and isolated (Online Resource 2 and 3). Dandenong NP is an area of low hills at the outer fringes of Melbourne with a complex pattern of *Eucalyptus* forest mixed with areas of human habitation, making it the most fragmented and disturbed of the four sites (Online Resources 2 and 3).

Experimental design

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142 The experiment was based on a factorial exclusion of ants and pollinators. The pollinator
143 exclusion incidentally allowed us to test for autogamous fruit set and for the relative effect of
144 large and small pollinators. We also made direct observations of floral visits, allowing statistical
145 identification of the most effective pollinators among all visitors.

146 We tagged 60 plants of similar size (1 m high) at each of the four sites. Half were
147 randomly assigned to an ant-exclusion treatment and half were left unmanipulated (ant control).
148 We excluded ants with a Fluon (polytetrafluoroethylene)-coated acetate disc 10 cm in diameter
149 fixed around the stem of a plant at 15 cm above the ground. The disc created an effective barrier
150 that prevented ants from reaching the upper portions of the plant. We removed any plants in
151 contact with *A. myrtifolia* plants in the ant-exclusion treatment to prevent alternative access
152 routes for ants. On subsequent visits, we again removed plants as necessary, and replaced any
153 exclusion discs that had become weathered. We checked for the presence of ants while collecting
154 data from the experimental plants to verify the efficacy of the exclusion treatment.

155 At each site, the plants in each ant visitation treatment were randomly assigned to one of
156 three pollination treatments. One branch on each of ten plants was enclosed in white fabric with a
157 mesh diameter of 3.7 mm in order to exclude large pollinators (especially the introduced
158 honeybee, *Apis mellifera*); one branch on another ten plants was enclosed in fabric with a mesh
159 diameter of 1.46 mm, small enough to exclude all flying insect visitors that were likely to be
160 pollinators; and one branch on each of the remaining ten plants was tagged as the focal branch
161 but left unmanipulated as a control (pollinator control). The pollinator exclusions allowed the
162 passage of air, water and light and were placed during the last week of June and the first week of
163 July, 2010, when the plants had only immature flower buds.

164

Ant presence on plants

To determine the extent of ant patrolling on *A. myrtifolia* and to assess the efficacy of the ant exclusion treatment, we examined each ant-control and ant-exclusion plant at every site for five or three minutes, respectively, on every sampling date between July 2010 and December 2011 (five sampling periods per site). We recorded every ant observed during these periods, noting whether it was tending EFN glands or patrolling the plant. Ants were then collected and identified to species and morphospecies in the laboratory using the keys of Shattuck (1999) and Andersen (1991) and later updated with Bolton's (2013) online ant catalogue.

Floral visitors

Patterns of floral visitation were assessed between the last week of August and early October 2010 (one sampling period per site). Each observation period from 10:00 to 15:00 consisted of 10 minutes observation per plant, rotating among plants at each site. We considered a floral visitor to be any insect that directly contacted anthers and assigned it to one of five groups: beetles, flies, native bees, *Apis mellifera* and wasps. To assess which taxa are most likely to be efficacious pollinators, we collected a subsample of the floral visitors after they left the inflorescences following the procedures of Kearns and Inouye (1993). These specimens were later scrutinized under a dissecting microscope to determine whether *A. myrtifolia* pollen (which consists of distinctive polyads) adhered to their bodies. Since all insects were killed together in a jar containing ethyl acetate vapor, an insect was considered a vector only if it carried 20 or more polyads, as the polyads are large and it seems unlikely that this level of pollen adherence would be due to cross-contamination among individuals. Collected specimens were identified by

A.M.B. to species or to morphospecies, which were then further identified by Dr. Ken Walker (Museum Victoria) and deposited in the Entomological Collection at the Melbourne Museum, Australia.

Plant reproductive success

We tested the effects of the ant and pollination treatments on fruit production. At the end of the reproductive season we counted mature pods on each plant and expressed female reproductive success as the proportion of inflorescences (racemes of capitulas) in which a fruit was produced. Across all sites, 82% of capitula on control plants produced a single mature pod, percentage calculated as an average of three consecutive years for control plants (with ant and pollinator access) suggesting that the inflorescence was the primary functional unit of female reproductive success. Since there were an average of 2.83 ± 0.7 flowers per capitulum ($n = 94$), the rate of fruit production per flower would be approximately one-third of the rate per capitulum.

Analysis

We used analysis of variance (ANOVA) as the principal analytical tool to detect effects of site, ant exclusion, and pollinator access treatment on mean floral visitation rates and on fruit set. Full three-way ANOVA designs including all interaction effects were tested for each of the dependent variables. Analyses were implemented in *R* version 3.0.1 (R Core Team 2013).

We wished to identify the effect of each major pollinator group on reproductive success in *A. myrtifolia*. To do this, we used multiple regression with visitation frequencies of each pollinator group as the independent variables and the proportion of capitula bearing fruit as the dependent variable in SYSTAT. We conducted this analysis with individual plants and with site means as the units of observation. This latter procedure assumes that measurement of pollinator visitation over periods of short duration at individual plants is subject to excessive sampling variation, so that differences among site-wide averages will be more informative. Because averaging leaves only four observations (the sites) and five independent variables (visitation rates for each pollinator type), we performed a forward stepwise regression (using $\alpha = 0.15$ as the criterion for both entry and subsequent removal) to find the optimal model.

Results

Ant activity on Acacia myrtifolia

Across all sites we recorded 57 species from five subfamilies of ants tending EFN glands or foraging on *A. myrtifolia* (Table 1). Plants at each site were visited by some ant species unique to the site (Table 1). The average activity of ants on the ant-control plants with free access was approximately 21 ants per hour per plant, pooling across all observation periods during the year of study. There was minor variation in visitation rates among sites, but overall there appears to be no shortage of ant visitors at any site. The potential for ant visitors to interfere with the reproductive interests of host plants was demonstrated by the frequent presence of ants on

inflorescences (Fig. 1). We happened to observe two instances in which bull-ants (*Myrmecia* sp.) attacked and captured flying insects that visited *A. myrtifolia* inflorescences.

The efficacy of the ant exclusion treatment was confirmed. Ants were completely absent from the ant-exclusion plants at the Croajingolong and Brisbane Ranges sites. On rare occasions at the other two sites a barrier was breached when an exclusion disc was damaged or an alternative access route had become available: two plants during the entire study at the Grampians site, seven plants at the Dandenong Ranges site. Even with these occasional imperfections, it is clear that ant activity was very sharply reduced in the exclusion treatment.

Floral visitation

There was substantial taxonomic diversity among floral visitors and variation in the composition of visitors among sites (Online Resource 4). Among the 57 morphospecies identified in total, there were 30 species from eight families of Hymenoptera, 22 morphospecies from 15 families of Diptera, and five morphospecies from four families of Coleoptera. Polyads were observed on at least one individual in 92% of the bee species, 82% of the wasp species, 77% of beetle species and 36% of fly species. Thus, flies may be poor pollinators despite their diversity as floral visitors. *Apis mellifera*, colletid bees and tenebrionid beetles were the most widespread taxa of floral visitors and the most consistent pollen carriers.

Visitation rates present a somewhat different picture from the taxonomic richness pattern (Table 2). Ant-exclusion treatments had no effect on visitation rates (ANOVA, $F_{1,882} = 0.38$, $P = 0.54$). There were, however, significant differences in mean visitation rate among the four sites (ANOVA, $F_{3,882} = 4.36$, $P = 0.005$), significant difference among the visitor groups ($F_{5, 882} =$

9.60, $P < 0.0001$), and, most importantly, a significant interaction between these two effects ($F_{15,882} = 978$, $P < 0.0001$), indicating that the taxonomic mix of visitors was not homogeneous among sites. This variation is relevant for understanding fruit set in *A. myrtifolia* (see below). The introduced honeybee, *Apis mellifera*, was a frequent visitor at the Grampians (15.2 visits hr^{-1} plant $^{-1}$) but nearly absent from Croajingolong (0.04 visits hr^{-1} plant $^{-1}$), the least fragmented and most remote site. In contrast, two morphospecies of small native *Leioproctus* (Colletidae) bees unique to Croajingolong were the most common visitors at that site (5.9 visits hr^{-1} plant $^{-1}$). Native bees visited much less frequently at the other sites. Beetles were especially common floral visitors at the Dandenong Ranges, the most fragmented site, but were common also at Croajingolong (Table 2).

Fruit production

The mean proportion of capitula that yielded fruit in control plants open to all pollinators differed significantly among sites (Table 3), with higher rates at Croajingolong (0.319) and Grampians (0.196), the least fragmented sites, and low rates at Brisbane Ranges (0.052) and Dandenong Ranges (0.078), the smaller and more disturbed sites (Fig. 2). Ant exclusion had only a small (Fig. 2) and statistically insignificant (Table 3) effect on fruit set. Exclusion of large pollinators, mainly *Apis mellifera*, substantially reduced but did not eliminate fruit production. Exclusion of all pollinators eliminated nearly all fruit set at the Grampians and Dandenong Ranges sites, allowed slight fruit set at the Brisbane Ranges, but yielded unexpectedly high fractions of fruit-bearing capitula among most plants at Croajingolong (site mean = 0.12) (Fig. 2). It is possible that the pollinator exclusion bags failed or that autogamous selfing occurred, which would imply that the Croajingolong population has evolved an anomalous breeding system, since

self-incompatibility has been thought to be a general characteristic of Australian *Acacia* (Bernhardt et al. 1984) and of *A. myrtifolia* (Kenrick 1994).

Regression analysis to assess the importance of the five pollinator groups indicated that native bees had the greatest effect on fruiting success in *A. myrtifolia*. Using individual plants as observations, the overall model was significant ($F_{5,47} = 2.55$, $P = 0.04$; $R^2 = 0.21$), but, more importantly, only native bee visitation had a significant independent relationship to fruit set ($t_{47} = 3.28$, $P = 0.002$). Using site-wide averages, stepwise regression allowed entry of only the native bee visitation rate as a significant predictor ($F_{1,2} = 37.6$, $P = 0.026$; $R^2 = 0.95$). These results are consistent with the high frequency of polyad presence on native bees, and less frequent polyad carrying by other groups, especially flies, despite their frequent flower visitation.

Discussion

Plant-ant associations can be a double-edged sword because the activity of ants may have inimical as well as beneficial consequences for plants. Some acacias face this dilemma, since ants that protect their hosts from herbivores also interfere with pollinators, and these acacias have evolved countermeasures to circumvent the conflict (Willmer and Stone 1997; Raine et al. 2002; Wagner and Kay 2002). *Acacia myrtifolia*, in contrast, seems uninvolved in such conflicts: neither presence nor absence of ants had much consequence for reproductive success of host plants at four sites across the range of the species. This is a curious difference in the apparent intensity of the plant-ant relationship.

We therefore surmise that either (1) the rate of ant activity on *A. myrtifolia* is insufficient to disrupt pollination and reduce fruit set (even in the absence of any adaptive countermeasure by

the host plant), or (2) ant activity could interfere with pollination but the threat is obviated in some way. We consider each of these possibilities in turn. Our present data do not point to one or the other as the definitive explanation, but by considering the evidence we can highlight the importance of phylogenetic history in shaping acacia-ant interactions and suggest future avenues to explore intercontinental and inter-lineage comparisons of these interactions.

Do ant visitors pose a threat to pollinators?

Many Australian ant species that visit extrafloral nectaries are aggressive predators (Stone et al. 2003; Lach et al. 2009). Thus, the potential to disrupt pollination exists, and the two fatal attacks we observed by bull-ants on floral visitors to *Acacia myrtifolia* illustrate the danger. But low visitation frequency by ants could make that danger remote. Our data suggest that an average of 21 ants visit an *A. myrtifolia* plant each hour. In the absence of similar data from acacias with domatia and resident colonies we cannot make quantitative comparisons. However, it seems likely that the total number of ants on a plant at any moment would usually be fewer than a colony of resident ants might provide. *Acacia myrtifolia* hosts a shifting assembly of ant species across the portion of its range that we studied (Table 1) and does not form obligate relationships with resident ants. The absence of resident colonies, then, may limit the extent of conflict between ants and pollinators, even though occasional encounters prove deadly.

Obligate mutualisms with resident colonies have evolved in Africa and America, but not in Australia (Knox et al. 1986). This geographic difference also has a phylogenetic context. Over 98% of Australasian acacia species are now known to form a distinct monophyletic group, *Acacia sensu stricto*, only distantly related to the African and American lineages that were, until recently, also assigned to the genus *Acacia* on the basis of morphological and ecological

similarity (Maslin et al. 2003, Brown et al. 2008, Murphy et al. 2010). Swollen stipules serve as domatia for the obligate mutualisms in Africa and America (Janzen 1966; Heil et al. 2009), but stipules are uncommon among species of *Acacia s.s.* Did the paucity of this morphological pre-adaptation for domatia prevent the evolution of obligate myrmecophytes in Australia? It is possible, but spinescent stipules occur among the tribe Ingeae and in smaller clades at the base of the radiation of *Acacia s.s.*, such as the *A. victoriae* and *A. pyrifolia* clade and the Pulchelloidea clade, suggesting that this condition may have been ancestral in Australian *Acacia* (Murphy et al. 2010). Furthermore, spinescent stipules do exist in some species like *A. paradoxa*, which is clearly derived well within the radiation of *Acacia s.s.* (Murphy et al. 2010). There is little known about ant-plant relationships among basal lineages of *Acacia s.s.* and among close outgroups in the tribe Ingeae, a deficit that limits what we can understand of the reasons myrmecophytes did not evolve in Australia. Our findings point to the need to collect such data to facilitate comparative approaches to understanding the phylogenetic context of mutualisms in acacias.

Does Acacia myrtifolia ameliorate ant-pollinator conflict?

The second possibility is that ant activity is sufficient to interfere substantially with pollination but that *A. myrtifolia* suppresses or obviates the conflict. In acacias on other continents, ant-pollinator interactions are limited by two mechanisms: volatile repellents and distraction by extrafloral nectaries. The former is unlikely to act among Australian *Acacia*. Nicklen and Wagner (2006) suggested that ant repellents may be a general feature of acacia flowers, but they had examined only species from Africa or the neotropics. The phylogenetically distant *A. myrtifolia* appears not to have chemical repellents. We observed several species of ants walking on *A. myrtifolia* inflorescences at all stages of development (buds, young open flowers, mature, and old

flowers) (Fig. 1). Unlike the case with other acacias, precision in suppressing ant-pollinator interactions with volatile repellents for the single day of anthesis is probably not possible in Australian *Acacia* species, because they produce long-lived inflorescences of non-synchronously blooming flowers (Bernhardt et al. 1984; Bernhardt & Walker 1985; Knox et al. 1985; Kenrick 1994; Stone et al. 2003). Formal testing for repellent floral volatiles in choice experiments among Australian species is needed, but the willingness of ants to patrol amongst flowers is evident in the field (Fig. 1).

A more plausible mechanisms to control ant pollinator conflict, if it exists in sufficient intensity, is the presence of EFN on phyllodes all year around. Extrafloral nectaries would tend to attract ants to vegetative parts of the plant and thereby reduce the time that ants are in contact with inflorescences of *A. myrtifolia*. Extrafloral nectaries of *Acacia constricta* in Arizona have been shown to have this effect (Wagner and Kay 2002), and Chamberlain and Holland (2008) identified a similar distraction strategy in *Pachycereus schottii* (Cactaceae). Our results are consistent with the EFN-distraction hypothesis, but direct support is required. As initial observational support, we would expect the proportion of time ants spend in contact with inflorescences to be less than the proportion of surface area that inflorescences represent relative to vegetative surface. Experimental ablation or masking of some of a plant's extrafloral nectaries should then increase the proportion of time ants spend at nearby inflorescences. Experimental addition of nectar at novel locations (while controlling the overall attractiveness of a plant by removing natural nectaries) should alter ant attendance at inflorescences in relation to the distance of novel nectar sources from inflorescences. Such experiments are feasible and needed.

It is interesting that the location of nectaries away from inflorescences seems not necessarily to be sufficient by itself to resolve ant-pollinator conflict between African and American acacias and their resident ants in domatia (Willmer and Stone 1997; Raine et al. 2002).

The distraction effect of EFN may diffuse ant-pollinator conflict only in the absence of strong ant-plant mutualism and abundant activity of resident ants. Among Australian *Acacia*, the lack of floral nectar and the spatial separation of flowers and nectar sources would reduce the incentives for ants to patrol inflorescences and may neutralize their potential for interfering with pollination services. Long-lived, coriaceous phyllodes among *Acacia* species may also reduce the need for herbivory protection, favoring modest rewards and generalist associations with multiple ant species.

Some non-myrmecophyte American acacias, such as *A. cochliacantha* and *A. farnesiana*, produce EFN only after damage to plant tissue (Heil et al. 2004; González-Teuber et al. 2009) thus limiting ant activity on flowers (and whole plants) when it is unnecessary. The timing of ant attendance in *A. pennatula*, in Mexico is further complicated by the presence of an alternative food source for ants: grass-feeding leafhoppers provide honeydew, an abundant food source that ants prefer to EFN, so that activity on *A. pennatula* is strong only during the late dry season when the grass hosts dry up and leafhopper populations crash (Moya-Ragoza and Larsen 2001). The diversity in these relationships suggests that any distraction effect of EFN in Australian *Acacia* may vary with geography and the particular suite of ant species and pollinators that are attracted at a given time and place. For example, we predict that experimental manipulation of EFN availability would have greater effect on fruit set at our Croajingolong site than at the other sites because of the greater importance of native bees as pollinators at that site.

Strength of the host-ant interaction

We have evidence from the ant-exclusion treatment that ant visitors provide small, long-term growth benefits for *A. myrtifolia* (Martínez-Bauer 2014). Given the small amount of EFN offered on phyllodes, *A. myrtifolia* appears to offer a low cost, low benefit interaction with ants. It seems likely that increased nectar secretion could elicit more intense ant activity on *A. myrtifolia* hosts were selection to favour it (cf. González-Teuber et al. 2012). However, the current interaction may provide some indirect protection against herbivory while posing little problem for pollination and reproduction, in contrast to many other Old World and neotropical species with obligate mutualisms involving domatia and resident ant colonies. This contrast seems to derive from different geographic and evolutionary histories of the several lineages called acacia.

In the meta-analysis of Chamberlain and Holland (2009), plant species that offered ants only EFN rather than domatia or food bodies received greater ant diversity and the smallest benefit in herbivory protection. Plant species offering only EFN did experience, on average, small but significantly positive effects of ant attendance on growth and reproductive performance (Chamberlain and Holland 2009). This is a mean effect across many studies, but plants offering only EFN may often not receive sufficient ant activity to interfere substantially with pollinators, unlike plants with resident ants that give sustained attention to their hosts to protect their food resource and domatia. Much more complete study of the strength of EFN-based ant-acacia relationships would be needed to assess the general strength of the interaction, especially for comparisons among continents and among major acacia clades.

Which pollinators are most important to Acacia myrtifolia?

Most flower visitors showed interest overwhelmingly in pollen and not towards EFN, except in some cases of beetles or parasitic wasps, as reported also by Bernhardt and Walker (1985). Australian *Acacia* and acacias elsewhere tend to be extreme pollination generalists (Bernhardt and Walker 1984; Bernhardt and Walker 1985; Tybirk 1993), although bees and wasps are the largest group of pollinators in *Acacia* s.s. (Bernhardt et al. 1984; Thorp and Sugden 1990; Stone et al. 2003). *Acacia myrtifolia* fits this pattern (Online Resource 4). We found that the visitation by native bees was the principal factor that significantly explained variation in fruit set across sites. However, other floral visitors may play an important role in a hierarchy of pollinators. The presence of polyads on several morphospecies of Coleoptera and Diptera (7 and 8 respectively) and their visitation frequency suggest that these insects could play a role of “insurance” pollinators, providing some degree of reproductive success to *A. myrtifolia* (and other *Acacia* species: Bernhardt et al. 1984; Bernhardt and Walker 1985; Stone et al. 2003). At the final, late winter stage of flowering by *A. myrtifolia*, other nearby flowering plants start to bloom, attracting Hymenoptera and apparently diverting them from the less attractive resource that *A. myrtifolia* provides (Bernhardt and Walker 1985). The benefits of “insurance” pollination may be particularly strong for late-season flowering plants. Furthermore, Romero *et al.* (2011) found in a meta-analysis that hymenopterans decrease their visitation rate and time spent on flowers in the presence of predators, but coleopterans and dipterans show no response. The role of coleopterans is also regarded as fundamental for successful pollination for acacia species from India, Africa and Brazil (*A. albida*, *A. nilotica*, *A. tortillis* and *A. (S.) senegal*: Tybirk 1993; *A. (Senegalia) senegal*: Tandon et al. 2001; *A. mearnsii*: Stiehl Alves and Marins-Corder 2009).

Overall, the introduced honeybee, *Apis mellifera*, appears to play a minor role in pollination, although it was present at all sites. Even at the Grampians, where the visitation rate by *A. mellifera* was over three times greater than at any other site, fruit set was one-third the rate

at Croajingolong. The 26 different native Hymenoptera species carrying polyads may account for the apparent lack of significant effect of *A. mellifera* on fruit set. Because *A. mellifera* carries pollen in the corbiculae on the hind legs, it is not clear if *Acacia* polyads are effectively transferred (Prescott 2005). In the case of native female bees, pollen is loose and carried on parts of the body that contact stigmas, and so the transfer of polyads is more effective (Prescott 2005).

Conclusion

Acacia myrtifolia is likely to be typical of Australian *Acacia* in having a generalized mutualistic relationship with many ant species, involving only modest resource investment. Nonresident ants are likely to provide some degree of anti-herbivore activity without much cost to the host plant in nectar production or in lost pollination. Experimental exclusion of ants did not improve fruit set in *A. myrtifolia*, possibly because its casual mutualism with nonresident ants poses little conflict with pollinators or because EFN tends to draw ants away from flowers. The absence of an ant effect on fruit set is unlikely to be a statistical effect of low power. The number of individual plants we tested, 240 in total, was large relative to previous studies of other host species that have found statistically significant effects of ant exclusion on reproductive success, including *Vaccinium reticulatum* (ant-exclusion vs. control branches on 10 plants) (Bleil et al. 2011), and *Hirtella physophora* (22 ant-exclusion inflorescences vs. 33 control inflorescences) (Malé et al. 2012). In general, ant-exclusion experiments have been able to reveal statistically significant reductions in herbivory or increases in growth and reproduction across a wide taxonomic range of host plants and attendant ants (Chamberlain and Holland 2009).

469 At least some acacias in Africa and America have evolved close and sometimes obligate
470 mutualisms with ants, involving the provision of high quality food resources and domatia. It is
471 interesting that such strong mutualisms seem not to have evolved during the radiation of
472 Australian *Acacia* into more than 1000 species (Murphy et al. 2010; The Legume Phylogeny
473 Working Group 2013), despite an abundant and diverse ant fauna. Although mutualisms
474 involving domatia are evidence of strong adaptation, we believe more study of facultative
475 associations in Australian *Acacia* and acacias on other continents is needed to take full advantage
476 of the independent evolutionary histories of the acacia lineages for comparative analysis.

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Tables

Table 1. Taxonomic distribution of ants tending EFN or foraging on *A. myrtifolia*, among the four sampling sites. Values indicate total number of species (sp) or morphospecies (msp), from which unique (u) sp or msp to sites are indicated in parenthesis. Sites: C = Croajingolong, G = Grampians, B = Brisbane Ranges, and D = Dandenong Ranges. The individuals from the subfamily Myrmeciinae, considered as specialist predators (Andersen 1995), are shown in boldface.

Subfamily	Pooled across sites		Per site			
	No.	No.	C	G	B	D
	Genera	sp	msp (u)	msp (u)	msp (u)	msp (u)
Dolichoderinae	6	12	6 (1)	8 (1)	6 (1)	3 (0)
Ectatomminae	1	2	1 (0)	1 (0)	1 (0)	2 (1)
Formicinae	9	22	16 (3)	7 (1)	4 (0)	11 (0)
Myrmeciinae	1	10	2 (1)	5 (3)	4 (1)	2 (0)
Myrmicinae	4	11	4 (2)	7 (1)	3 (0)	2 (1)

Table 2. Rate of visitation of five groups of flying insects to *A. myrtifolia* flowers (mean no. visits hr⁻¹ plant⁻¹ ± 1 standard deviation among individual plants). Sites: C = Croajingolong, G = Grampians, B = Brisbane Ranges, and D = Dandenong Ranges.

	Site			
	C	G	B	D
<i>Apis mellifera</i>	0.04 ± 0.3	21.8 ± 23.7	6.2 ± 9.3	0.3 ± 1.3
Native bees	5.9 ± 10.4	1.8 ± 3.6	0.2 ± 1.0	0.4 ± 2.3
Wasps	0.7 ± 3.8	2.1 ± 4.7	0.8 ± 2.3	1.1 ± 2.8
Beetles	2.9 ± 7.2	0.1 ± 0.9	0 ± 0	6.1 ± 13.2
Flies	0.8 ± 2.9	1.4 ± 2.6	2.2 ± 4.7	0.7 ± 1.7

Table 3. ANOVA of experimental effects on fruit set in *Acacia myrtifolia*. Fruit set was square-root transformed to homogenize the variances. Significant effects are shown in boldface.

Effect	df	MS	<i>F</i>	<i>P</i>
Site	3	0.371	95.51	0.000
Ant exclusion	1	0.010	2.72	0.101
Pollinator exclusion	2	0.257	66.09	0.000
Site × Ant exclusion	3	0.001	0.35	0.786
Site × Pollinator exclusion	6	0.039	10.02	0.000
Ant exclusion × Pollinator exclusion	2	0.008	2.1	0.125
Site × Ant exclusion × Pollinator exclusion	6	0.001	0.26	0.953
Error	216	0.004		

Fig. 1 Ants walking on young and old *Acacia myrtifolia* flowers. A) *Myrmecia* spp. B) *Notoncus* spp. C) Unidentified Dolichoderinae or Formicinae

Fig. 2 Proportion of *Acacia myrtifolia* capitula producing fruit, by site, ant and pollinator treatment. Lines represent medians, boxes are interquartile ranges, lines show total range with outliers as filled circles. Ant treatment: A = ants present (ant control plants) and N = ant-excluded plants. Pollination treatment: panels a to d = open pollination, e to h = total pollinator exclusion and i to l = large pollinators excluded.

Figure 1.

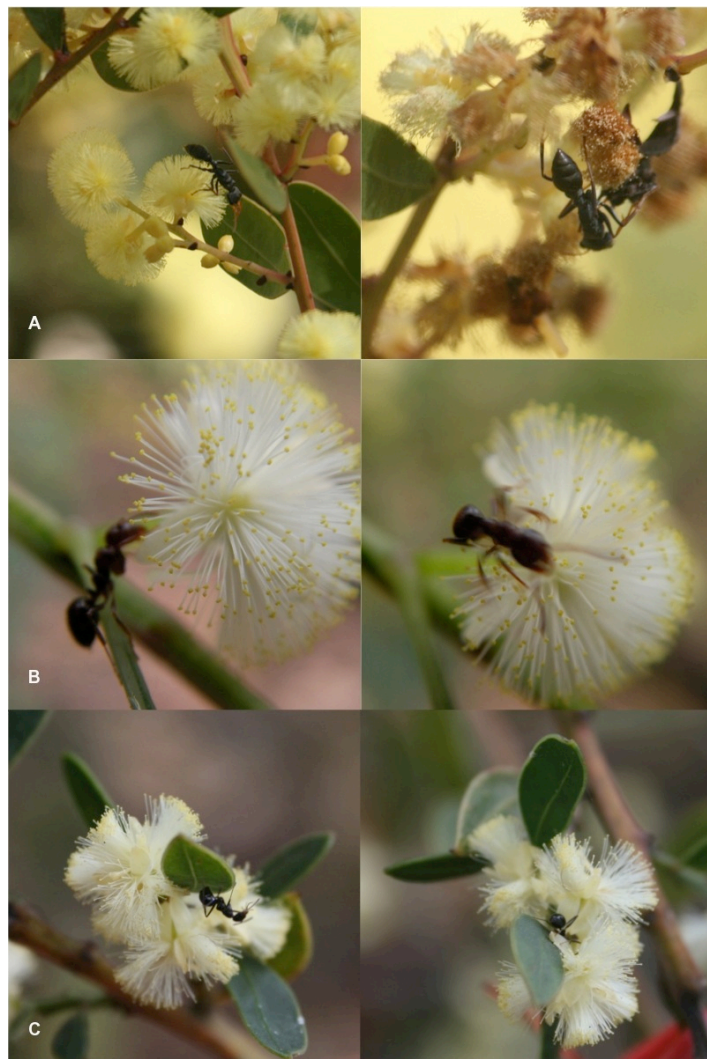


Figure 2.

