

## FLORAL SCENT IN A WHOLE-PLANT CONTEXT

## Floral volatiles controlling ant behaviour

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## Summary

1. Ants show complex interactions with plants, both facultative and mutualistic, ranging from grazers through seed predators and dispersers to herders of some herbivores and guards against others. But ants are rarely pollinators, and their visits to flowers may be detrimental to plant fitness.
2. Plants therefore have various strategies to control ant distributions, and restrict them to foliage rather than flowers. These ‘filters’ may involve physical barriers on or around flowers, or ‘decoys and bribes’ sited on the foliage (usually extrafloral nectaries - EFNs). Alternatively, volatile organic compounds (VOCs) are used as signals to control ant behaviour, attracting ants to leaves and/or deterring them from functional flowers. Some of the past evidence that flowers repel ants by VOCs has been equivocal and we describe the shortcomings of some experimental approaches, which involve behavioural tests in artificial conditions.
3. We review our previous study of myrmecophytic acacias, which used *in situ* experiments to show that volatiles derived from pollen can specifically and transiently deter ants during dehiscence, the effects being stronger in ant-guarded species and more effective on resident ants, both in African and Neotropical species. In these plants, repellence involves at least some volatiles that are known components of ant alarm pheromones, but are not repellent to beneficial bee visitors.
4. We also present new evidence of ant repellence by VOCs in temperate flowers, which is usually pollen-based and active on common European ants. We use these data to indicate that across a wide range of plants there is an apparent trade-off in ant-controlling filter strategies between the use of defensive floral volatiles and the alternatives of decoying EFNs or physical barriers.

**Key-words:** ant guards, *E,E*- $\alpha$ -farnesene, evolutionary filters, extrafloral nectar, floral repellence, morphological floral barriers, pollen volatiles

## Introduction: costs and benefits of ants on flowers

Many plant species are able to attract ants for defence against herbivores. Some plants possess extrafloral nectaries (EFNs) on their foliage, which attract nectar-gathering ants, while a subset of these species (myrmecophytic plants) offer the ants specialized structures as shelter (domatia) and sometimes protein bodies as food (Bentley 1977; Heil & McKey 2003). Often, ants protect the plant from herbivores that graze the photosynthetic tissues of leaves, but theoretically ants could also protect a plant's flowers from florivores. However, ants are usually unwelcome as flower visitors for several reasons.

First, they have a limited potential as pollinators (Janzen 1977; Hölldobler & Wilson 1990; Peakall, Handel & Beattie 1991) because they are typically of small size, making them a poor physical fit for the sexual parts of most flowers; their smooth, hairless cuticles are poorly suited for pollen adhesion; and their low mobility due to winglessness makes them less likely to effect cross-pollination. Moreover, most ants possess metapleural glands that produce anti-microbial agents, necessary for nest hygiene (Fernández-Marin *et al.* 2006), but detrimental to pollen longevity and fertility (e.g. Beattie *et al.* 1984; Galen & Butchart 2003).

Second, ants may interfere with the plant's effective pollinators. Aggressive ants may deter some incoming flower visitors, including legitimate pollinators (e.g. Altshuler 1999; Galen 1999; Tsuji, Hasyim & Nakamura 2004; Gaume, Zacharias & Borges 2005; Ness 2006; Junker, Chung & Blüthgen

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2007); notably, the invasive ant *Linepithema humile* is known to significantly reduce the diversity of floral visitors to a wide range of plants (Lach 2008). Such effects will usually be detrimental, but can enhance outcrossing if ants make pollinators move more frequently between plants (see Maloof & Inouye 2000). In addition, ants may act as nectar 'thieves', thereby reducing the attractiveness of flowers to effective pollinators by removing nectar rewards, which may reduce pollinator visit frequency or duration (Galen & Geib 2007), and thus reduce seed set.

Third, ants can act as florivores themselves by harvesting certain floral structures or constituents. For example, ants have been observed cutting the styles in *Polemonium* (Galen 1983), partly destroying flower buds in the semi-myrmecophyte *Humboldtia* (Gaume, Zacharias & Borges 2005), or destroying *Cordia* flowers (Edwards & Yu 2008). Izzo & Vasconcelos (2002) demonstrated that *Hirtella myrmecophila* plants produce flowers only on branches that lack the structures (leaf-pouch domatia) that the plant otherwise uses to attract ants for protection against herbivory.

Overall, ants are potentially deleterious to flowers, and reducing these harmful effects on reproduction can exert substantial selective pressures that favour adaptive responses by plants. Perhaps unsurprisingly, therefore, many plant species have adaptations to protect their valuable floral structures, which can be seen as 'filters' for ants (cf. Kautz *et al.* 2009). We propose that there are three main types: architectural barriers; decoys and bribes (e.g. food or lodging located some distance from the flowers); and chemical deterrents, often using floral volatile organic compounds (VOCs).

In common with other plant defences, such filters can have variable properties: they may be permanent features, or transient to coincide with flowering; they may be constitutive (varying mainly with plant age), or inducible and only appearing in response to ant presence or herbivore damage (e.g. Heil 2002, 2004); and they may be broad-spectrum filters against any ants, or highly specifically targeted (especially in myrmecophytes). Currently, the roles and interactions of plant defensive filters in floral protection are relatively unexplored. Here, we review the current state of knowledge about the filters that may protect flowers against ants. Initially, we briefly survey the ecology of architectural barriers and decoys and identify their limitations as filters, before turning to our main theme, which is the role of VOCs.

#### ARCHITECTURAL FILTERS

Physical defences come in many varieties (Kerner 1878; Guerant & Fiedler 1981; Beattie 1985), and tend to be permanent and broad-spectrum filters. Some act as barriers, and these include: water moats in bracts or calyces (e.g. *Heliconia*, *Lathraea*, some *Tillandsia*); mucilage in the calyx (e.g. *Commelina*, *Malvaviscus*); dense pubescence around the corolla base (e.g. *Witheringia*); sticky surfaces preventing access for walking ants (e.g. *Chamaecrista desvauxii*); or waxy surfaces too slippery to be negotiated (Harley 1991), present in some zoophilous flowers and acting against non-specialist ants (e.g.

Federle & Rheindt 2005). Dense leathery calyces (e.g. *Dianthus*) and inflated calyces (e.g. *Silene*) may also restrict ant access to flowers, while thin and/or pendant flower stalks that bend easily may also deter most larger ant species.

Other barrier defences include physically damaging structures such as small thorns, and penetrating or secretory trichomes. Some *Dalechampia* species have moveable bracts that close around the flowers at night, preventing ~90% of nocturnal florivory (Armbruster *et al.* 1997). Other floral movements may have similar effects, and many diurnal or post-visitation changes (flower shape, orientation, scent and colour) could be interpreted in this light, making a flower inconspicuous to ants and other enemies after pollination.

In addition to barriers on stems, sepals and calyces, the corolla itself may be defended internally with rings of fine hairs, an extremely narrow tube or a specific constricted zone, or with nectary 'lids' so that only the tongue of a pollinator can reach the nectar.

For myrmecophytic plants, another architectural option is to locate domatia well away from the flowers (Izzo & Vasconcelos 2002; Raine, Willmer & Stone 2002), a physical feature that is unusual in being inducible since domatia production can be triggered by the presence of ants on foliage (Blüthgen & Wesenberg 2001).

#### DECOYS AND BRIBES

Extrafloral nectaries (EFNs) occur in more than 90 angiosperm families and in some ferns (e.g. bracken, Tempel 1983), and can attract ants (and other predators) onto plants where they act as guards, with the EFN nectar regarded as fuel for generalist plant protectors (Koptur 2005). Some recruited ants are so effective that they can provide biological control of herbivores (e.g. *Oecophylla* weaver ants, Tsuji, Hasyim & Nakamura 2004).

Herbivory (and hence potential selection for guards) is much older than insect pollination, so EFNs may well predate floral nectaries. In this light, we hypothesize that EFNs may have evolved an important secondary role as attractive decoys to keep ants away from the floral nectar and out of flowers in both myrmecophytes and non-myrmecophytes (Wagner & Kay 2002). To tailor the bribe to its target, ant-defended plants sometimes offer EFN nectar with reduced sucrose (resulting from high invertase levels) and/or higher amino acid levels relative to floral nectars, both features that are often preferred by ants (Wagner & Kay 2002; Heil, Rattke & Boland 2005). EFNs are normally broad-spectrum filters, but may be either permanent and constitutive, or inducible. In either case, the prevalence of EFNs may be related to the vulnerability of the plant's structures. For example, there may be greater EFN production on the youngest (most valuable) leaves of both myrmecophyte and non-myrmecophyte plants (Heil *et al.* 2000; Radhika *et al.* 2008), and amino acid levels in EFNs may rise after simulated herbivory (Smith, Lanza & Smith 1990). There is also evidence of selection for increased EFN nectar production at peak periods of herbivore activity in *Macaranga* (Heil

*et al.* 2000), in populations exposed to higher levels of herbivore damage in *Chamaecrista fasciculata* (Rios, Marquis & Flunker 2008), and for synchrony with peak ant activity periods in some Malpighiaceae (Pascal & Belin-Depoux 1991). Additionally, there has been a clear shift to constitutive secretion in the myrmecophytic ant-acacias where ants are always present (Heil *et al.* 2004). However, we are not aware of published evidence for increased EFN secretion during flowering, which would be necessary to support the hypothesis that EFNs act to decoy ants from flowers.

Nevertheless, the use of architectural barriers and EFNs as filters against ants does have its problems, because ants may still be present on or close to flowers. How does the plant achieve pollination, seed-set and seed-dispersal, which commonly rely on other animals gaining access, without excessive ant interference (cf. Bronstein, Huxman & Davidowitz 2006)? In acacias the fruits are large, pendant and tough, mainly bird- or mammal-dispersed, and these agents are little bothered by ants. In fact ant-guarded plants often have higher seed-set than unguarded congeners (e.g. Wagner 1997; Willmer & Stone 1997), though the converse can also occur with pollinators deterred by particularly aggressive ants like *Solenopsis* (Ness 2006). But pollination is a very different matter. Where resident ants are timid, pollinators may still function normally, and in one case (semi-myrmecophytic *Humboldtia*) ants and pollinating *Braunsapis* bees can co-occupy domatia on the plant (Shenoy & Borges 2008). But aggressive ants pose more substantial problems. Physical defences and EFNs are potentially long-lasting once the plant has invested in them, whereas flowers lacking any potential architectural barriers, irrespective of whether they have EFNs, might need to use chemically-based behavioural filters operating on a shorter time scale for full control of such ants. Potentially these could be transient, inducible and highly specific, thus reducing the overall costs of ant management, which brings us back to our main topic.

#### CHEMICAL DETERRENCE: VOCS AS FILTERS AGAINST ANTS

Volatile organic compounds can originate from flowers, foliage or seeds. Some cases of non-floral VOCs that control ant behaviour and distribution in mutualisms are already known. For example, young leaves of *Leonardoxa* produce an ant-attractant VOC blend (perhaps involving methyl salicylate) (Brouat *et al.* 2000), ensuring that ants mainly patrol there and provide maximum benefit against herbivores and, presumably, minimal interference with flowers. Foliage VOCs can also be used to recruit ants to damaged leaves (e.g. *Hirtella*, Romero & Izzo 2004; *Macaranga*, Inui & Itioka 2007), and they may be important for initial recruitment to myrmecophytes (e.g. *Cordia* (Edwards *et al.* 2006); and *Macaranga* (Jürgens *et al.* 2006) where foundress ants could distinguish the volatile profiles of different potential host plant leaves). Seed VOCs elicit ant-carrying behaviour of elaiosomes (Brew, O'Dowd & Rae 1989) and mediate specific *Peperomia* seed-collection by ant-garden *Camponotus* ants (Youngsteadt *et al.*

2008). More non-floral examples of this kind would be very valuable in establishing the range of chemical components that can influence the behaviour of ants.

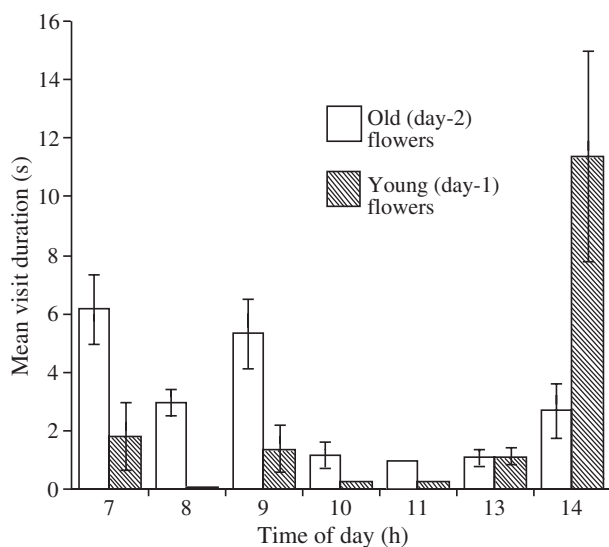
Floral VOCs are taxonomically extremely widespread and commonly act as flower scents attractive to pollinators. Plants often manipulate their release in time or space; for example floral emissions of the thistle *Cirsium arvense* are maximized when pollinators are abundant, and reduced when folivores are active (Theis, Lerdau & Raguso 2007). Furthermore, differential effects of floral odour can be seen across populations in relation to ant control in *Polemonium viscosum*, which at higher (alpine tundra) altitudes is bumblebee-pollinated with larger corollas and a sweet scent, but below the tree line is visited by flies and small bees and has smaller corollas with sticky calyx trichomes producing a 'skunky' odour that repels abundant *Formica* ants (Galen, Zimmer & Newport 1987).

Flowers commonly contain the same secondary plant compounds used as defences in the rest of the plant (Heil 2008), though often at reduced concentrations or altered proportions. These can deter foliage or flower feeders, and in some cases once released at the right time or site as VOCs can also attract appropriate parasitoids and predators that reduce plant damage. Responses induced by VOC signals generated by damage to nearby plants (Heil, Lion & Boland 2008) may also affect flowering time or frequency (e.g. Strauss *et al.* 1999). These same plant defence compounds could be either repellent or attractant to ants. For example in lima beans both VOCs and EFNs are inducible defences and both attract ants, though the EFNs have a more substantial effect (Kost & Heil 2008).

However there are instances of specific chemical defences against ants in flowers. Preliminary suggestions that ant-repellent floral nectar might be widespread (Janzen 1977) are now discounted (Feinsinger & Swarm 1978; and discussion in Junker, Chung & Blüthgen 2007), although occasional examples occur (see Adler 2000). Chemical defences could occur in floral tissues instead, either as cytoplasmic feeding deterrents or as VOCs, and could be inducible. *Nicotiana* corollas develop more nicotine when the plant is under attack by folivores (Euler & Baldwin 1996), and *Raphanus* flowers have higher glucosinolate levels when leaves are damaged (Strauss, Irwin & Lambrix 2004). More direct effects on flower defences when a plant undergoes florivory are poorly documented, but *Nemophila* flowers damaged artificially or by caterpillars can develop increased resistance, and suffer less subsequent petal damage (McCall 2006).

Willmer & Stone (1997) presented the first evidence of ant-repellence in flowers, using East African ant-defended acacias (*Vachellia zanzibarica*\*), summarized in Fig. 1. Here pollinators were able to access young inflorescences during a brief 2–3 h interval when dehiscence was occurring, and when the aggressive resident ants (*Crematogaster* spp.) were deterred

\*Acacia Classification. In 2005 the long-standing genus *Acacia* was formally revised and split into several new genera including *Vachellia*, *Senegalia* and *Acaciella*, with *Acacia* to be used only for Australian species.



**Fig. 1.** Mean visit durations of ants to young and old flowers of *Vachellia (Acacia) zanzibarica*, with extremely short visits to young flowers during the dehiscence window (from Willmer & Stone 1997). Means  $\pm$  SEM.

from normal patrolling activities. Since repellence could be transferred from young flowers to old (normally non-repellent) flowers by superficial contact, it was apparently due to chemicals present on the flowers, and/or emitted from them.

Besides the example of *Vachellia-Crematogaster*, which demonstrates the existence of VOC filters against ants, how widespread are volatile-mediated specific ant and flower interactions? To address this question, we review the published studies of VOC filters that have been postulated in several ant-plant systems and identify limitations in the evidence that supports their existence, so that our ability to assess the prevalence of VOC filters against ants is greatly constrained. We therefore critically review the methodologies used in the study of VOC filters and present new data both for myrmecophyte ant-acacias from Africa and Central America, and for northern European plants.

#### APPROACHES TO ESTABLISHING VOCS AS ANT-REPELLENT FILTERS THAT PROTECT FLOWERS

Following Willmer & Stone's (1997) work, various kinds of evidence have been cited as indicating the presence of repellent VOCS in flowers. In a few instances the evidence is largely circumstantial, describing the temporal coincidence of ant absence with anthesis. Otherwise, studies have been based on behavioural tests, usually in artificial conditions with enclosures or olfactometers, but occasionally *in situ*. We argue that in many cases the evidence produced by behavioural studies in artificial conditions is largely inconclusive, as the following examples show.

Several studies have examined the behaviour of ants in simple arenas with sections treated by contact with floral tissues. Ghazoul (2001) reported ant-repellence as extremely com-

monplace in tropical flowers, by recording ant positions in Petri dishes where one half had been 'wiped' with flowers and showing that ants avoided the wiped area. Ness (2006) used similar protocols to examine effects of *Ferocactus wislizeni* petals, and found that only one of four ant species tested spent more time in the non-wiped half, correlating well with the ants' observed occurrence on flowers. Agarwal & Rastogi (2008) also used the same technique to implicate floral repellents in *Luffa* plants, which deterred five of six visiting ant species, with only the smallest sized ant (*Tapinoma* sp.) being unaffected.

Other studies have compared the responses of ants to flowers vs. 'flower-like' objects. For example, Jaffé *et al.* (2003) studied encounter rates of ants with flowers of Venezuelan plants *in situ*, compared with 'control sticks' of similar size to the flowers, and found lower 'ant-repellency' in forest canopy flowers compared to savanna flowers. Junker, Chung & Blüthgen (2007) also studied behavioural effects of whole flowers, but presented in an artificial arena with a similar-sized stick as control; they reported ant-repellence for 8 of 18 species from Borneo, using groups of five *Dolichoderus* ants, with greater repellence in canopy flowers than in forest understorey flowers.

Overall, we have some reservations as to whether these behavioural studies have fully established floral VOCS as filters against ants. Our reasons are as follows. First, most of the studies did not allow separation of 'contact' vs. volatile ant-repellence; and where floral compounds were presented to ants after floral tissue was wiped on a surface, the contact between surface and flower could yield both chemical and textural cues. Furthermore some approaches lacked clear controls for chemical effects from cut surfaces or damaged floral tissue. Additionally, single ant responses were often not quantified; but when multiple ants are tested together there are potential confounding effects from individuals following each other (using visual cues, surface chemical trails or airborne pheromones), thus magnifying apparent effects. Finally, repellence was always recorded merely in terms of ant location relative to floral cues.

Most recently, Junker & Blüthgen (2008) used four-way olfactometric assays with 30 flower species, excluding any possibility of contact chemoreception mediating repellence and with cut stalks immersed in water to minimize contaminating volatiles from wounds. They exposed ants in groups of ten, recording the numbers found in each odour field after 2–5 min (thus again suffering possible magnification effects from group behaviours). *Camponotus floridanus* was repelled from 20 of the 30 flowers tested, and *Lasius fuliginosus* from 8 of 26. The ants were also repelled by some individual floral scents, notably linalool, geraniol,  $\alpha$ -pinene and limonene, among the most ubiquitous floral scent compounds (Knudsen *et al.* 2006). Despite our reservation about the testing of ants in groups, this study nevertheless raises interesting questions as to why such ubiquitous floral VOCS should be ant-deterrent when most VOC-mediated communication in ants is based on rather specific ranges of compounds.

Overall, we cannot yet evaluate the prevalence of VOC-based filters against ants. Hence we present below our own case studies, where some or all of the confounding effects that we identified above have been eliminated.

Our first case study concerns myrmecophytic ant-acacias. An ideal test flower would be one with no physical floral barriers, where rewards (pollen and/or nectar) are freely available to any visitors. Acacia inflorescences exemplify this situation, being composed of many tiny individual corollas with abundant protruding anthers forming a pompom-like 'flower surface', and visited by a wide range of insects. Furthermore, visiting ants are known to be unwelcome, as they reduce pollen viability (Wagner 2000). We studied (1999–2006) an acacia community in a Kenyan savanna, where at least 12 acacia species co-occur and 6–8 species may co-flower, but each releases pollen in a different diurnal 'dehiscence window' (Stone, Willmer & Nee 1996; Stone, Willmer & Rowe 1998).

Our second case study concerns temperate plants and ants. Here we exposed *Formica aquilonia* to flower volatiles of 67 temperate plants and *Lasius niger* to a subset of these species.

In both cases, we utilized a method of administering species-specific VOCs to individual ants using an air-puffing method, and we record stereotyped alarm behaviours as a proxy for floral repellence.

## Materials and methods

### ACACIA STUDIES

Acacia studies were conducted at Mpala Research Centre (Laikipia Province, Kenya), 1999–2006, in a mixed-species acacia savanna. *Vachellia drepanolobium* trees bear domatia inhabited by one of four ant species: *Crematogaster sjostedti*, *C. mimosae*, *C. nigriceps*, or *Tetraponera penzigi* (Stanton, Palmer & Young 2002). Domatia also occur on *V. seyal* subspecies *fistula*\* (though not on subspecies *seyal*<sup>†</sup>), and are occupied by *C. sjostedti*. Other local acacias generally lack 'resident' ants, but most are invaded by vagrants (Fig. 5b), commonly two *Camponotus* species (Young, Stubblefield & Isbell 1997).

### Volatile collection and analysis

Volatiles were obtained from acacia inflorescences with head-space collection techniques (Raguso & Pellmyr 1998), using at least four inflorescences per tree, and 2–4 trees per species. On the evening before anthesis buds were enclosed in polyacetate bags to avoid early visitation or access by ants. From 07.00 the next day head-space samples were collected using battery-operated membrane pumps (air flow 100 mL min<sup>-1</sup>). Teflon cartridges containing 80 mg Tenax GR (mesh-size 60/80) provided the adsorbent, and were collected and replaced every 2 h to obtain diel profiles of scent release. 'Blank' samples of buds and vegetative parts were collected in parallel. Following scent collection, the adsorbent tubes were stored frozen until extraction. Adsorbed scent was eluted with 300 µL diethyl ether, and eluates were concentrated at room temperature and analysed by GC-MS using a 30 m medium polar column (HP-INNOWAX) (inner diameter

0.25 mm, d.f. 0.25 µm). GC-programming was 3 min at 40°, then 8° min<sup>-1</sup> to 230 °C, and steady for 10 min (see Knudsen 1999). Quantifications were made against the mean of two internal standards (methyl stearate, furfuryl octanoate) added to samples directly after elution.

### Assessment of flowers and pollen availability

For each acacia species used the timing of pollen grain availability ('dehiscence window') was assessed from pollen slides (see Stone, Willmer & Nee 1996). Acacia anthers consist of locular tissue forming the cup in which polyads mature, a stalked lid-like 'anther gland' which 'unzips' at dehiscence and stands above the anther until knocked off by visitors, and the polyads themselves; each anther contains 8 polyads (diameter 27–40 µ) and one gland (diameter 45–100 µ). Polyads cannot be lifted or shaken from the inflorescence (experimentally, or by visiting insects) without also gathering many glandular lids; thus different anther components cannot be tested on ants individually. Instead, we used a magnifier (x30, Lumagney, Revel Ltd., Harrow, UK) to score '% lids up' and '% pollen depletion' as measures of dehiscence progression *in situ* for each inflorescence. Mean pollen available was the difference between these scores (availability 0% until the lid rises, then 100% until some is removed by visitors); this allowed direct comparisons of pollen availability, lid availability, and ant responses.

### Behavioural bioassays

Single acacia inflorescences were picked with clean forceps and placed in 10 mL syringes (slightly larger diameter than the flower heads, so avoiding damage and pollen disturbance). The syringe was sealed and stored for 5 min. An 'air-puff' with volatile-loaded air was then ejected gently from the syringe (5 mL in 5 s) at ants resting on twigs or foliage, or feeding at EFNs, with the syringe tip at ~50 mm range. A flower-loaded syringe allowed two trials, each releasing 5 mL of volatile-laden air. Response scores were: 0 = no response; 1 = antennal response; 2 = 1 plus abdominal cocking or mandibular biting; 3 = 1 or 2 plus running activity; these behaviours are known indicators of aggressive and/or alarm responses (Brian 1977; Hölldobler & Wilson 1990; Witte, Attygalle & Meinwald 2007). Control syringes with no flowers were then tested on the same ants within 2 min, and corrected responses derived by subtraction.

### Statistical analyses

Behavioural responses of each ant species to each acacia species were incorporated into a GLM analysis, testing responses to flowers and differences between floral and control responses. Dehiscence timing was also included, as % pollen available (with proportional data arcsine square root transformed), and as time of day (divided as 'in' or 'out' of the known population dehiscence window). Tukey's post-hoc tests were run as sub-commands to assess specific differences between ant responses. Rank order correlations between ant species and floral emissions were compared using the Kendall coefficient of concordance (*W* values; Siegel & Castellan 1988).

### TEMPERATE PLANT SPECIES

Temperate flowers were assessed in St Andrews, using *Lasius niger* ants from local gardens and *Formica aquilonia* ants collected from Scottish pine forests, maintained in a formicarium with standard ant food.

<sup>†</sup>*V. seyal seyal* and *V. seyal fistula* were formerly regarded as subspecies, but will be properly reclassified as full species (Dr Stephen Harris, Dept. of Plant Sciences, Oxford, pers. comm).

### Behavioural assays

The flowers were tested in a laboratory setting again with air-puffs, the loaded and blank syringes connected to a Y-junction in the lid of a Petri dish. Single ants were 'settled' in the dish for 5 min before testing, again with floral and then blank samples. Then standardized 'volatile reaction score' for ant alarm/aggression in a 1 min exposure was calculated from the duration of mandible opening (M) or abdomen cocking (A), each converted to rank values of 0–6 and 1–7 respectively (giving greater weight to the more specific abdominal response), and the number of head up responses (H). The score used was  $(M + A + H) \times (\%/10)$  of all ants showing any aggressive response). This proportional correction recognized that fewer ants giving a small response was more informative than just one or two ants responding strongly. Final mean scores thus calculated ranged from 0 to ~90. For some species, anthers alone were used in syringes, comparing dehiscent and post-dehiscent phases.

### Assessment of flowers, pollen availability and plant barriers

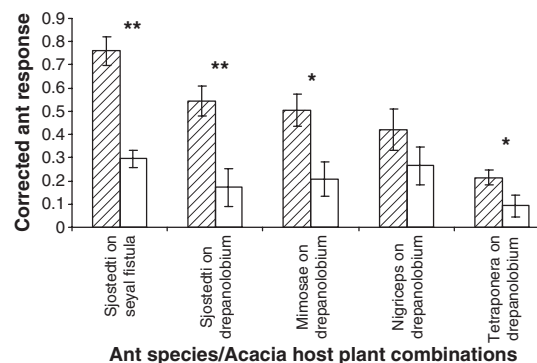
For temperate flowers, pollen availability was assessed by direct inspection of anthers. Each temperate plant species was assigned a defence score from 0 to 3, based on the number and degree of traits restricting ant access to flowers. 0 = no restrictive traits; 1 = one trait that partially restricts ants; 2 = two or more traits that partially restrict ants; 3 = morphology that completely excludes ants. Presence of EFNs as decoys was also included as a defensive trait here.

## Results and discussion

### ACACIAS

Using the behavioural bioassay *in situ* we found that temporary ant-repellency resulting from VOC emission during dehiscence is common in young acacia flowers, but varies greatly in intensity between species. The flower volatiles tested also elicited different response levels in different resident ant species, with the rank-order being *Crematogaster sjostedti* most responsive, *C. mimosae* and then *C. nigriceps* intermediate, and *Tetraponera penzigi* least responsive (Fig. 2); these rank-orders were the same for all six acacia species providing adequate data for testing ( $H_0$  = rankings independent:  $W = 0.543$ ,  $P < 0.05$ ), and they match, in order of decreasing aggression, the dominance rank order for the ants from these acacias (Stanton, Palmer & Young 2002). Responses of non-resident ants (*Camponotus*) were usually undetectable above control levels. Matching of aggression and repellence hierarchies supports the hypothesis that floral repellents have been selected to keep the most aggressive ants away from flowers that require pollinating insect visitors.

The ant-acacia *Vachellia seyal fistula* had the most ant-repellent flowers, eliciting responses significantly ( $n = 223$ ,  $\chi^2 = 3.99$ , d.f. 1,  $P < 0.05$ ) above the average level from all resident ant species, and with especially high responses when assayed within the dehiscence window. More generally, the responses for every instance of resident ant species tested on its own myrmecophyte acacia were always stronger when tested within the specific dehiscence windows, compared to

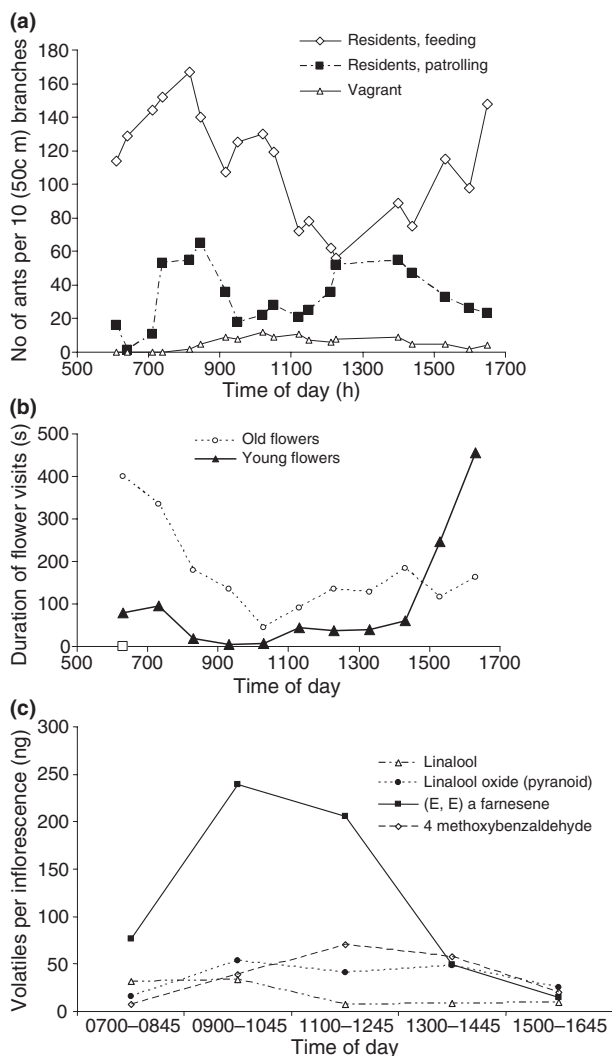


**Fig. 2.** Behavioural responses of different ants to flower volatiles from the eight acacia species collectively, showing both the hierarchy of response levels between ant species, and the greater responses during dehiscence. Responses of ants are means, corrected by subtraction of responses to clean air puffs (see Methods). The first four comparisons show different species of genus *Crematogaster*, the last one shows *Tetraponera penzigi*; and they derived from two different domatia-bearing host trees, *Vachellia seyal fistula* and *V. drepanolobium*. Hatched bars show mean responses during the dehiscence windows, and open bars are responses outside the windows; error bars are  $\pm 1$  SEM. Significant differences between these two periods ('in' vs 'out' dehiscence window) from GLM analyses are indicated: \*\*  $P < 0.01$ , \*  $P < 0.05$ .

using pre- or post-dehiscent flowers (see Fig. 2); and for 17 out of 26 other ant/acacia combinations tested the dehiscing flowers gave significantly stronger responses than the non-dehiscing flowers (with no significantly weaker responses). More specific time-based data from *V. seyal fistula* are shown in Fig. 3, including its volatile scent profile in successive 2-h samples.

Given that acacia flowers were more ant-repellent when pollen was dehiscing, the volatile signal is likely to derive from the anthers. This is confirmed by ant behaviour patterns; patrolling ants that walked onto the inflorescence 'surface' provided by the massed anther heads made long visits (mean  $126 \pm 27$  s,  $n = 74$ ) to old flowers, but only short visits to young flowers (mean  $17 \pm 9$  s,  $n = 146$ , for all recording times). Specifically in the dehiscence window, the few ants seen on the surface of young flowers were visibly agitated and rapidly departed (mean visit  $1.8 \pm 1.1$  s,  $n = 19$ ). However, occasional ants (< 10% of patrollers) instead foraged deep *within* a young inflorescence at the basal corollas, made longer visits (mean  $245 \pm 72$  s,  $n = 34$ ), and were unaffected by dehiscence. These patterns are consistent with repellent volatiles being emitted outwards from the peripheral cup-shaped anthers, rather than from the basal corollas. Two further lines of evidence locate the repellent signals specifically to the compound pollen grains (polyads):

(1) Timing of ant-repellence differed across species, but always matched the diurnal course of polyad availability for each of eight acacia species tested. For example, on *V. seyal fistula*, ants were more strongly repelled by inflorescences with a higher pollen standing crop so there was a strong positive correlation between percentage pollen availability and the magnitude of the ant response ( $R^2 = 0.368$ ,  $n = 302$ ,



**Fig. 3.** Numbers (a) and durations (b) of ant visits to flowers of the myrmecophytic *Vachellia seyal fistula*, showing the few and very short visits to young flowers in the dehiscence window (09.00–11.00); and (c) the volatile emissions profile for young flowers of this species in 2-h sampling slots, with the peak of *E,E*- $\alpha$ -farnesene corresponding temporally with ant deterrence from flowers.

$P < 0.01$ ). The only other possible volatile source in anthers is the anther gland 'lids' (see Methods); but for all eight species the correlations between % lids present and ant responses were weak and in nearly all cases non-significant.

(2) Ant-repellence persisted when polyads were artificially retained on inflorescences by bagging from dawn to exclude visitors. *Vachellia etbaica* inflorescences with 78% polyads retained, but with 87% anther glands shed (their stalks withered, and shrunken glands fallen from the muslin bag), elicited significantly higher ant responses ( $0.88 \pm 0.12$ ; see Methods) compared to the normally-visited inflorescences (10% polyads retained, 95% anther glands shed: ant response  $0.18 \pm 0.04$ ).

We conclude that volatiles derive from polyads, so that sequential loss of pollen to visitors structures the time course of repellence, beginning when the 'lids' lift and expose the

polyads and potential pollinators start arriving (Stone, Willmer & Nee 1996) and diminishing as the polyads are progressively removed by visitors. A pollen-based odour signal automatically and precisely provides the appropriate time-course to ant-deterrence. This specific floral VOC emission provides a transient, highly-focused protection for the sparse and valuable young inflorescences, but allows ants to return and protect older post-pollination flowers as seed-set commences.

These results generalize our specific findings from 1997, and have been augmented by analysis of the VOCs concerned. GC-MS confirmed that old acacia inflorescences had much lower volatile emission levels than young ones (Table 1). The mean total volatile outputs detected per young inflorescence varied from a maximum of 1070 ng in *V. etbaica* to just 4 ng in *V. drepanolobium*. The mean ranks of repellence and of volatile output showed no match, so mere quantity of VOC emissions did not influence ant responses. However, the acacias showed qualitatively very different scent profiles, as did conspecific young and old inflorescences (notably *V. seyal fistula* where old inflorescences retained very little of the complex VOC profile of young ones). Several VOCs occurred in more than one acacia species, especially linalool and its derivatives, 2-ethylhexanoic acid, and pinenes (all being common floral volatiles: Knudsen *et al.* 2006). However, the only conspicuous *temporal* VOC effect occurred in *V. seyal fistula*, with a strong peak of *E,E*- $\alpha$ -farnesene dominating the 0900 and 1100 h samples (see Fig. 3c), and thus coincident with the dehiscence window and with maximum bioassay repellence. This was also the only VOC to show a significant negative correlation with recorded ant responses through time, as expected for an effective repellent (ant numbers,  $R^2 = 0.49$ ,  $P < 0.1$  NS; ant visit duration,  $R^2 = 0.59$ ,  $P < 0.05$ ; all other correlations NS).

*E,E*- $\alpha$ -farnesene is already known as a signalling molecule. In plants it is associated with anthers in several genera (Jürgens & Dotterl 2004), and is potentially inducible via the jasmonate pathway in response to herbivory (Rodriguez-Saona *et al.* 2001). In insects it is a known component of alarm pheromones in several taxa including aphids and some myrmecine and formicine ants (e.g. D'Ettore *et al.* 2000). Related volatiles from fruit extracts can repel *Crematogaster opuntiae* (Russell *et al.* 1994); and synthetic farnesol is repellent to the ant *Linepithema humile* (Shorey *et al.* 1996). Conversely, *E,E*- $\alpha$ -farnesene is attractive to bees, including *Apis mellifera* (Blight *et al.* 1997), and *E,E*-farnesol is a component of the foraging recruitment pheromone used by *Bombus terrestris* (Mena Granero *et al.* 2005). Thus our observations add a further nuance to the ways that plant VOCs can act as filters, manipulating insect behaviour by chemical mimicry and serving as dual function floral traits (Herrera *et al.* 2002).

Floral repellence is now also known in some Central American acacias (*V. collinsii*, Ghazoul 2001; *V. hindsii*, *V. macracantha*, *Acaciella angustissima*, Raine, Willmer & Stone 2002; and *V. constricta*, Nicklen & Wagner 2006). The last authors found that ants avoided protracted interactions with the youngest dehiscing flowers (though apparently only being

**Table 1.** Volatiles present (ng per inflorescence) in different acacia species, as detected by GC-MS. (a) Samples gathered as five sequential aliquots from young inflorescences, then averaged; (b) Single samples gathered over 8 h, for young and old inflorescences. (Values are from at least 10 inflorescences, taken from 2 to 4 different trees)

	A						B			
	<i>Vachellia seyal seyal</i>	<i>Vachellia seyal fistula</i>	<i>Senegalia mellifera</i>	<i>Vachellia etbaica</i>	<i>Vachellia brevispica</i>	<i>Vachellia drepanolobium</i>	<i>Vachellia seyal seyal</i>		<i>Vachellia seyal fistula</i>	
							Young	Old	Young	Old
Terpenoids										
Monoterpenes:										
α-pinene	–	–	80	–	–	–	–	–	384	163
β-pinene	–	–	9	–	–	–	–	–	944	189
myrcene	–	–	27	–	–	–	–	–	–	–
β-phellandrene	–	–	7	–	4	–	–	–	–	–
Ocimene Z + E/ ocimenol	–	–	95	–	163	–	–	–	–	–
Linalool	18	11	50	56	246	–	121	–	252	–
Linalool oxide pyranoid Z + E	21	23	23	22	65	–	–	–	272	–
Linalool oxide furanoid Z + E	2	–	15	82	339	–	183	31	62	–
Chrysanthenone	–	–	15	–	–	–	–	–	–	–
α-terpineol	–	–	–	–	7	–	–	–	–	–
Verbenone	–	–	13	–	–	–	–	–	–	–
Sesquiterpenes										
<i>E,E</i> -α-farnesene	–	73	8	–	–	–	–	–	1412	–
Irregular terpenes										
4,8 dimethyl 1,3,7 nonatriene Z + E	–	–	–	–	92	–	–	–	–	–
Geranylacetone	–	–	–	702	–	–	–	–	–	–
Fatty acid derivatives										
2-ethylhexanol	–	–	25	–	–	–	–	–	–	–
2-ethylhexanoic acid	26	2	12	4	2	4	–	–	–	–
Benzenoids										
Cinnamic aldehyde/ alcohol Z + E	–	2	–	10	–	–	–	–	288	–
4-methoxy benzoate/ aldehyde	–	24	–	21	–	–	–	–	292	–
2-phenylethanol	–	–	9	–	–	–	–	–	–	–
N-containing compounds										
Indole	–	–	–	173	–	–	–	–	–	–
Total ng per inflorescence	67	135	388	1070	918	4	304	31	3906	352

repelled on contact), and concluded that repellence resided in pollen or anther glands. Intriguingly, *E,E*-α-farnesene has also been identified in *V. collinsii* from Costa Rica (NE Raine & D Edwards, unpublished data).

Thus at least 14 acacia species show some degree of floral ant-repellence, comprising six myrmecophytes and eight non-myrmecophytes. Patterns are beginning to emerge, since ant-repellence is generally higher in the myrmecophytes within related co-flowering communities (e.g. high in *V. seyal fistula* and *V. hindsii*, but lower in other species in Kenya and Mexico respectively). However the common East African *V. drepanolobium*, though heavily ant-defended, has low overall floral repellence and low volatile output per inflorescence (Table 1). This anomaly may relate to flowering regime; most species studied have sporadic sparse flowering, but *V. drepanolobium* shows intense mass-flowering, with old and young inflorescences in crowded contact and presumably indistinguishable by scent. High repellence from young inflorescences

might then preclude ant-guard protection of older (seed-setting) flowers; in fact patrolling frequency of ant-guards was almost zero on heavily-flowering branches. Further investigation of the interactions of flowering regime, inflorescence density and repellence should clarify these effects.

We note also that volatile repellence is not the only tactic employed by acacias; some ant-guarded species use temporal and spatial patterning of their rewards to manipulate ant distributions and keep ants away from young inflorescences (Raine, Willmer & Stone 2002; Gaume, Zacharias & Borges 2005). Thus it is evident that ant-repellent 'filters' are not the whole story, and interact with other key aspects of the adaptive plant phenotype.

#### TEMPERATE PLANTS

*Formica aquilonia* was more sensitive than *L. niger*, showing clear alarm/aggressive responses to about half of all floral



**Table 2.** Volatile reaction scores of *Formica aquilonia* (means,  $n = 6-22$  for different species) to various floral volatiles, and the morphological score for physical barriers to ants in the same flowers (see Materials and Methods)

Clade	Order	Genus and species	Volatile reaction score	Morphological score		
Magnoliids	Magnoliales	<i>Magnolia x soulangeana</i> *	66	0		
Monocots	Alismatales	<i>Aponogeton x crispus</i>	0	3		
		<i>Clivea miniata</i>	38	0		
	Asparagales	<i>Galanthus nivalis</i>	25	1		
		<i>Narcissus</i> 'Minnow'	91	0		
		<i>Narcissus</i> 'Tete-a-tete'	43	0		
		<i>Narcissus</i> 'Topolino'	29	0		
		<i>Chionodoxa forbesii</i>	0	0		
		<i>Hyacinthus orientalis</i> *	30	1		
		<i>Muscari racemosum</i>	8	1		
		<i>Crocus chrysanthus</i>	15	0		
		<i>Iris stylosa</i>	71	1		
		<i>Tulipa turkestanica</i> *	97	0		
		Eudicots	Ranunculales	<i>Mahonia aquifolium</i>	45	1
				<i>Corydalis solida</i>	0	2
<i>Anemone appennina</i>	13			0		
<i>Anemone blanda</i>	0			0		
<i>Anemone nemorosa</i>	17			0		
<i>Anemone ranunculoides</i>	13			0		
<i>Eranthis hyemalis</i>	0			0		
<i>Helleborus foetidus</i>	0			1		
<i>Helleborus niger</i> *	58			1		
<i>Ranunculus ficaria</i>	8			0		
Caryophyllales	<i>Silene vulgaris</i>			2	1	
	<i>Ribes sanguineum</i> *			9	0	
	<i>Saxifraga splendens</i>			16	0	
	<i>Plumbago auriculata</i>			9	3	
	<i>Ulex europaeus</i>		2	3		
Fabales	<i>Polygala chamaebuxus</i>		6	2		
	<i>Vicia faba</i>		3	3		
	Malpighiales		<i>Viola odorata</i>	43	1	
<i>Viola riviniana</i>			6	1		
Rosales	<i>Fragaria vesca</i>		0	0		
	<i>Potentilla fruticosa</i>		53	0		
	<i>Prunus cerasifera</i>		9	0		
	<i>Prunus avium</i>		49	0		
	<i>Prunus spinosa</i>		16	0		
Malvales	<i>Daphne bholua</i>		29	1		
	<i>Daphne blagayana</i>		39	1		
Ericales	<i>Erica carnea</i>		9	1		
	<i>Rhododendron praecox</i>		10	0		
	<i>Cyclamen purpurascens</i> *		19	0		
	<i>Primula denticulata</i>		20	1		
	<i>Primula vulgaris</i>		5	2		
	<i>Camellia japonica</i>		2	0		
	Boraginales		<i>Brunnera macrophylla</i>	38	1	
<i>Pentaglottis sempervirens</i>			8	1		
<i>Pulmonaria officinalis</i> *			7	2		
<i>Pulmonaria rubra</i>			0	2		
<i>Symphytum x uplandicum</i>			21	1		
Gentianales	<i>Hoya carnosa</i> *		81	1		
	<i>Vinca minor</i>		8	0		
Lamiales	<i>Ajuga reptans</i>		4	0		
	<i>Lamium purpureum</i>		0	2		
	<i>Rosmarinus officinalis</i>		17	0		
	<i>Forsythia x intermedia</i>		35	0		
	<i>Lathraea clandestina</i> *		38	2		
	<i>Veronica officinalis</i>		8	0		
	<i>Veronica penuncularis</i>		14	0		
	<i>Hedera helix</i>		20	0		
	Asterales		<i>Petasites albus</i>	38	0	
			<i>Petasites hybridus</i>	90	0	
<i>Taraxacum officinale</i>			37	0		
Dipsacales	<i>Menyanthes trifoliata</i>		2	3		
	<i>Lonicera fragrantissima</i>		26	1		
	<i>Viburnum bodnantense</i>		71	0		
	<i>Viburnum tinus</i> *		36	0		

\*Also eliciting strong response from *Lasius niger*.

species tested (Table 2), and with differences between overall responses to flowers for the three key behaviours ('mandibles open', 'head-up' and 'charge'; see Methods). Again, responses were always greatest when flowers were at peak dehiscence, and were elicited to pollen alone when this was tested (Table 3), even though much smaller volumes of tissue were then used.

For these plants, Fig. 4 indicates some trade-off between morphological barriers and the strength of volatile repellence in the flowers. No species tested showed high levels of both kinds of defence (i.e. none occur in the 'upper right' portion of the plot). This supports the original speculation on trade-offs by Guerrant & Fiedler (1981).

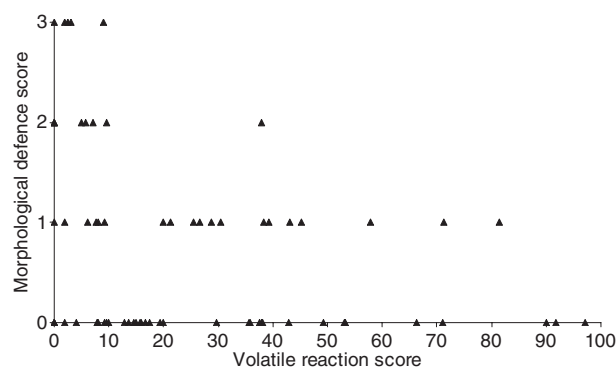
As with acacias, it is likely that other variables influence both physical/decoy defences and VOCs in relation to ants, such as timing of anthesis and of nectar presentation (if any), and accounting for these might in practice reveal a multivariate trade-off (Agrawal & Fishbein 2006).

#### GENERAL IMPLICATIONS

Floral volatile signals clearly have multiple functions as do all floral traits (Irwin, Adler & Brody 2004), including attracting pollinators, deterring casual visitors or thieves, and sometimes the specific deterrence of ants. Thus floral scents may function as allomones to deter enemies as well as being synomones to attract pollinating mutualists. But whilst it may be desirable to keep ants away from flowers, as with any mutualism there are associated costs for all the mechanisms described here, and in some cases there may be additional inherent risks of exploitation and cheating, so that the balance of costs and benefits will inevitably vary with circumstance and may change across evolutionary time. By keeping ants away, 'windows of opportunity' are provided for florivores, especially flower-feeding beetles. In an American ant-acacia, caterpillars of an unidentified moth exploit ant-repellency by constructing a protective case of acacia filaments so they can eat the foliage without being attacked by resident ants (Raine & Stone, pers. obs., Fig. 5a). Repelling the normally guarding ants may also alter the mutualism's costs and benefits by allowing in enemies (predators and parasitoids) of herbivores, the enemies themselves often being

**Table 3.** Mean volatile reaction scores of *Formica aquilonia* ( $n \geq 7$ ) to volatiles from whole flowers and to separated anthers, either dehiscent or post-dehiscent, from five temperate plant species

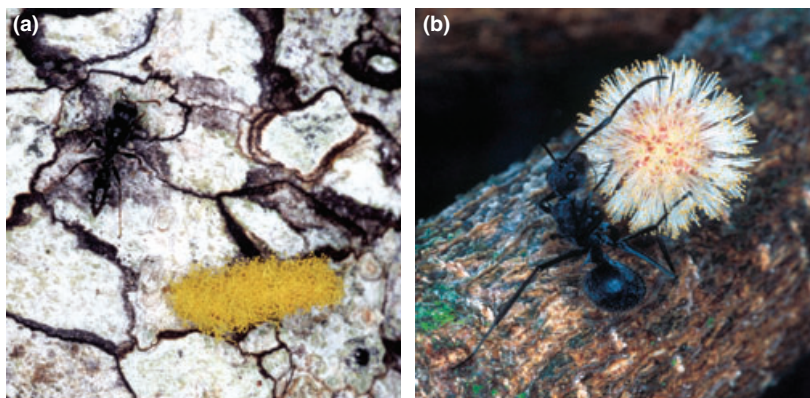
	Volatile reaction score			Flower/ inflorescence with anthers removed
	Whole flower	Dehiscent anthers	Old anthers	
<i>Petasites hybridus</i>	90	30	–	1
<i>Viburnum tinus</i>	36	44	2	–
<i>Vicia faba</i>	2.5	2.4	1	1
<i>Potentilla fruticosa</i>	53	29	11	–
<i>Plumbago auriculata</i>	9.5	11	2	–



**Fig. 4.** Trade-off effects between morphological defences and volatile floral repellence in 67 plants tested. While many species had low scores in both respects, those with high morphological defence scores never had strong VOC repellence, and those with strong repellence never had substantial morphological defence. (There were significant decreases in repellence between groups 0 and 2 ( $W = 35.0$ ,  $P = 0.015$ ); and between 2 and 1 ( $W = 40.5$ ,  $P = 0.018$ ); though not between 0 and 1 ( $W = 425.5$ ,  $P = 0.825$ ); with no tests using group 3 due to small sample size).

recruited by plant leaf VOCs, so that the plant experiences multiple and conflicting selective pressures. Any ant-repellent filter is also likely to select for ants that can circumvent it, and 'parasitic' non-defensive ants commonly do exploit the normal ant-plant mutualisms (e.g. Gaume & McKey 1999; Raine *et al.* 2004; Clement *et al.* 2008). More specifically, Junker, Chung & Blüthgen (2007) found some ants resistant to the repellent effects of certain flowers; for example *Dolichoderus thoracicus* ants regularly foraged in *Ipomea cairica* flowers despite these eliciting strong repellence in other ant species; *D. thoracicus* thereby gain access to an otherwise underutilized resource. Further studies of ant behaviours in natural encounters with living flowers whose repellence has been assayed would clearly be valuable and are underway.

Until recently, little was known about floral or pollen volatile effects except as attractants. Existing compilations of floral and/or pollen volatiles (Dobson & Bergström 2000; Knudsen *et al.* 2006) do contain some compounds that are known to affect some ant behaviours or to act as components of ant pheromones or defensive secretions. But our studies show that floral volatiles with a generalized role as filters against ants are far from characteristic of plants in general; they may be associated principally with species that recruit ants for defensive purposes or for seed-dispersal services, and/or with species lacking architectural defences for their flowers. Pollen-based compounds with defensive functions have been recorded in a few wind-pollinated plants (Jürgens & Dotterl 2004), but compounds deterrent to animal pollen-vectors were largely unreported until our work on acacias, and it is intriguing that one of the most effective compounds appears to be a pheromonal 'mimic'. Since ants substantially pre-date much of the explosive radiation of flowering plants, they may have played a major role in selecting for dual-function VOCs that still attract pollinators, whether by influencing chemistry, dosage, or both. It has often been noted that ants' pheromones can be perceived by other insects and so



**Fig. 5.** Two contrasting ant responses to floral volatiles. (a) Hijacking a behavioural filter: the larva of an unidentified moth caterpillar obtaining protection from aggressive ant guards on the myrmecophyte acacia *Vachellia hindsii* by concealment within a case made from ant-repellent filaments and anthers. (b) A non-resident *Camponotus* ant foraging from the inflorescence of an East African acacia *Vachellia reficiens*, with no detectable impact of floral volatiles.

contribute to the ants' protection of plants against herbivores (Offenberg *et al.* 2004); it now seems likely that floral release of pheromonal mimics can have similar effects, giving additional benefit to plants. Thus flower VOCs can be targeted such that particular animals are manipulated in time and space as either friend or enemy to a plant. Flowers are therefore emerging as hotspots for research into convergent mutualism management, with a diversity of subtle and interacting strategies that can be employed when simple morphological features are not enough.

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