The Ecology and Evolution of Ant- Aphid Interactions

THOMAS HENRY OLIVER

Division of Biology, Silwood Park campus, Ascot, Berkshire, SL5 7PY, U.K.

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Supervisors: Dr James Cook
Dr Simon Leather



Lasius niger ants tending aphids on ornamental daisies.

Photo taken by the author at Wye College, Kent, UK, June 2006.

Abstract

The evolution of species interactions is a fascinating subject, and one of vital importance if we are to understand how biological communities change over time. This thesis considers the interaction between aphids (Homoptera) and ants (Formicidae). Ants tend aphids for sugary honeydew and in return provide a variety of protective services. A literature review in Chapter 1 introduces the subject and provides background information. Chapter 2 considers ant- aphid interactions in a community setting. Specifically, I consider the fitness effects of the ant- aphid interaction on host plants. Net benefits or costs to plants depend on the densities of ants and aphids; these densities may themselves change depending on context dependent factors. Chapters 3 and 4 consider how semiochemicals can allow species to respectively maintain or avoid synchrony in space and time with mutualists or antagonists. Chapter 3 shows ladybirds avoid prey patches guarded by ants by reducing oviposition in response to ant semiochemicals. Chapter 4 shows that aphid walking dispersal can be limited by ant semiochemicals. This may be adaptive for aphids to remain in areas of enemy- free space. Alternatively, if levels of kin competition are high limited dispersal could be costly to aphids. In Chapter 5 I consider interactions between invasive and native ants. Ecological dominance in ants may be mediated by the ability to monopolise honeydew- producing resources. Chapter 6 explores ants' decisions whether to tend or prey upon aphids. Predation of aphids depends on colony demand (e.g. through cues from the presence of larvae) as well as the quality or quantity of supply (e.g. increased predation of unproductive aphids). Finally, Chapter 7 deals with macroevolutionary patterns in the interaction between ants and aphids. Specifically, I identify ecological traits that characterise aphid- tending ants. A final discussion chapter summarises how ant-aphid interactions fit into existing mutualism theory.

Declaration

I hereby confirm that all the work within this thesis is my own, with the exception of a small number of replicates of the experiment in Chapter 3, which were conducted by a student, Ian Jones, under my supervision and also collaborative work with Ms. Alla Mashanova in Chapter 4. Dr. Tim Pettitt aided the research published in Chaper 7 by providing ant cultures from the Eden Project, Bodelva, Cornwall, UK. In addition, my supervisors James Cook and Simon Leather helped by reading draft copies of published manuscripts mentioned herein.

Signed: **T H OLIVER**

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Chapter 1

Literature Review

Aphids

There are about 4200-4500 species of aphid worldwide (Dixon, 1998, Grimaldi and Engel, 2005), more than 500 of which are found in the British Isles (Blackman, 1974). Like most Hemiptera, aphids feed on plant phloem sap using piercing mouthparts. Phloem fluids are typically rich in sugars but low in nitrogen, therefore aphids consume large volumes, filtering out required metabolites and excreting waste from the anus as sugary 'honeydew'. This honeydew is attractive to ants and other insects, such as wasps, which require high levels of carbohydrate to fuel foraging activities (Stadler and Dixon, 2005). Aphids show prodigious rates of increase unparalled in other herbivorous insects (Stadler and Dixon, 2005). They achieve this through very short generation times due to a parthenogenetic, viviparous mode of reproduction which allows embryos to develop inside an unborn nymph while it still resides within its mother (Blackman, 1974). In addition, aphids are iteroparous with a single female adult able to give birth to many offspring over time. In holocyclic heteroecious species, sexual generations typically occur in autumn, with overwintering in the egg stage on the primary host (Blackman, 1974). Polyphenism is often also apparent throughout the spring and summer, however: when conditions become too crowded or host plant quality deteriorates sufficiently, alate virginoparae are produced to disperse and colonise new host plants (Blackman, 1974, Dixon, 1998). Aphid colonies rapidly reach large sizes but often do not persist for long periods due to deterioration of host plant quality or location by natural enemies (Dixon, 1998). Thus, throughout the spring and summer, dispersal is important, with species persisting as dynamic metapopulations. Attendance by ants may increase the persistence time of colonies (Addicott, 1978, Dixon, 1998, Fischer et al., 2001). Ants tend to increase the populations of small tended colonies, but may limit the growth of larger colonies, thus having a stabilising effect on aphid abundance (Addicott, 1979, Cushman and Addicott, 1989).

Ants

The ants comprise some 8000 species worldwide (Hölldobler and Wilson, 1990) and, although significantly more diverse in the tropics, they are still an highly important fauna in temperate ecosystems. There are almost 50 species in Britain (Skinner and Allen, 1996), many of which form associations with aphids. All ants are eusocial, forming highly organised societies where generations overlap, adults care for young and there is reproductive division of labour (Hölldobler and Wilson, 1990). In addition, non-reproductive workers show temporal division of labour (polyethism), with the youngest workers performing 'nurse' duties within the nest: tending to the queen and brood, and the older workers carrying out more dangerous tasks such as foraging and defence (Hölldobler and Wilson, 1990). This polyethism is evident in ant- aphid interactions, where younger workers may tend aphids while older workers act as predators and may attack aphids not close to- , or marked with pheromones by, their more pastoral sisters (Weir, 1958a, Weir, 1958b, Way, 1963, Sakata, 1994).

Evolutionary Interactions

The interaction between aphids and ants dates back to at least the early Oligocene (c. 30 Mya) (Wheeler, 1915), and probably long before (Grimaldi and Engel, 2005). Aphids evolved at least 210- 220 Mya in the Triassic (Shcherbakov and Wegeriek, 1991), and ants some 115- 135 Mya in the lower Cretaceous (Brady *et al.*, 2006). The ant-aphid interaction along with the adaptive radiation of angiosperm plants have been suggested as drivers of such rapid evolutionary diversification of both taxonomic groups (Grimaldi and Engel, 2005, Moreau et al., 2006). At the species level, aphids show highly variable degrees of association with ants (Stadler *et al.*, 2003), yet at higher taxonomic levels the frequency of ant attendance does not appear to vary greatly between groups (Blackman and Eastop, 1994, Dixon, 1998), suggesting multiple origins of myrmecophily (Stadler and Dixon, 2005). In addition, within an aphid lineage ant attendance may be an evolutionary labile trait, which is evolved and lost several times (Shingleton and Stern, 2003). Thus, in contrast to the view that

control of the interaction is entirely asymmetric, with domination by the ants, aphids may be flexible in their association, entering and leaving the association when it is advantageous (Stadler and Dixon, 2005).

In the Rocky Mountain region of the United States only one quarter of all aphid species are tended by ants (Bristow, 1991). In Europe, the proportion is slightly higher, with one third being obligate myrmecophiles (Stadler, 1997). What factors cause such large variability in ant- aphid interactions and limit the extent of ant- attendance in aphids? Several hypotheses have been proposed. Firstly, there may be constraints by host plant characters that prevent relationships forming. The 'permissive plant hypothesis' states that plant type can have a large effect on whether aphids are ant attended (Bristow, 1991). Certain plant families (e.g Anacardaceae, 100%) support considerably higher proportions of ant tended aphids than others (e.g. Fagaceae, 2%) (Bristow, 1991). The fact that only 37% of species on permissive plants are ant-attended, however, shows that other factors are important (Dixon, 1998). There may be phylogenetic constraints preventing associations forming, or simply an unavailability of ants (Bristow, 1991). In a comparative analysis, for 112 aphid species, of the predictive power of different morphological and ecological traits for explaining the strength of the association between ants and aphids, Stadler *et al.* (2003) identified four key aphid traits that explained the most variation: feeding on woody plant parts, a low degree of mobility, feeding in dense aggregations and the lack of wings in the adult stage, were all positively associated with ant attendance. Dixon (1998) and Shingleton *et al*. (2005) describe aphids feeding on deeper phloem elements , as well as those feeding on roots, as more likely to be ant attended. For Hölldobler and Wilson (1990), honeydew- producing stages present throughout the year are an important factor. The emerging view, is that no single hypothesis can account for the diversity of ant- aphid interactions. Rather, a wide range of physiological, ecological and evolutionary constraints determine each interspecific relationship (Stadler and Dixon, 2005). We can state, however, that the degree of association of a particular aphid species with an ant depends: a) on the coevolutionary history of the two species and, b) the current ecological context. Given that there is enough genetic variation and phylogenetic contraints can be overcome, then selection for adapations favouring mutualism depend on the net benefit of the interaction over evolutionary time. If this is consistently positive in both species, we can expect to see the emergence of mutual coadaptations, facilitating symbiosis. The paragraphs below

explain the different interaction costs and benefits, discovered by studies to date, that may act upon ant- aphid coevolution.

Costs and benefits

Aphids

Costs

1. **Predation of aphids by ants**. Ants remove dead aphids and take them to the nest to use as food (Banks, 1962, Nault et al., 1976). In some cases ants kill living, tended aphids (Brian, 1977, Edinger, 1985, Sakata, 1994). Way (1963) suggests that ants maintain enough Homoptera to satisfy their sugar requirements and any excess are killed and used as a source of protein. Indeed, providing an alternate sugar solution leads to a shift from mutualism towards exploitation, due to increased predation of ant-attended homopterans (Way, 1954, Offenberg, 2001). High aphid abundances may result in some not being pheromone-marked by tending ants (Sakata, 1994); these unmarked aphids may be predated by older, more predatory ants (Way, 1963, Hölldobler and Wilson, 1990). Depriving aphids of phloem fluid, by girdling vascular tissues of the host plant, also leads to increased predation by ants (Edinger, 1985). Thus, it appears ants assess the productivity of individual aphids and attack them if their honeydew production rate falls below a certain threshold. In contrast, Pontin (1958) hypothesised that ants maintain a balanced protein-carbohydrate intake and predate aphids when other sources of prey are scarce. This idea is not supported by a study in which alternative prey was offered to ants; no effect on the predation rate of aphids was observed (Offenberg, 2001). It is likely that ants need a constant supply of sugar to maintain all activities in the colony whereas a protein supply becomes more important for the queen and larvae in times of colony growth (Carroll & Janzen, 1973).

2. **Increased Parasitism**. Some specialised parasites and parasitoids can avoid ant aggression through rapid oviposition before ant detection or using

chemical mimicry (Dettner and Liepert, 1994, Price, 1997). For example the monophagous parasitoid *Lysiphlebus hirticornis* Mackauer is not attacked by ants and thus can exploit ant attended colonies of *Metopeurum fuscoviride* Stroyan (Fischer *et al.*, 2001). Ant-tended aphids may face costs if these parasites preferentially target ant attended aphid colonies. This may occur through the parasites facing reduced competition for aphid hosts from other parasites which cannot employ chemical cloaks or rapid oviposition to escape ant detection. Alternately, the parasites may benefit from ant protection through reduced hyperparasitism and occupancy of 'enemy free space' (Völkl 1992).

3. **Costs in life history parameters**. Even if there are benefits at the colony level, at the individual level, aphids may incur physiological costs from ant attendance. Costs in life history parameters include prolonged developmental time, delayed offspring production, proportionally smaller gonads, fewer well developed embryos and reduced mean relative growth rate (Stadler and Dixon, 1998a, Yao et al., 2000, Yao and Akimoto, 2001, Stadler et al., 2002). These effects are similar to those observed when aphids feed on poor quality plants (Stadler and Dixon, 1998a). To minimise predation by ants, aphids may feed at higher rates in order to appease ants with honeydew (Banks and Nixon, 1958, Del-Claro and Oliveira, 1993). This may result in suboptimal nutritional uptake (Stadler and Dixon, 1998a). Additionally, honeydew composition may be altered in response to ant presence (Yao and Akimoto, 2001), which may incur costs. Whether ant-attendance imposes a physiological burden varies greatly between aphid species. For example *Aphis fabae cirsiiacanthoides* (Scop.) individuals tended by the ant *Lasius niger*, tend to develop more slowly and allocate less energy to their gonads (Stadler & Dixon, 1998). In contrast, *L. niger* tended individuals of *Symydobius oblongus* (von Heyden) develop faster, are bigger, and have more well developed embryos in their gonads (Stadler and Dixon, 1999).

4. **Indirect costs in the absence of ants.** *Evolved dependence* occurs when a species in symbiosis loses the ability to perform well in the partner's absence (De Mazancourt et al., 2005). Aphids that come to depend too heavily on ant

attendance through evolutionary adaptation, will face costs if habitats are spatially or temporally heterogeneous in terms of occupancy by ants (Stadler *et al.*, 2001). The result of such evolved dependence is that obligate aphids may be limited by the abundance and distribution of their host ants (Stadler and Dixon, 2005).

Morales (2000) noted that the trophobiotic organ (a group of anal setae that retain honeydew until it is collected by ants) may reduce homopterans' ability to freely eject honeydew in the absence of ants. In aphids this trophobiotic organ often replaces a large cauda (tail piece) that helps in the ejection of fluid (Brian, 1977). Defences against natural enemies are also much reduced in attended aphids (Way, 1963, Hölldobler and Wilson, 1990). For example, the lack of thick wax coverings, hard sclerotized cuticles, cornicle secretions and alarm pheromone triggered- evasive behaviour in attended aphids that rely on ant protective services (Buckley, 1987). Such obvious changes in morphology, revealing the dependence on ants, are sure to also be matched by less apparent behavioural changes. The overall result is an evolutionary dependence on ants and, hence, costs suffered in their absence. For example, honeydew accumulating on the plant in the absence of ants increases the probability of homopteran infection by pathogens (Way, 1963), and may also reduce host plant photosynthesis, thereby indirectly harming homopterans (Morales, 2000). The tendency for aggregation and conspicuousness of ant- tended colonies may also lead to increased predation risk from natural enemies in the absence of ants (Ives et al., 1993, Stadler et al., 2001).

5. **Ant mediated transmission of pathogens.** Ants can be vectors for entomopathogens that affect aphids. For example, the ant *L. niger* may carry *Lecanicillium longisporum* (Zimmerman), a fungal pathogen of the aphid *Dysaphis plantaginea* (Passerini) (Bird *et al.*, 2004). To date, only one recent study has documented such ant mediated pathogen transmission (Bird *et al.*, 2004), although if this is simply due to lack of research effort, such ant-aphid-parasite interactions could potentially be quite common.

6. **Reduced Dispersal.** Although fitness is often measured using individual life history parameter surrogates such as reproductive rate, growth rate etc., the long term fitness of an aphid clone may depend on its ability to disperse and colonise new hosts, thus maintaining a viable metapopulation. The production of alate forms has been shown to be reduced in the presence of ants (Banks, 1958, El Ziady, 1960). This may be by a 'tranquillising' effect of ants reducing aphid restlessness and thus the tactile stimulation necessary to induce alate formation (Way, 1963). Dixon (1998) suggests there may be a trade off between dispersal and reproduction, with reproduction (achieved most effectively through apterous forms (Dixon et al., 1993)) favoured when ants are present and reducing natural enemy pressure. Ant semiochemicals, however, can have direct effects inhibiting alate aphid development (Kleinjan and Mittler, 1975). For example, mandibular gland extracts of the ant *Formica fusca* Wheeler applied to *A. fabae* significantly reduced alate production (Kleinjan and Mittler, 1975). Thus, the reduced aphid dispersal in the presence of ants may not be adaptive, but rather, a chemical manipulation of aphid physiology by ants, allowing them to maintain a constant honeydew supply. Another explanation is that long wings on aphids render it more difficult for ants to collect honeydew from aphids, therefore ants benefit from biting them off (Hölldobler and Wilson, 1990). A similar, but physical, manipulation occurs where the wings of alate aphids are bitten and crumpled by guarding ants (Kunkel 1973, as cited by Hölldobler and Wilson, 1990).

Benefits

1. **Protection from natural enemies**. Ants confer significant indirect benefits on aphids through defence from natural enemies (Way, 1954, Banks and Macaulay, 1967). Ownership behaviour of food sources by ants, such as aggregates of tended aphids, results in aggression (increased attacks) towards intruders which, away from the food source, would be tolerated (Brian, 1955). For example, three aphid species: *Metopeurum fuscoviride* (Stroyan), *Brachycaudus cardui* (L.) and *Aphis fabae*, were all significantly less heavily attacked by predators when attended by *L. niger*, in comparison to unattended

control colonies (Fischer *et al*., 2001). It is clear that tending ants may have different responses to different types of predators (Way, 1963). Some natural enemies may employ chemical mimicry to bypass ant defence (Dettner and Liepert, 1994). The protection conferred by ant attendance varies between different ant species (Way, 1963, Ness et al., 2006). Also, the degree of protection afforded by ants may decrease with the distance from the nest and with increasing food supply (Way, 1963), and ant aggressiveness may vary with the density of nestmates (Harmon and Andow, 2007). In addition, certain types of aphid benefit more from ant protection. Larger aphid species that feed on branches or trunks using a long proboscis are particularly vulnerable to natural enemies and often are ant attended (Dixon, 1998).

2. **Removal of competitors.** Mutualistic aphids providing high quality honeydew can distract ants from tending competing neighbouring colonies (Addicott, 1978, Cushman and Addicott, 1989, Fischer et al., 2001). Furthermore, ants respond to the relative difference in aphid value by increasing predation on the poorer honeydew-producing species or colonies (Sakata, 1995, Sakata, 1999).

3. **Living within Ants' Nests and Shelters**. Some species of root aphids cannot survive during winter except in ants' nests (Zwolfer 1958, cited by Way, 1963). These lodgers obtain protection from natural enemies and adverse weather conditions. Overwintering active stages are protected against fungi, excessive moisture and very low temperatures. Overwintering eggs may be licked by ants, as they do their own brood, thereby protecting them from pathogenic microorganisms (Matsuura and Yashiro, 2006). Ants also build protective tents over aphid colonies away from the nest (Anderson and McShea, 2001). These shelters serve to defend aphids from natural enemies and probably also provide ideal microclimates for aphid colony growth (Stopes and Hewitt, 1909).

4. **Transport**. In addition to some ants collecting aphid eggs and carrying them back to their nests, there is evidence of ants transporting aphids to new feeding sites (Jones, 1929, Sudd, 1967). *Lasius fuliginosus* (Latreille) may transport young fundatrices of *Stomaphis quercus* (L.) from the trunk bases of oak trees,

where they overwinter as eggs, to the upper branches (Goidanich, 1959; as quoted by Way, 1963). *Lasius niger* has been demonstrated to transport *Pterocomma salicis* (L.) aphids from low quality host plants to plants of higher quality (Collins & Leather, 2002).

5. **Direct Physiological Benefits.** As stated earlier, in many cases ant attendance has been shown to impose physiological costs on aphids (e.g. Stadler and Dixon, 1998a). In other cases aphids appear to gain direct fitness benefits through increased feeding rates (e.g. Stadler and Dixon, 1999, Flatt and Weisser, 2000). It is unclear why aphids should directly benefit from honeydew solicitation by ants- why have facultative aphids not evolved to feed at this optimum rate when unattended? (Stadler & Dixon, 1998). Perhaps there is a trade-off between feeding rate and sanitation (see *reduced pathogen contamination* below), thus ant- dependent aphids feed at a lower rate in the absence of ants because the cost of entomopathogen contamination by fungi from uncollected honeydew is so high (Morales, 2000).

   There is likely to be interspecific variation in the physiological effects of ant attendance, and even variation within individual aphid clones, mediated by host plant type (phenotypic plasticity)(Cushman, 1991). Careful experimental design is needed, however, to discount the subtle indirect effects of ants. For example, ants may increase the aggregation of aphid colonies which can impose indirect costs or benefits (Way, 1963).

6. **Reduced Pathogen Contamination.** Ants solicit honeydew directly from aphids and clean up droplets of honeydew from surfaces (Sakata & Hashimoto, 2000), thus reducing contamination by pathogens (Way, 1963). Dead individuals and exuviae are also removed by ants (Banks, 1962). Uncollected honeydew may lead to the formation of sooty moulds (Fokkema *et al.*, 1983), which may block excretion (Way, 1954), and also decrease photosynthesis in the host plant (Wood et al., 1988). The presence of honeydew can also attract predators. The hoverfly *Syrphus corollae* lays twice as many eggs on leaves contaminated with honeydew as on clean ones (Bombosh 1962, cited by Sudd, 1967), and ladybirds use honeydew as an attractant foraging cue (Ide et al.,

2007). It has even been suggested that the decreased aphid feeding rates when unattended are a mechanism to prevent honeydew contamination of themselves and hostplants (Morales, 2000).

7. **Ants protect plants.** Ants may defend plants from herbivores that are competitors of homopterans (Fritz, 1983, Beattie, 1985). By expelling other herbivores ants may preserve plants for Homoptera. For example, *Formica* ants defend tall goldenrod *Solidago altissima* (L.) plants from *Trirhabda* spp. beetles thus indirectly benefiting *Publilia concave* (Say) membracids (Messina, 1981).

Ants

Costs

The costs faced by ants of interacting with aphids have not, to date, been well studied. There are inherent difficulties in measuring fitness costs for such a long lived, widely dispersed 'organism' as the ant colony. Costs are likely to include the time and energy invested in tending aphids. Honeydew must be transported, often long distances, back to the nest. Indirect evidence for this cost is the observation that aphid tending declines with increasing distance from the nest (Way, 1963). Thus, ants seem able to assess the cost of foraging with regards to the time or energy spent versus the potential gains (Hölldobler and Wilson, 1990). The protection of aphid colonies from natural enemies also has associated time and energy costs, and may increase the risk of worker mortality (Stadler and Dixon, 2005). Ants appear to be able to balance the costs of making attacks with the costs of losing aphids to a predator (Phillips, 2005).

It has been claimed that aphids are a poor food resource (Toft, 1995) and not usually the preferred prey of ants (Stadler and Dixon, 2005). Instead, honeydew is used as fuel to provide workers with energy while they forage for protein- and lipid-rich prey (Carroll and Janzen, 1973, Offenberg, 2001). A large proportion of the diet of some ants, however, comprises aphid prey (Pontin, 1958, Rosengren and Sundström, 1991). Whatever their use, there may be costs for ants whose foraging strategies rely too heavily on these transient and patchily distributed resources (Dixon, 1998, Stadler and Dixon, 2005). Considering macroevolutionary patterns, however, it

appears that honeydew gathering is a highly successful ant trait, and has even been attributed to the explosive radiation of particular ant groups in the early Tertiary (Grimaldi and Engel, 2005).

Benefits

Honeydew provides an readily available source of carbohydrates for foraging ants. Aphids may excrete, as honeydew, up to 90% of the food energy they obtain from ingesting plant phloem sap (Dixon, 1998). Honeydew comprises not only sugars, but also many amino acids and amides, proteins and minerals (Way, 1963, Völkl et al., 1999, Fischer et al., 2005). The composition of honeydew, and the quantity produced, varies depending on aphid age (Llewllyn et al., 1973), aphid species (Völkl et al., 1999, Fischer et al., 2001), temperature (Way, 1963, Dixon, 1998), intracellular symbionts (Dixon, 1998), parasitoid infection (Völkl 1992) host plant species (Fischer and Shingleton, 2001), host plant condition (Cushman, 1991) and part of the plant on which aphids are feeding (Way, 1963, Dixon, 1998). In addition, the quantity and composition of honeydew may be modified in the presence of ants (El Ziady, 1960, Takeda et al., 1982, Fischer and Shingleton, 2001, Yao and Akimoto, 2001, Yao and Akimoto, 2002), making it more attractive to the aphids' fickle guardians. Ants benefit most from the highest quality honeydew (presence of preferred amino acids and sugars, such as melezitose (Kiss, 1981)), produced in the highest volumes (Völkl *et al.*, 1999). Also predictability of the honeydew source may be important (Krebs & McCleery, 1984). Nutritional requirements of the ant colony are likely to change throughout the year. For example, *L. niger* workers forage mainly on protein food such as insect prey during the growing season (larval growth and egg production by the queen), but mainly sugary foods after most of their larvae have pupated (Edwards, 1951, Sakata, 1994) There seems to be a upper limit on the amount of honeydew a colony can use. If honeydew is in excess ants often switch to predating aphids rather than tending them (Way, 1963).

Context dependence and ecological dynamics

Mutualisms are reciprocally beneficial relationships between organisms (Herre et al., 1999), i.e. the net effect of the interaction (total benefits minus costs) on both species is positive. Costs and benefits are dynamic, however, and, depending on the biotic and abiotic context, the net effect of the interaction on a species may change in magnitude and even in sign (Bronstein, 1994). There may exist not only geographic mosaics in abiotic and biotic aspects affecting the cost/ benefit balance (Thompson, 1994), but also temporal dynamics, as environmental conditions change and populations of interacting organisms wax and wane. Factors causing changes in costs and benefits include abiotic (Kersch and Fonseca, 2005) and biotic effects such as: variation in density of ants and aphids (Banks, 1962, Banks and Macaulay, 1967, Addicott, 1979, Cushman and Whitham, 1989, Morales, 2000, Oliver et al., 2007a), age-specific effects (Cushman and Whitham, 1989), alternative food sources (e.g. extrafloral nectaries) (Way, 1963, Becerra and Venable, 1989, Engel et al., 2001, Offenberg, 2001), host plant mediation (Cushman, 1991), presence of competing mutualists (Addicott, 1978, Cushman and Addicott, 1989, Fischer et al., 2001) and density of natural enemies (Sakata and Hashimoto, 2000). The spatial and temporal variation of cost and benefits may present problems in qualifying mutualistic relationships simply by measuring costs and benefits at one location, at one moment in time. Furthermore, there may be delays in net interaction effect- changes being manifested as measurable changes in gene frequencies (i.e through the presence of measurable phenotypic traits). Similarly, presuming a mutualistic relationship simply from observation of mutualistic traits (e.g decreased defence structures in aphids), may be erroneous if costs and benefits have since changed and we are simply observing an evolutionary time lag.

Adaptations for mutualism

If mutualistic conditions persist for significant periods over evolutionary time, then we can expect to see the emergence of adaptations which facilitate the symbiosis between the two organisms. In ants and aphids these comprise a wide range of

structural, behavioural and lifecycle adaptations that maintain the interaction. Such mutual adaptations are most apparent in specific, obligate relationships. Most ant-aphid interactions, however, are non- specific and facultative (Bristow, 1991, Delabie, 2001). The ant genus *Formica*, for example, has been recorded to tend 483 different species of aphids comprising 208 different genera (Jones, 1929). This low specificity continues at the species level, *e.g.*, the ant *L. niger* tends a number of different aphid species including the willow aphid *Pterocomma salicis*, maple aphid *Periphylus acericola*, thistle aphid *Aphis fabae cirsiiacanthoides*, bean aphid *Aphis fabae* and chestnut aphids *Lachnus tropicalis* and *Myzocallis kuricola*. Interactions with ants have a similarly low degree of specificity for aphids. For example, the aphid *Aphis helianthi* is tended by 32 different ant species (Jones, 1929). In these faculative interactions, ants are usually present around the aphid colonies, but aphids may also survive well without the presence of ants. Despite these loose associations, it is apparent that diffuse coevolution has led to an impressive level of adaptation facilitating the ant-aphid interaction.

Aphid adaptations

Structural and behavioural adaptations in aphids, which facilitate ant attendance, tend to increase the honeydew available to ants or reduce defence mechanisms. Myrmecophilous aphids may be conspicuously coloured and gregarious, thus forming a brightly coloured 'herd' easy to find by the ants (Dixon, 1998), (although aggregation may also be a strategy for reducing predation risk from natural enemies (Turchin and Kareiva, 1989)). The quality, quantity and rate of honeydew excretion can all be controlled by aphids (Fischer and Shingleton, 2001, Yao and Akimoto, 2001, Yao and Akimoto, 2002). For example, honeydew may be eased out in symbiotic aphids, rather than the droplets 'kicked off' (Sudd, 1967, Addicott, 1978). In addition, if no ants are present to accept the honeydew from myrmecophilous aphids, they may withdraw the droplets back into the abdomen (Hölldobler and Wilson, 1990). In some aphids, e.g. *A. fabae*, an ant will palpate the aphid's abdomen with its antennae and in response the aphid raises its abdomen and a honeydew droplet is emitted and held at the tip of the abdomen while the ant imbibes it (Hölldobler and Wilson, 1990). The hind legs are seldom lifted as they are when the

aphids are unattended (Way, 1963). In other aphid species, for example *Lachnus roboris*, hind legs are raised and waved when ants approach. Kloft (1959, as cited by Hölldobler and Wilson, 1990) speculated that this behaviour may mimic the head of another worker ant waving its antenna; thus the solicitation that follows being reminiscent of the trophallaxis that occurs between sister workers. Honeydew solicitation by ants, however, also occurs in other homopterans, e.g. coccids and pseudococcids (scale insects and mealybugs), whose rear ends look nothing like those of sister ants (Way, 1963). To hold the honeydew droplet at the tip of the abdomen, myrmecophilous aphids tend to have a group of anal setae (fine hairs), dubbed the "trophobiotic organ" (Way, 1963), which replaces the long cauda of unattended species (Brian, 1977). Defensive structures, such as waxy coverings, may also be reduced in ant attended species, as mentioned earlier (Way, 1963, Buckley, 1987, Hölldobler and Wilson, 1990).

Myrmecophilous aphids also often have lifecycles conducive to the ant mutualism. The ideal lifecycle for development of mutualism with ants may be one in which stages capable of producing honeydew are available throughout the year (Way, 1963, Hölldobler and Wilson, 1990). Aphid taxa possessing this trait are more likely to become trophobiotic (Way, 1963). In addition, the lifecycle of some aphid species may evolve such after symbiosis has begun (Hölldobler and Wilson, 1990).

Ant adaptations

Documented adaptations in ants regarding the aphid mutualism are primarily behavioural, but some physiological adaptations have been described: for example, the production of semiochemicals which influence alate aphid development (Kleinjan and Mittler, 1975). Also, the proventricular valve, which prevents liquids in the crop passing to the midgut, is best developed in the Formicinae and it is in this subfamily that most honeydew-soliciting ants are found (Sudd, 1967). Such gut morpology, facilitating the storage and transport of liquid food, may have been a key innovation that allowed ants to exploit of a whole new range of carbohydrate resources, greatly contributing to the ecological success of those groups (Davidson, 1997, Davidson et al., 2004). Behavioural adaptations include collecting honeydew (Way, 1963), removing dead individuals (Banks, 1962, Nault et al., 1976), carrrying aphids to new

feeding sites (Collins & Leather, 2002) or to- and from nests (Sudd, 1967) and caring for aphid eggs (Pontin, 1960). In addition, there may be well developed interspecific communication between ants and aphids. For example, ants may signal to aphids to produce honeydew by palpating their abdomens with antennae (Hölldobler and Wilson, 1990), and aphid alarm pheromone signals to ants to enhance protective behaviour (Nault *et al.*, 1976).

Pre-adaptation

Many of the ant and aphid traits described above may have evolved through enhancement of mechanisms that existed before the ant- aphid association formed (Way, 1963). For example, it is easy to see how the larval carrying behaviour of ants, where eggs, larvae, pupae and other workers are moved from place to place, could evolve to become interspecific carrying of aphid partners. Way (1963) even describes the transport and nursing behaviour of *Lasius flavus* to its attended aphids to be mediated by a 'virtually unmodified brood care instinct'. Trophallaxis (liquid food exchange) in ants, made possible by the stucture of the proventriculus, may pre-adapt them for 'milking' aphids (Grimaldi and Engel, 2005). In addition, the defence of aphid colonies from natural enemies is thought to simply be an extension of 'ownership' behaviour of resources against competitors (Way, 1963). In aphids, the cibarial pump muscle and the piston valve in the pharynx, that allow food intake to be regulated (Dixon, 1998), may be preadaptations, which, in combination with the cardiac valve (Auclair, 1963) and anus muscles, allow honeydew excretion rate to be modified.

Such predaptations and the 'by-product' value of rewards (*sensu* Connor 1995). could potentially explain the facultative nature of many ant-aphid interactions. General non-specific adaptations allow dynamic partner switching, which is likely to be advantageous in ranges where previous partners are absent, or when invading new habitats (e.g. Mondor and Addicott, 2007). It is possible that some kind of clade selection could promote facultative mutualism, rather than obligate specialisation. Facultative interactions could also be prevalent simply due to the dynamic metapopulation structure of Homoptera, however, making stable obligate interactions unfeasible.

Phenotypic plasticity

Given that the costs and benefits for ants and aphids are dynamic and dependent upon the particular biotic and abiotic context (Cushman and Addicott, 1989, Bronstein, 1994, Kersch and Fonseca, 2005), the ability to evolve plastic traits that facilitate partners under mutualistic conditions but defend against them under exploitative conditions, would be of great benefit (Offenberg, 2001). Phenotypic plasticity (the ability of a genotype to produce distinct phenotypes when exposed to different environments) (Pigliucci, 2005) is now widely accepted to have a genetic basis and can be adaptive (Agrawal, 2001). The highly facultative nature of ant- aphid interactions, and the wide range of ecological factors affecting costs and benefits, make them a likely place to find phenotypic plasticity, which would allow the maximisation of fitness in such variable environments. Indeed, as stated earlier, aphids can alter the rate and quality of honeydew in response to ant presence, thereby reducing the costs of producing enriched honeydew at higher rates when ants are absent (e.g. Fischer and Shingleton, 2001). Phenotypic plasticity may also be apparent as reduced alate production in response to ant presence, if it is indeed the case that the phenomenon is adaptive for aphids as Dixon (1998) has suggested. In ants, there is flexibility in the foraging strategy, with the number of foragers recruited to a resource related to total production of honeydew or aphid colony size (Völkl et al., 1999, Oliver et al., 2007a). In addition, the decision to tend or predate an aphid may depend upon the nutritional demands of the colony (Carroll and Janzen, 1973). It has been suggested that species interactions may involve escalating reciprocal phenotypic changes in ecological time (Agrawal, 2001). For example, in a mutualistic interaction such as that of ant and aphids, individuals may adjust rewards in response to changes in levels of services from a partner (Agrawal, 2001). In ant- aphid interactions this could occur if ant presence induces more- or better quality honeydew from aphids (e.g. Fischer and Shingleton, 2001) and this, in turn, causes increased defensive behaviour by ants, as the value of the food source increases (e.g. Way, 1963).

Summary

The association of an ant with any particular aphid species depends on a) the coevolutionary history between the two species and, b) the current ecological context. The coevolutionary history involves the adaptations which species have evolved to cope with each other and is determined by the myriad factors affecting costs and benefits over an evolutionary timescale. In ants and aphids, the result is often a suite of adaptations facilitating facultative mutualistic interaction, not necessarily with a specific partner (i.e. coevolution may be diffuse). In addition to fixed adaptations, however, species may also evolve plastic traits that are flexible over ecological time. These traits allow 'fine tuning' of the interaction, enabling an organism to gain the maximum possible benefit in its current context. Ant- aphid interactions provide an ideal system to study the evolutionary and ecological dynamics of facultative interactions.

Chapter 2

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When are ant-attractant devices a worthwhile investment? *Vicia faba* extrafloral nectaries and *Lasius niger* ants

Summary

Most studies aiming to determine the beneficial effect of ants on plants simply consider the effects of presence or exclusion of ants on plant yield. This approach is often inadequate however, as ants interact with both non-tended herbivores and tended Homoptera. Moreover, the interaction with these groups of organisms is dependent on ant density and these functional relationships are likely to be non-linear. A model is presented here which segregates plant herbivores into two categories depending on the sign of their numerical response to ants (myrmecophiles increase with ants, non-tended herbivores decline). The changes in these two components of herbivory with increasing ant density and the resulting implications for ant-plant mutualisms are considered. It emerges that a wide range of ant densities needs to be considered as the interaction sign (mutualism or parasitism) and strength is likely to change with ant density. The model is used to interpret the results of an experimental study which varied levels of *Aphis fabae* infestation and *Lasius niger* ant attendance on *Vicia* faba bean plants. Increasing ant density consistently reduced plant fitness and thus, in this location, the interaction between the ants and the plant can be considered parasitic. In the *Vicia faba* system, these costs of ants are unlikely to be offset by other beneficial agents (e.g. parasitoids) which also visit extrafloral nectaries.

Introduction

A wide range of different insects may visit extrafloral nectaries and have direct or indirect effects on plant fitness, thus mediating selection for extrafloral nectary traits (Koptur, 1991, Koptur, 1992, Pemberton and Vandenberg, 1993, Gentry, 2003, Heil and McKey, 2003, Azzouz et al., 2004, Rudgers and Gardener, 2004). Multiple interaction studies are important as different organisms often have non-additive effects on the plant (Cuautle and Rico-Gray, 2003). This study considers how ants, the most common visitors to extrafloral nectaries, interact with tended (myrmecophilous) and non- tended herbivores to determine plant fitness. The frequent and wide occurrence of ant-attended plants with extrafloral nectaries (including many economically important crops such as cotton, cashews and beans) make the study of these interactions particularly relevant.

Extrafloral nectary plant-ant relationships are often regarded as mutualisms (Bentley, 1977, Beattie, 1985, Ness, 2003, Rudgers, 2004). Plants provide food rewards and in return ants provide protection from herbivores. (O'Dowd, 1979, Stephenson, 1982, Ito and Higashi, 1991, Del-Claro and Oliveira, 1993, Koptur et al., 1998). Contemporary views of species interactions recognise that the net effect of the interaction on each species may change in magnitude and even sign over space and time (Bronstein, 1994, Thompson, 1994). Costs and benefits are dynamic and affected by a multitude of biotic and abiotic factors (Cushman and Whitham, 1989). For example, temporal or spatial heterogeneity in herbivore density may mediate the benefits that ants provide plants. This type of heterogeneity is a very direct cause of conditionality in the mutualism. The services that the ants provide the plant (herbivore protection) are altered, directly affecting the cost: benefit balance. A more indirect cause of conditionality involves factors that change the density of the interacting organisms. For example, if Homoptera become established on a plant this can encourage more ants. This is likely to alter the interaction between the ants and the plant, but not necessarily in a simple manner (more ants do not always equate to more benefits). This study is the first to explore how a range of ant densities affects plant fitness payoffs, enabling us to better understand conditionality in extrafloral nectary plant: ant relationships. In the empirical section we utilize a ‘crossed’ design that manipulates aphid density and the presence of ants. In a recent review Styrsky and

Eubanks (2007) exhort this as the best design for determining if ant predation is independent or truly facilitated by honeydew-producing homopterans. In spite of this only two out of 30 ant-hemipteran interaction studies have used this design to date (Styrsky and Eubanks, 2007).

Ants often form mutualistic associations with myrmecophilous herbivores, such as lycaenids or homopterans. Myrmecophiles are species which benefit from association with ants, being more or less adapted to live with them in a relationship which need not be obligatory or mutually beneficial, following Way's (1963) definition. Tended homopterans may benefit from ant attendance in many ways. These include: protection from natural enemies (Way, 1963; Dixon, 1998; Fischer et al. 2001), living within ant nests and shelters (Zwolfer, 1958, cited by Way 1963), transport to higher quality patches (Goidanich, 1959, cited by Way, 1963; Collins & Leather, 2002), direct physiological benefits (Banks and Nixon, 1958, El Ziady, 1960, Yao et al., 2000) and reduced pathogen contamination (Sakata and Hashimoto, 2000). Ants benefit principally by harvesting honeydew from homopterans to obtain a sugar source, and occasionally predating them when protein is required (Buckley, 1987). The mutualism between ants and myrmecophilous herbivores introduces extra costs for plants, in addition to the production costs of extrafloral nectar (Southwick, 1984, Mondor and Addicott, 2003). In this paper we develop a simple graphical model to explore the plant fitness effects of the relationships between ants and myrmecophilous herbivores versus ants and non- tended herbivores. We then use empirical data from *Vicia faba* L. bean plants, associated herbivores and *Lasius niger* L. ants to test the model.

The model

Herbivory results in a loss of fitness to plants, which can be quantified by comparing the fitness of plants from which herbivores are excluded with that of untreated control plants. The difference in fitness can be expressed as a *total percentage fitness cost* of herbivory (C_t). We can split this into two components: the cost from non ant-attended herbivores (C_h), and the cost from ant-attended, myrmecophilous herbivores (C_m). A simple equation can be written thus:

$$C_t = C_h + C_m \qquad [1]$$

Both C_h and C_m will vary with ant density. As the number of ants on a plant increases the efficiency of herbivore exclusion is likely to increase, causing a reduction in the damage caused by non-ant attended herbivores (i.e. C_h) (Koptur, 1984, Beattie, 1985). Ants may directly interrupt herbivore feeding or oviposition, or remove eggs from plants (Letourneau, 1983, Koptur, 1991, de la Fuente and Marquis, 1999). If, however, ants also deter natural enemies of non-tended herbivores this will tend to increase C_h (Fritz, 1983). Increased numbers of ants will also benefit myrmecophilous herbivores, facilitating an increase in their numbers and therefore the damage to the plant caused by them (i.e. C_m) (Cushman and Addicott, 1989, Cushman and Whitham, 1989) (Fig. 1).

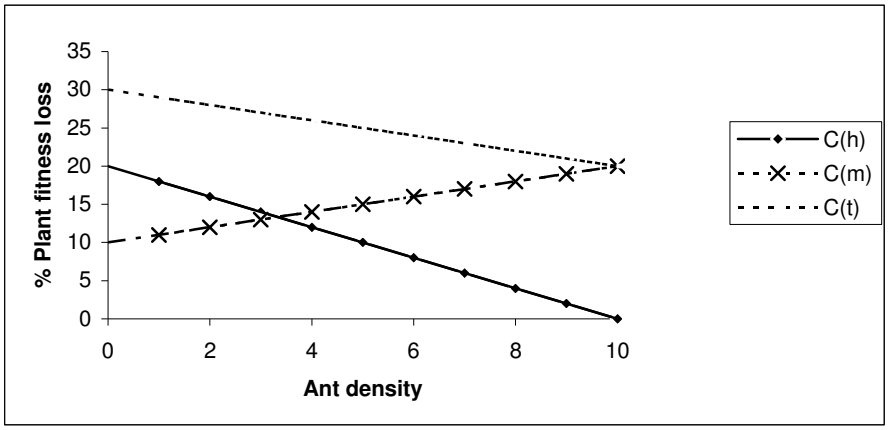


Fig. 1, Hypothetical relationships of the two components of herbivory with increasing ant density. Damage due to myrmecophilous herbivores (C_m) increases with ant density ($y = x + 10$, in this example). Damage due to non-ant tended herbivores (C_h) decreases with ant density ($y = -2x + 20$). The total fitness cost (C_t) is the sum of the two components of herbivory ($y = -x + 30$).

For ants to give a net benefit to plants the total cost of herbivory (C_t) must decrease as ant density increases i.e. the slope of C_t must be consistently negative. Many previous experiments however, simply compare ant- visited plants with plants from which ants are excluded. Unfortunately, without explicitly considering a range of ant densites, it is impossible to conclude that the total cost of herbivory, C_t, always declines with ant density. In fact, the slope of C_t may change in sign rather then follow a simple monotonic relationship with ant density.

If C_h and C_m are simple linear functions, then C_t is also linear with a constant positive or negative slope (Fig. 1) Thus we can describe the ant- plant interaction as exclusively parasitic or mutualistic respectively. A commensalistic relationship would simply be a line of slope zero. In reality however, C_h and C_m are unlikely to be linear. C_m might show a positive correlation with ant density, but only up to a saturation level, after which homopterans are saturated by tending ants and no further benefits of ant presence are conferred. Such saturation effects are analagous to those seen in predator: prey functional responses (Holling, 1959). For example, Morales (2000) found that individuals of the membracid bug *Publilia concava* Say tended by *Formica obscuriventris* Mayr ants benefitted more when part of smaller aggregations, indicating a negative density dependent benefit of this mutualism. Similar negative density dependent benefits in ant- aphid interactions have been found in two other studies (Banks and Macaulay, 1967, Addicott, 1979), although there has been one report of positive density dependence (Cushman and Whitham, 1989). In addition, if compensatory mechanisms exist in the plant then herbivory at low intensity may have little effect on fitness (Trumble et al., 1993). Thus, although increasing ant density induces a corresponding increase in myrmecophiles, the effect on plant fitness may be reduced at low ant/herbivore densities due to herbivory compensation effects. This would cause the slope of the curve to increase with ant density. Combining the above two effects could result in a sigmoidal function (Fig. 2).

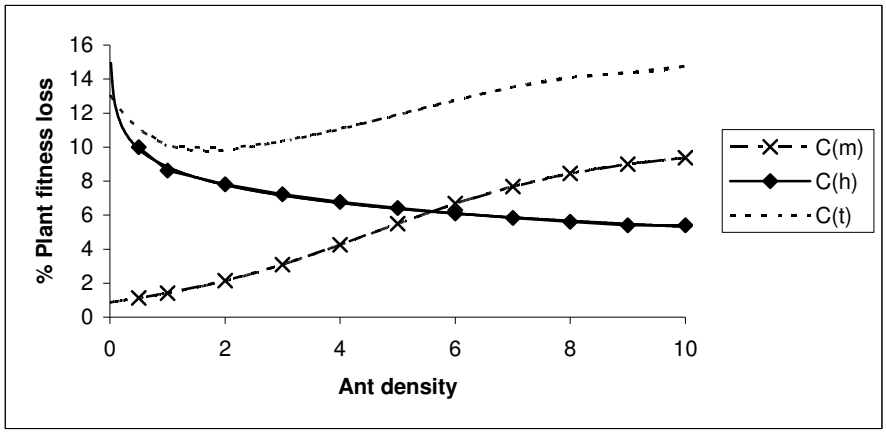


Fig. 2, Fitness cost of myrmecophilous herbivores is modelled as a sigmoid function and cost of non-tended herbivores as a natural logarithm. The resulting change in total plant fitness with ant density now describes a 'u' shaped curve indicating that intermediate ant densities are optimal for minimising plant fitness loss.

Similarly C_h may also be non linear. The overall efficiency of herbivore exclusion by ants is likely to be a function combining encounter rate with herbivores, attack rate, and success rate of each attack (Katayama and Suzuki, 2005). Given that the attack rate is a sigmoid function, this is likely to result in a non linear overall efficiency of herbivore exclusion (Katayama and Suzuki, 2005), resulting in a non linear function for C_h.

As examples of non-linearity, using a natural log function ($y = a \ln(x) + b$) to describe the two curves, C_h and C_m, produces similar results to the linear models: the function of C_t is now a curve but its slope is still consistently positive or negative. If we use a sigmoid curve, however, to describe one of the functions, combined with a natural log function for the other, then this results in the slope of C_t changing sign as ant density increases (Fig. 2). The total cost of herbivory (C_t) with ant density appears as an 'n' or 'u' shaped curve. In this event it is much more difficult to summarise the interaction as exclusively mutualistic or parasitic. An 'n' shaped curve implies intermediate ant densities are optimum for plants. A 'u' shaped curve implies very low, or high ant densities best for plants. To determine the net interaction effect, i.e. a 'mutualism' with ants or 'parasitic relationship' would require knowledge of the probablility of each specific ant density occuring on plants in the field. This change of sign of the C_t slope can also occur if we use natural log function to describe one curve but a linear function for the other.

To summarise: Without empirical data we cannot predict how C_h and C_m will change with ant density. In theory, however, the benefit of ants to a plant might not simply increase or decrease with ant density but may show an 'n' or 'u' shaped curve. Thus it may be inappropriate to seek to define a specific ant- plant relationship as simply 'mutualistic' or 'parasitic', as fitness effects are context dependent and conditional on ant density. The average number of ants on the plant over daily and seasonal cycles, or more specifically the overall probability of each specific ant density occuring, will determine the net interaction effect and whether the initial conditions required for extrafloral nectaries to evolve are met.

The Experiment

Study system

Extrafloral nectaries of *Vicia faba* bean plants in the UK are commonly visited by *Lasius niger* ants. Extrafloral nectaries alone attract ants, but ant attendance is higher when the aphid *Aphis fabae* Scop. is on the plant (Engel *et al.*, 2001). Other aphids, e.g. *Acyrthosiphon pisum* Harris and *Megoura viciae* Bukt., are also occasional pests on the plant, but *A. fabae* has by far the biggest economic impact on yield (Cammell and Way, 1983). Through exclusion experiments, Banks and Macaulay (1967) found that *A. fabae* had a negative effect on plant fitness and ants exacerbated this negative effect. Bean plants without aphids yielded 56 seeds per plant, those with aphids gave 17 and those with ant-attended aphids only eight seeds per plant. *Lasius niger* presence may increase the negative impact of aphids on bean plant fitness because: (a) Ants increase the feeding rate of aphids, thereby increasing per capita plant damage (Banks and Nixon, 1958), and (b) ants protect aphids from coccinellid, syrphid, anthocorid and chrysopid predators (Banks, 1962), reduce aphid dispersal (El Ziady, 1960) and induce faster reproduction rates (Banks, 1958), thereby increasing *A. fabae* abundance. Considering our model, these factors may cause a rapid rate of increase in C_m and this could offset the benefits of ants reducing C_h, therefore leading to a reduction in total plant fitness with ant density. The relationship between *L. niger* ants and *V. faba* may therefore be antagonistic, begging the question: why do bean plants invest in extrafloral nectaries to attract harmful ants? Without considering a range of ant densities, however, it is impossible to conclude that *L. niger* ants are consistently harmful to *V. faba*. For example, it may be that *L. niger* ants are beneficial to the plant at intermediate densities, yet Banks and Macaulay (1967) only studied damaging, high ant densities (i.e. the function of total herbivory cost, C_t, might describe a 'u' shaped curve).

The aim of this experiment is to gain insight into how C_t changes with ant density. By manipulating the level of *A. fabae* aphid infestation, different numbers of *L. niger* ants can be attracted to bean plants, and the resulting effects on plant fitness elucidated; an approach not yet undertaken. In addition, by measuring leaf damage (caused by non-ant attended leaf chewers, rollers and miners) we can investigate the rate of change of C_h with increasing ant density, providing further novel insights into ant- plant interactions.

Materials and Methods

Vicia faba (broad bean)(Vicieae: Leguminosae) plants, cv. the Sutton, were infested with four densities of aphids at Silwood Park, Berkshire, U.K. and the number of ants on the plants and levels of herbivore attack were monitored. Thirty-two one month old (*c*. 12cm tall with 5 leaf nodes) *V. faba* plants, grown in 12cm pots, were selected and grouped into four replicate groups of eight similar sized plants. Within each group, plants were randomly allocated into one of four treatments: no aphids, light, medium or heavy *A. fabae* infestation (0, 15, *c.*100 or >250 aphids/plant respectively). To achieve the different degrees of infestation, plants were placed into different mesh cages, containing increasing numbers of other bean plants heavily infested with *A. fabae*, for 48h. Plants were examined frequently and moved around within the mesh cage to try to minimise variation in aphid number within treatments. Within each group of eight plants, four were then randomly allocated to the 'ant- treatment', in which ants were allowed access to the plant. Ants were excluded from the remaining four plants in each group by applying Oecotak® around the base of the pots. The plants were then arranged in two 4 x 4 Latin squares, two replicates per square, with the constraint that each ant-treatment plant was adjacent to a non-ant-treatment plant. The Latin squares were set up 50cm apart on a strip of soil close to woodland and fields and adjacent to a building and where *L. niger* ants had been observed foraging. The experiment began in mid June and after an initial period of two days for ants to find the plants, the plants were checked once a day at 12:00 and the number of *L. niger* ants per plant were counted. Ant counts continued for 21 days after which, due to very hot summer temperatures and high levels of parasitism causing nearly all aphids to die, fewer ants were visiting the plants. At this time the herbivore damage to plants was assessed by counting the total number of leaves per plant and the number of leaves with herbivore damage. Herbivore damage was divided into three feeding guilds: leaf chewers, miners and rollers. The plants were kept under the same conditions until the end of the experiment, in late-August, when they were harvested and the number of seed pods, total seed count, numbers of seeds parasitised and dry seed mass, were recorded.

STATISTICAL ANALYSIS

ANOVA and ANCOVAs were carried out using the statistical program 'R' (Ihaka and Gentleman, 1996) to test for the effects of ants and aphids on plant performance. Data on proportion leaf damage were arc-sine square root transformed prior to analysis. Before the experiment, plants were grouped into blocks of similar sized plants. To see if there was an effect of plant size, each analysis was first performed as a simple non-factorial ANOVA or ANCOVA. Block was never found to significantly affect response variables and therefore the blocks could be treated as true replicates. Analyses were then able to be repeated as full factorials, as there were four replicates at each combination of factor levels. Insignificant terms were removed in a stepwise fashion to find the minimum adequate model. Likewise, factor levels were collapsed to reduce factor levels into significantly different groupings. Red spider mites had infested some of the plants prior to the experiment. We expected that this would affect results so spider mite damage was subjectively assessed as one of three qualitative levels (light, medium or heavy) and included as an explanatory variable in order to reduce unexplained variation. Midway through the experiment one of the heavily aphid infested plants died. This was excluded from all analyses, including plant yield, as it would be unreasonable to compare the mean ant density of this plant with others, in case there were overall seasonal fluctuations in ant presence. For seed count data a generalised linear model with Poisson errors was used. In cases where the data were overdispersed the 'quasi' function was used to define a different family of error structures (Crawley, 2005).

Results

Ant counts

Ant density increased with the level of *A. fabae* infestation (Table 1). There was no significant effect of block on ant count ($n = 15$, $F_{1,10} = 0.045$, $P = 0.84$), therefore the groups of plants could be considered true replicates. Level of aphid infestation significantly affected the number of ants per plant ($n = 15$, $F_{2,12} = 5.67$, $P = 0.0018$).

Table 1. Average *Lasius niger* ant counts from late June – early July on bean plants with varying levels of aphid infestation. Different letters in the final column indicate significantly different means. A Tukey test was inappropriate as the experimental design was unbalanced through the loss of a heavily aphid infested plant midway through the experiment. Factor levels were therefore collapsed to find significantly different groups (ANOVA).

A. fabae infestation	*n*	Mean	SE	*P*(0.05)
None	4	0.70	0.19	a
Light	4	1.16	0.25	a
Medium	4	4.39	1.00	b
Heavy	3	11.76	6.56	b

Herbivore damage

Plants with higher ant densities suffered significantly *more* damage by leaf chewing herbivores (n = 31, $F_{1,26}$ = 17.17, P = 0.0003) (Fig. 3). In addition, there was a significant interaction between the initial level of *A. fabae* infestation and spider mites on the damage by leaf chewing herbivores ($F_{1,26}$ = 16.29, P = 0.0004) (Fig. 4). There was no significant effect of ant density, *A. fabae* or spider mite infestation on the proportion of leaves damaged by leaf mining herbivores (ants: $F_{1,24}$ = 0.12, P = 0.73; aphids: $F_{2,27}$ = 0.30, P = 0.75; mites: $F_{3,27}$ = 1.44, P = 0.25). Similarly, neither aphids nor spider mites significantly affected damage by leaf rollers (aphids: $F_{3,27}$ = 0.47, P = 0.71; mites: $F_{2,27}$ = 0.17, P = 0.84). Ant density was found to have a significant effect on leaf rolling damage ($F_{1,29}$ = 5.00, P = 0.033), but because only four plants (three of which were ant treatment plants) suffered damage by leaf rollers, more replication is needed to increase confidence in this result. No damage to any plants by pod-feeding herbivores occurred throughout the course of the experiment.

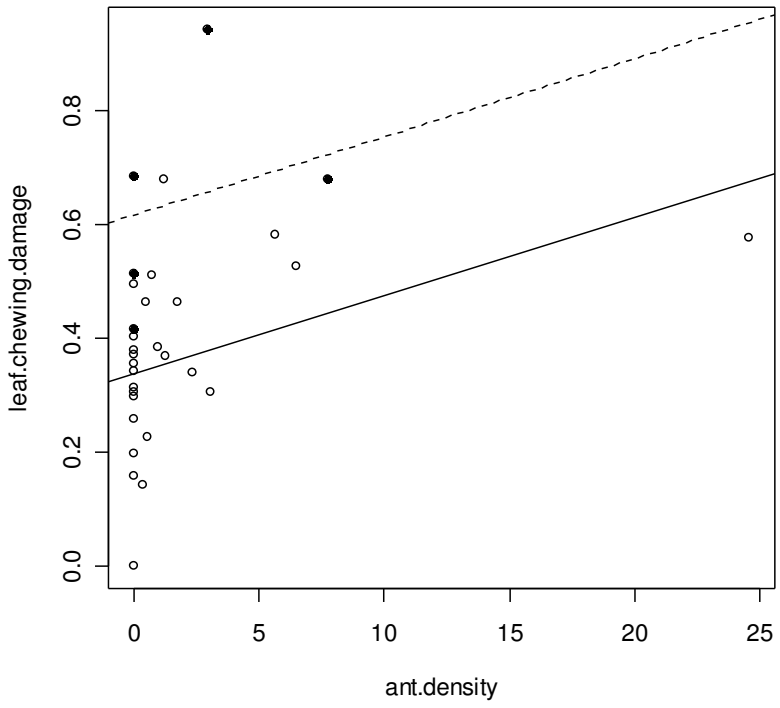


Fig. 3, Relationship between ant density and damage by leaf chewing herbivores (arcsin square root-transformed data). The five filled data points are plants with high levels of infestation by both aphids and spider mites. These plants suffer significantly more leaf damage by leaf-chewing herbivores $F_{1,28}$ = 15.02, P = 0.0006. The dashed line has the equation $y = 0.014x + 0.618$. The solid line represents the change in leaf damage with ant density for plants with lower aphid or spider mite infestation (n = 26) ($y = 0.014x + 0.338$) R^2= 0.43, $F_{2,28}$ = 10.52 P = 0.0004.

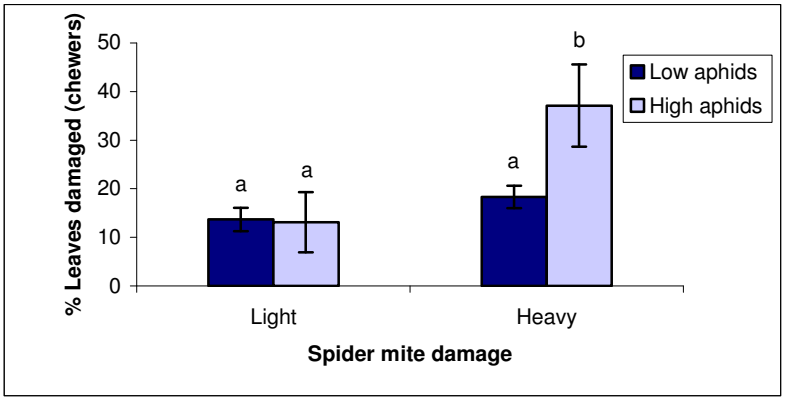


Fig. 4, Effect of spider mite damage and initial level of *A. fabae* infestation on the proportion of leaves damaged by leaf chewing herbivores. Neither aphids nor spider mites affected leaf chewing damage if either were at low densities. When both are at high densities, however, the level of leaf chewing damage significantly increased. Different letters above the means indicate significant differences (n = 31, P<0.05; Tukey's honestly significantly difference test).

Plant fitness

The number of seed pods was not significantly affected by the level of aphid or spider mite infestation (n = 31, aphids: $F_{3,24}$ = 0.21, P = 0.89; mites: $F_{2,24}$ = 0.87, P = 0.42), nor by the density of ants ($F_{1,24}$ = 0.07, P = 0.79). The total number of seeds was also not significantly affected by the three variables (aphids: $F_{3,27}$ = 0.59, P = 0.62; mites: $F_{2,27}$ = 1.15, P = 0.32; ants: $F_{1,30}$ = 1.34, P = 0.25). The total weight of seeds from each plant, however, was found to be significantly negatively correlated with ant density ($F_{1,29}$ = 4.39, P = 0.045) (Fig. 5), but not with aphids ($F_{1,27}$ = 0.17, P = 0.69) or spider mites ($F_{1,29}$ = 0.16, P = 0.70), although the interaction between ant density and aphid infestation was close to significance ($F_{1,26}$ = 3.48, P = 0.074). When only ant-treatment plants were considered, the level of aphid infestation had a significant effect on yield (n = 15, $F_{2,11}$ = 4.80, P = 0.032). Comparing the effect of ants on only those plants without aphids: the mean total seed mass (± SE) on plants with ants was 1.16g ± 0.26 (n = 4), compared with 0.92g ± 0.24 (n = 4) without ants. This difference is not significant (t = 0.44, df = 3), but the replication level is clearly low.

The overall average total mass of seeds from ant-treatment plants was 0.65g ± 0.13 (n = 15), whereas plants from which ants were excluded yielded a total seed mass of 1.11g ± 0.19 (n = 16),(t.test: df = 26.96, P = 0.0553). Given that the average number of seeds between ant-treatment (2.32 ± 0.41 seeds/plant) and ant-excluded plants (2.25 ± 0.38 seeds/plant) was almost equal, we can deduce that the lower total seed mass of ant-treatment plants was a result of a lower average seed size rather than fewer seeds. Indeed the average seed mass of ant-treatment plants was 0.28g ± 0.055 (n = 37) compared with 0.49g ± 0.067 (n = 36) for ant-excluded plants (t.test: df = 68.14, P = 0.017). There was also a greater number of very small, shrivelled seeds, weighing less than 0.2g (unlikely to germinate sucessfully (Banks and Macaulay, 1967)) produced by the ant-treatment plants (21 in total) compared with the ant-excluded plants (12 in total).

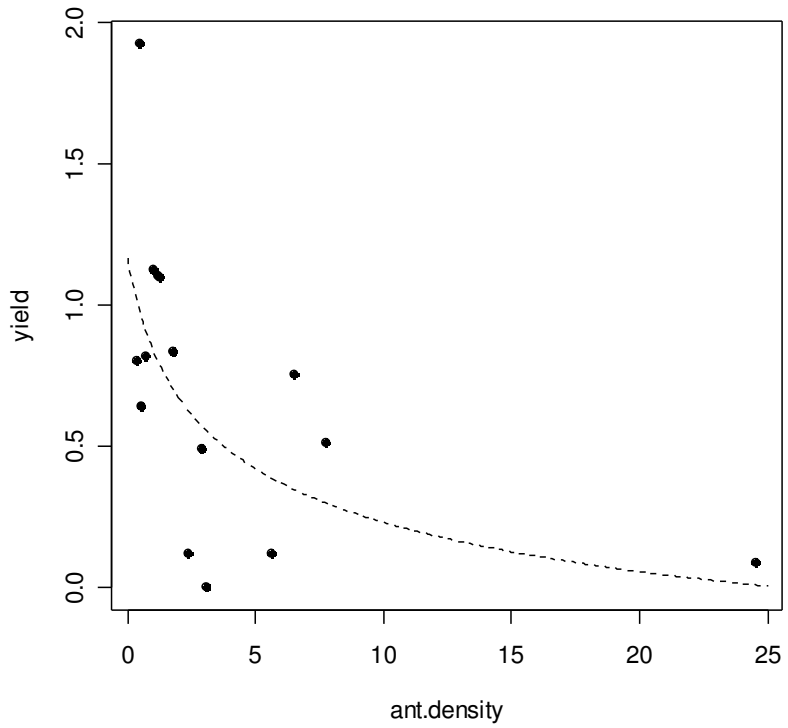


Fig. 5. Relationship between mean ant density and yield (total seed mass in grams) of ant-treatment bean plants (n = 15). The curve has the equation $\log(y+1) = 0.78 - 0.24\log(x+1)$, $R^2 = 0.41$, $F_{1,13} = 8.87$, $P = 0.011$).

Discussion

The number of *L. niger* ants on bean plants was positively correlated with *A. fabae* density, as expected (Engel *et al.*, 2001). In terms of our model (with ants as the explanatory variable), it is clear that as observed ant density increases this is correlated with an increase in the number of myrmecophilous herbivores and so to the fitness cost to the plant (C_m). Because *A. fabae* attract ants and, in turn, the ants have a positive effect on the aphids (Banks, 1958, Banks and Macaulay, 1967), there may be positive feedback driving ant density upwards. This positive feedback mechanism in ant : aphid abundance may explain the patchy occurrence of large aphid colonies tended by many ants, when surrounding plants and even branches of the same plant are empty. Although an additional, non-mutually exclusive explanation may be the differential resistance to herbivore attack between individuals and even branches on the same tree (Buckley, 1987).

Plant damage by leaf mining and rolling guilds of herbivores was not significantly influenced by the density of ants. This may be because concealed feeders are largely immune to ants during their active feeding stages (Heads and Lawton, 1985, Rashbrook et al., 1991). Damage by leaf chewers however, showed a significant positive correlation with ants. Increasing the number of ants actually *increased* C_h- the plant fitness loss by non-attended herbivores. This result is completely unexpected and in contrast to the predictions of our conceptual model (and other studies; e.g. Koptur (1979) found ant visitation reduced damage by folivores in the vetch *Vicia sativa* in California). It implies ants were not effectively defending the plant against non-tended herbivores. A possible explanation for this is that the positive effect of ants on aphids causes increased plant damage (Banks and Nixon, 1958, Way, 1963), leading to stressed plants which are more susceptible to herbivory (White, 1969, Rhoades, 1983, White, 1984, Ohgushi, 2005). Evidence consistent with this is the fact that plants heavily infested with both aphids and spider mites suffered significantly greater attack by leaf chewing herbivores (Fig. 4). Such aphid-mediated effects on plant chemistry could also explain the indirect mutualism between ants and gall making midges found by Savage and Peterson (2007). Greater numbers of galls occurred on plants with ants, yet this was not simply due to enemy-free space provided by the ants; ants did not significantly influence parasitism rates nor galler survival. Savage and Peterson (2007) speculate that ant-tended aphids may elevate

nutrient concentrations near the feeding sites, thereby promoting higher gall densities. An alternative explanation for the indirect positive effect of ants on folivores found in this experiment, is that ants, attracted by aphids, are deterring beneficial natural enemies of the herbivores (Fritz, 1983). Pod feeding lepidopterans have been shown to cause greatest damage to the related *V. sativa* when ants were abundant, because their larval parasitoids were deterred (Koptur and Lawton, 1988). Although, in this case, this does not explain why heavy spider mite infestations were also associated with leaf chewing damage. If both components of herbivory, C_m and C_h, increase with ant density, then so too will the total cost of herbivory (C_t), making the relationship between ant and plant parasitic.

The results of our plant fitness study confirm the parasitic relationship; *L. niger* ants did not reciprocate benefits to *V. faba* plants. Yield in total seed mass per plant was measured as a surrogate for plant fitness and ant density was found to have a significant negative effect. The relationship between yield and ant density was monotonic (Fig. 5), i.e. increasing ant density further always reduced yield. The interaction between ants and aphids on plant yield was close to significance. This hints at the interdependent effect of these explanatory variables, perhaps reflecting the reciprocal benefits between the two organisms. When only ant treatment plants were considered, there was a significant negative relationship between aphid infestation and plant yield. In contrast, when ants were absent, aphid infestation had little effect on plant yield. Thus, the negative effect of aphids is mediated through the presence of ant mutualists. Without ants, aphid colonies suffered and declined (aphid infestation refers to the initial level of aphid infestation at the start of the experiment).

The parasitic relationship between *L. niger* ants and *V. faba* contradicts the hypothesis of the plant using extrafloral nectaries to attract these ants for protection. Becerra and Venable (1989) suggest that, rather than receiving herbivore protection from ants, plants may secrete extrafloral nectar in order to distract ants from tending Homoptera. Engel (2001), however, found no evidence for this distraction hypothesis in the *V. faba* system. Aphid growth did not increase when extrafloral nectaries were removed and there were no detectable changes in nectar composition or volume in response to aphid presence. Plants could use extrafloral nectar to distract ants from flowers (Kerner, 1978, Wagner and Kay, 2002) or perhaps extrafloral nectaries have functions other than attracting ants (Kawano et al., 1999, Heil et al., 2004, Choh et al.,

2006). Pollinators (Koptur, 1992), predatory insects (Pemberton and Vandenberg, 1993, Heil and McKey, 2003) and parasitoids, which attack herbivores, may all visit extrafloral nectaries (Koptur, 1991, Koptur, 1992, Gentry, 2003, Azzouz et al., 2004, Kost and Heil, 2005). Indeed, Cuautle and Rico-Gray (2003) showed extrafloral nectary- visiting wasps to have positive fitness effects on the plant *Turnera ulmifolia* (Turneraceae). In the *V. faba* system, however, parasitic wasps of *A. fabae*, the main aphid pest of *V. faba*, are positively associated with *L. niger* ants, probably using a semiochemical camoflage to avoid ant aggression (Völkl 1992). Given that ants are attracted by extrafloral nectaries (Engel *et al.*, 2001), the benefit of parasitic wasps to the plant would need to offset the large negative effect of ants. (Unpublished) preliminary data do not support such a view.

The herbivory component model has certain limitations in application because it considers the average fitness loss of plants across all situations. In reality particular populations of plants will differ due to geographic mosaics in conditions that can alter certain interaction benefits (Thompson, 1994). Geographic mosaics in plant resistance to herbivores (Cushman, 1991), herbivore and mutualist identity (Horvitz and Douglas, 1984) and in ant behaviour to tended homopterans and other herbivores due to distance from nest (Sudd, 1983), starvation and colony protein requirements (Offenberg, 2001), may all affect the exact trends of the herbivore components of plant fitness loss with ant density. Therefore care should be taken generalising results from only one particular time or location (Rashbrook et al., 1991, Rosengren and Sundström, 1991). In this study we considered the interaction between ants, myrmecophilous aphids, non-myrmecophilous herbivores and bean plants in the UK. Changes in the identity and abundance of these players could change the result of a parasitic relationship between the plant and ants. For example if aphids are absent then the C_m will be zero and the positive effect of ants on folivores, if mediated indirectly through the presence of aphids as suggested in this study, will also be absent. Thus, in the absence of aphids the relationship between plant and ants could be mutualistic. This mutualism was not supported in this study as seed mass was similar between ant-treatment and ent-excluded plants in the absence of aphids, although further work on this system in different locations with different suites of interacting herbivores would clearly be useful. As mentioned above, selection pressures for the evolution of extrafloral nectaries will depend on average fitness

effects across space and time, mediated by gene flow between populations. One problem with the hypothesis that extrafloral nectaries have evolved because of the benefits to plants when aphids are absent, is that evolutionary patterns show a positive association between plants with extrafloral nectaries and ant-tended aphids (Offenberg, 2000). Also on an ecological scale, aphid colonies have been shown to survive better on plants with ants (Dixon, 1998) and ant presence drives aphid abundance upward in a positive feedback loop. Thus plants take a risk investing in ant-attractant mechanisms because if aphids become established then plant fitness can rapidly suffer.

Using the herbivory component model, if fitness cost curves are to be inferred from plant damage-yield functions, then it is important to remember that herbivores can have fitness effects on plants other than the physiological damage caused by herbivory. Diseases introduced by herbivores comprise part of their overall impact on plant fitness and cannot be neglected when considering herbivore impact over evolutionary timescales. Sap-sucking insects are infamous for introducing fungal infections and transmitting a wide range of plant pathogens including bacteria, viruses, mycoplasmas, mollicutes, rickettsias and related organisms (Buckley, 1987), and these would tend to increase the slope of the myrmecophilous component of fitness cost with ant density (C_h), thus making an ant-plant mutualism less likely. Finally, it should be noted that some species of plants use ants as seed dispersers (Beattie, 1985) (N.B. not *Vicia faba*) and in these cases ant-attractant mechanisms confer fitness benefits other than those mediated through herbivore defense.

The herbivory component theory of ant-plant interactions contributes to understanding the significance and evolutionary implications of conditionality in mutualisms. In this case an intriguing relationship between the two herbivore components, potentially mediated through plant chemistry, results in an overall negative effect of ants on plants that is robust over a wide range of ant densities.

Chapter 3

In press as:

Oliver, T.H., Jones, I., Cook, J.M., Leather, S.R. (in press) Avoidance responses of an aphidophagous ladybird, *Adalia bipunctata*, to aphid- tending ants. *Ecological Entomology*

Avoidance responses of an aphidophagous ladybird, *Adalia bipunctata*, to aphid- tending ants

Summary

Insect predators often aggregrate to patches of high prey density and use prey chemicals as cues for oviposition. If prey have mutualistic guardians such as ants, however, then these patches may be less suitable for predators. Ants often tend aphids and defend them against predators such as ladybirds. Here, we show that ants can reduce ladybird performance by destroying eggs and physically attacking larvae and adults. Unless ladybirds are able to defend against ant attacks they are likely to have adaptations to avoid ants. We show that *Adalia bipunctata* ladybirds not only move away from patches with *Lasius niger* ants, but also avoid laying eggs in these patches. Furthermore, ladybirds not only respond to ant presence, but also detect ant semiochemicals and alter oviposition strategy accordingly. Ant semiochemicals may signal the extent of ant territories allowing aphid predators to effectively navigate a mosaic landscape of sub-optimal patches in search of less well-defended prey. Such avoidance probably benefits both ants and ladybirds, and the semiochemicals could be regarded as a means of cooperative communication between enemies. Overall, ladybirds respond to a wide range of positive and negative oviposition cues that may trade-off with each other and internal motivation to determine the overall oviposition strategy.

Introduction

Many ladybirds (Coccinellidae) are predaceous, feeding primarily on Homoptera (e.g aphids, scales and mealybugs) (Hodek, 1973, Majerus, 1994), which are agricultural pests that impose considerable economic losses to crops (Buckley, 1987). Ants (Formicidae) too, prey upon Homoptera, but also tend them to obtain sugary honeydew rich in amino acids and vitamins (Stadler and Dixon, 2005). Approximately 45% of ant genera contain homopteran-tending species (Oliver, in preparation) and, correspondingly, about one-quarter of aphid species appear to be always tended by ants, with many more species tended facultatively (Bristow, 1991, Stadler and Dixon, 1998b). Thus, there is likely to be conflict between many ants and ladybirds for homopteran resources. Ants are able to effectively defend resources and often show increased aggression to intruders closer to food sources (Brian, 1955, Way, 1963, Offenberg, 2001, Dejean, 2002). Regardless of whether such defensive behaviour has evolved primarily in response to competition by other ants (e.g. Blüthgen et al., 2004), or in response to aphidophagous predators, the defences appear effective at repelling ladybirds (e.g. Banks and Macaulay, 1967, Nault et al., 1976, Harmon and Andow, 2007). Conversely, some species of ladybird can avoid or defend against ant attacks and are more able to exploit ant-attended resources than other less well defended ladybird species (for a comprehensive review see Majerus *et al.*, 2007).

The two-spot ladybird *Adalia bipunctata* L. regularly feeds on aphids such as *Aphis fabae* that are facultatively tended by ants (Banks and Macaulay, 1967, Hodek, 1973). Given that this competitive use of shared resources has continued for sufficient evolutionary time, we may expect *A. bipunctata* to have either: a) adaptations to defend against ants, or, b) mechanisms to avoid ants. In this study, we investigate the mechanisms *A. bipunctata* uses to avoid costly conflict with ants. First, we test whether *A. bipunctata* can effectively defend itself against ants in order to exploit ant-tended aphids. We then test for any adaptations to avoid ant presence. Ladybirds may avoid ants by evading direct encounters; they may also use non-physical cues, however, as signals of ant presence. Ants actively lay semiochemical trails by touching exocrine glands onto a substrate surface. These actively laid chemical marks are often used to recruit nestmates to profitable food sources (Hölldobler and Wilson,

1990). Semiochemicals can also be applied to substrates passively through shedding of cuticular hydrocarbons (Yamoaka and Akino, 1994, Depickière et al., 2004). These cuticular hydrocarbons are important in colony nestmate recognition and, when transferred onto the ground, may also mark out territories (Devigne and Detrain, 2002). We test whether *A. bipunctata* uses ant semiochemicals as an oviposition cue to assess patch quality, avoiding laying eggs where ants have been present.

Ladybirds respond to many different cues to select patches that will maximise their own and their offspring's fitness. Oviposition cues for coccinellids reported to date include prey species (Blackman, 1967) and abundance (Dixon, 1959, Wratten, 1973, Oliver et al., 2006) , presence of- and chemicals from- competing females (Mishra and Omkar, 2006) and conspecific larval tracks (Hemptinne et al., 1992, Ružicka, 1997, Doumbia et al., 1998, Yasuda et al., 2000, Hemptinne et al., 2001, Ružicka, 2001). There is some evidence that certain heterospecific larval tracks may also inhibit oviposition (Ružicka, 2001). Furthermore, oviposition responses to patch quality cues may not be fixed, but flexible, and may depend upon the individual experience of the gravid female, along with internal physiological state (Fréchette *et al.*, 2004). There is growing evidence that ant semiochemicals are often used as information signalling patch quality to other insects. Ant-induced oviposition has been observed in membracids (Hemiptera) (Hölldobler and Wilson, 1990) and lycaenids (Lepidoptera) (Wagner, 1997). Chrysomelid beetles (Coleoptera), in contrast, have found to be deterred by ant semiochemicals (Offenberg, 2004). There are no current reports, however, of ant semiochemicals affecting the oviposition strategy of coccinellids.

Materials and Methods

Insect culture

Two-spot ladybirds, *A. bipunctata,* were reared at 20°C ± 1°C and a photoperiod of LD 16:8h, in five-litre plastic containers which also included a piece of corrugated filter paper, on which the ladybirds often laid eggs. The ladybirds were fed daily an excess of pea aphids *Acyrthosiphon pisum* Harris and black bean aphids *Aphis fabae*

Scopoli, which were reared on broad beans, *Vicia faba* L. Once a week the adults were transferred to clean containers to stimulate egg laying.

Expt. 1: Efficiency of aphid-defence by Lasius niger ants

The effectiveness of defence by ants of tended aphid colonies was investigated by placing coccinellid predators onto plants and observing patch residency times. The aim of this experiment was not to consider the avoidance response of coccinellids to ant semiochemicals, but rather to investigate whether coccinellids could tolerate ant presence. A *V. faba* bean plant, heavily infested with *A. fabae*, was placed outdoors close to a natural *L. niger* colony. After 24h there were *c*.35 ants on the plant. A final instar *A. bipunctata* larva was then placed onto a lower leaf. The amount of time the larva remained on the plant without walking away or dropping-off was recorded, up to a maximum of 180 seconds. The experiment was repeated 12 times, each time using a different larva. All ants were then removed from the plant using a fine paintbrush and the experiment was repeated another 12 times as a control, again using different larvae, but placing them onto the same leaf as before. The experiment was repeated at a later date using *A. bipunctata* adults.

Expt. 2: Oviposition response to ant semiochemicals

Adalia bipunctata adults were offered a choice of four oviposition sites contaminated by varying levels of ant semiochemicals. Inside four 4.5cm Petri dishes pieces of 4.25cm filter paper were placed. Two, five or ten *Lasius niger* ants were placed into each Petri dish, with one left empty as a control. After three hours the filter papers were removed, folded to corrugate them, and a small piece of Blu-tac® attached to the centre of one side. The filter papers were then secured in a square formation onto the base of a sterile 15cm Petri dish. To mitigate any bias from spatial positioning, the light source was directly above and filter papers were placed an equal distance from the edge of the Petri dish. Ten satiated *A. bipunctata* adults were placed into the large Petri dish with an excess of *A. pisum* aphids. A preliminary investigation ensured that aphids did not aggregate disproportionately to either filter paper ($n = 20$, $t = 0.22$, d.f. = 19, $p = 0.83$). The number of coccinellid eggs on each

filter paper was counted at intervals of 5h and 10h and then again after 24h. The test was replicated 24 times.

Expt. 3: Avoidance and oviposition response to ant presence

Adalia bipunctata ladybirds were given a choice to lay eggs in one of two adjacent arenas: one empty, the other occupied by *L. niger* ants. Two ten-litre closed plastic boxes were placed together with an open window in the upper half of the adjoining wall. Fluon® was applied in a horizontal line, below the window, halfway up the inside of the boxes. This prevented ants crossing from one arena into the other, yet ladybirds could still access both arenas by flying over the wall. Occasionally, ladybirds were also able to climb across the Fluon® barrier and thus occupy the upper half of the box and cross the dividing wall in this way. Ants, however, were always unable to traverse the Fluon® line. Before the experiment, the arenas were cleaned with hot water and dried to remove any residual semiochemicals. In the centre of each arena a 9cm Petri dish was placed, raised above the base of the arena with a plastic stopper. In the centre of each dish a corrugated 4.25cm filter paper was attached using Blu-tac® and an excess of *A. pisum* aphids placed onto the paper. The inside and outside rim of the Petri dish was coated with Fluon® to prevent aphids escaping or ants climbing in. At the start of the experiment 50 *L. niger* ants were placed into one arena and six *A. bipunctata* adults placed into each arena (12 in total). At 4h intervals, over a period of 24h, the number of eggs laid in the raised Petri dishes or on the base of the arenas was counted and also the number of *A. bipunctata* in each of the two arenas. The experiment was replicated 12 times.

Expt. 4: Fitness effects of ants on coccinellid eggs

Batches of *A. bipunctata* eggs recently laid on filter papers were counted (range: 5-40 eggs) and then randomly allocated to one of two treatments. For the first treatment, eggs were placed in plastic boxes containing unqueened colonies of *c.*200 *L. niger* for 24h. Eggs were then left in a 9cm Petri dish with Fluon® coated sides and upon hatching the number of larvae emerging were counted. The second treatment comprised a control with no ants present. Sixteen replicates were conducted in total.

STATISTICAL ANALYSIS

Analysis of deviance, using the program 'R' (Ihaka and Gentleman, 1996), was used to compare the number of eggs laid on filter papers contaminated by different levels of ant semiochemical. Eggs were counted after 24h, but also twice during the experiment because eggs can be cannibalised by adults. For this reason, the maximum count of the three recordings was used for analysis. Egg cannibalism was very rare during the experiment, however, as an excess of aphid prey was provided. The Poisson family of error structures was used to deal with the count data and overdispersion was accounted for where necessary by using the 'quasipoisson' family of error structures (Crawley, 2005). Factor levels were collapsed to test for differences between them and obtain the minimum adequate model.

A standard t-test was used to compare mean times ladybirds remained on ant-covered versus control plants. As the sample variances were different (as determined using an *F* test), the Welch approximation for degrees of freedom was used. A paired Students t-test was used to test for differences in ladybird numbers and eggs in the two arenas of the ant presence experiment. Again, maximum egg counts were used for analysis. Similarly, a t-test was used to compare the hatch rate of ladybird eggs on control and ant-treatment filter papers. Because the starting number of eggs on each filter paper varied, however, the proportion of eggs that hatched was used for analysis, with appropriate square root-arcsine transformation. All mean values listed are followed by ± one standard error.

Results

Expt. 1: Efficiency of aphid-defence by Lasius niger ants

Ants attacked both ladybird larvae and adults and ladybirds spent less time on plants when ants were present. *Adalia bipunctata* larvae remained on plants for 105.8 ± 15.7 s when ants were present, compared with 169.2 ± 10.8 s when ants were absent ($t = 3.32$, $df = 22$, $p < 0.005$). Ladybird adults remained on plants for 103.9 ± 24.1 s when ants were present, compared with 166.6 ± 13.3 s when ants were absent ($t = 2.28$, df =

17.2, $p < 0.05$). Only larvae were observed to 'reflex bleed' (Pasteels *et al.*, 1973) in defence.

Expt. 2: Oviposition response to ant semiochemicals

Ladybirds avoided laying eggs in the presence of *L. niger* semiochemicals. Oviposition was significantly reduced on filter papers contaminated with the highest level of *L. niger* semiochemicals compared with lower concentrations and the control ($F_{1,94} = 6.88$, $p < 0.05$) (Fig. 1). Oviposition on filter papers with lower concentrations of semiochemical was not different to the control (*L. niger*: $F_{2,92} = 0.05$, $p = 0.95$).

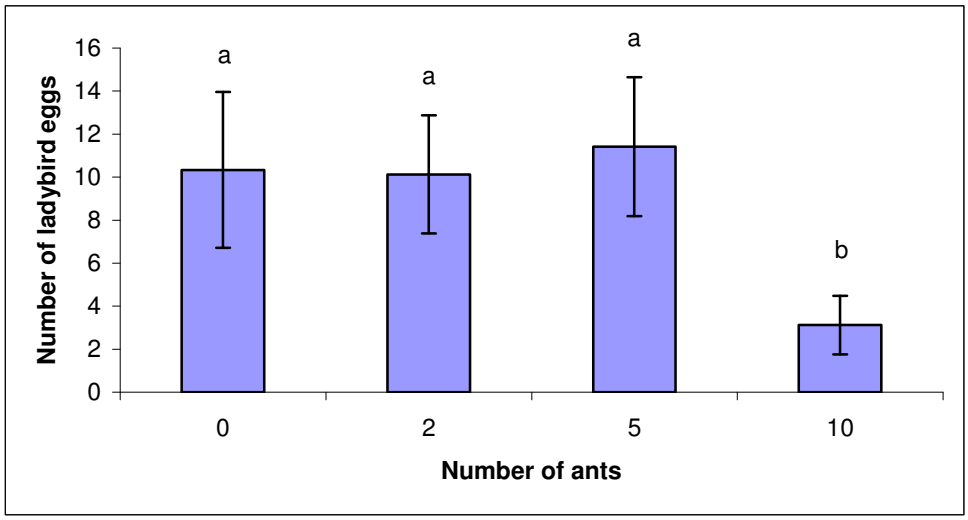


Fig. 1, The mean number of eggs laid by ten *A. bipunctata* adults over 24h on filter papers contaminated with semiochemicals from different numbers of *L. niger* ants. Different letters represent significantly different means ($p<0.05$). Bars represent standard errors ($n = 24$).

Expt. 3: Avoidance and oviposition response to ant presence

Ladybirds avoided the presence of ants and, correspondingly, fewer eggs were laid in the arenas containing ants. Ants often attacked the *A. bipunctata* ladybirds by biting them and arching the abdomen underneath the thorax to face the gaster forwards, a behaviour typically associated with the spraying of formic acid (Sloggett et al., 1998). Throughout the experiment, there were on average 5.02 ± 0.29 ladybirds in the arena with *L. niger ants*, compared with 6.98 ± 0.29 in the arena without ($t = 3.37$, $df = 11$,

$p < 0.01$). After 24h, 64.08 ± 9.42 eggs were laid in arenas with *L. niger*, compared with 21.92 ± 3.55 eggs in arenas without ants ($t = 4.93$, $df = 11$, $p < 0.001$). Thus, almost three quarters of all ladybird eggs were laid in the arena without ants.

Expt. 4: Fitness effects of ants on coccinellid eggs

Ant attacks reduced the viability of ladybird eggs (Fig. 2). *Lasius niger* were observed to occasionally bite and damage the eggs, although never to feed on the contents. As a result, significantly fewer eggs hatched succesfully in the ant treatment ($t = 4.04$, $df = 30$, $p < 0.001$).

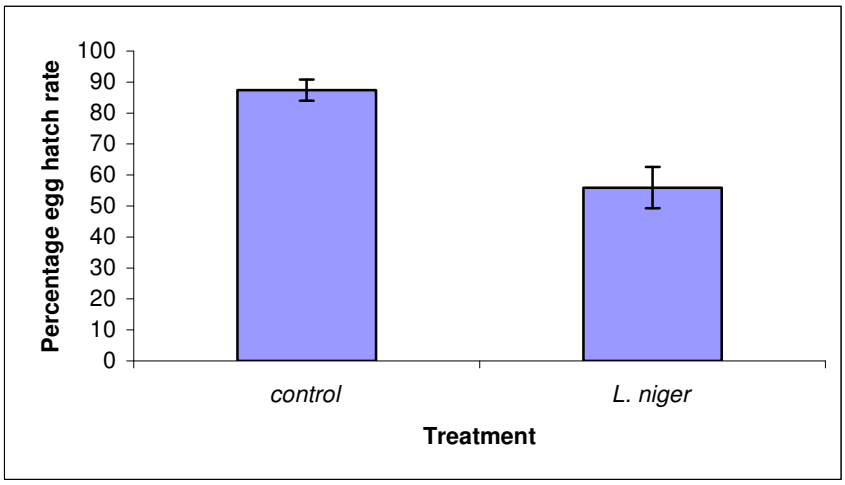


Fig. 2, Damage by *L. niger* reduces the percentage hatch rate of *A. bipunctata* eggs. Bars represent standard errors about the means (n = 16).

Discussion

Adalia bipunctata ladybirds avoided *Lasius niger* ants, but also responded to ant semiochemicals alone, by reducing oviposition. Thus, *L. niger* effectively repelled *A. bipunctata* from homopteran resources, as in other studies (e.g. Banks and Macaulay, 1967, Harmon and Andow, 2007). There were some defences observed against ant-attacks, including withdrawal of appendages and tilting of the carapace in adults and reflex bleeding in larvae. Despite these stationary defences, however, ladybirds were often forced to evade ants. For example, in the arena experiments, ladybirds actively avoided the presence of the ants. More eggs were laid in the arena without ants and this could simply be due to interference of oviposition behaviour by ants and a consequence of more ladybirds present in the arena without ants. Semiochemicals, in the absence of ants, however, were also found to inhibit *A. bipunctata* oviposition. Thus, a combination of active avoidance of ants, oviposition interference by ants and oviposition inhibition by ant semiochemicals, may determine the overall oviposition strategy of ladybirds in patches with ants. Harmon *et al*. (2007) showed that ladybirds avoid foraging in patches with ants by assessing ant density relative to aphid abundance. This is the first demonstration, however, of coccinellids using ant semiochemicals as an oviposition cue. Laying fewer eggs near ants is likely to be adaptive: ants can sometimes damage ladybirds, especially larvae, during attacks; time and energy are lost if ladybirds are forced to drop from plants and, finally, ants chew and destroy ladybird eggs. Indeed, we found that there was a greater than 35% reduction in egg hatch rate, due to ant attack (Fig. 2). Ants did not appear to feed on egg contents; indeed, they often contain toxic alkaloids (Pasteels, 2007). This attack of ladybird eggs could suggest specific adaptation in ants against ladybird antagonists, rather than some extension of aggression towards moving insects close to food resources (e.g. Way, 1963).

The avoidance of ant-guarded prey patches is likely to benefit ants as well as ladybirds themselves. Defending aphids from natural enemies has time and energy costs for ants, and there may be also be increased risk of mortality (Stadler and Dixon, 2005). Ladybird responses to ant semiochemicals could thus be viewed as a form of cooperative communication between enemies, similar to the conspicuous communication between predators and prey, where both parties benefit from avoiding unsuccessful, but costly, chases of strong individuals (e.g. gazelle 'stotting' signalling

agile prey to predators) (van Baalen and Jansen, 2001). In these cases, however, both predator and prey evolve to facilitate the communication: prey perform conspicuous signals, to which predators 'tune in' and respond appropriately. Ant semiochemicals, in contrast, are likely to have multiple functions including intraspecific communication with nestmates and competitors (Hölldobler and Wilson, 1990, Devigne and Detrain, 2002), and mediation of aphid dispersal (Oliver *et al.*, 2007b). Thus, the evolution of these chemicals may be somewhat constrained, and we propose it is more likely that ladybirds have evolved to receive and respond to the ant chemicals, rather than ant chemicals evolving to manipulate ladybird responses.

We have found that ladybirds avoid ovipositing in ant patches in search of better sites, yet the searching experience and internal physiology of ladybirds is also known to affect oviposition behaviour (Fréchette *et al.*, 2004). Thus, it would be of interest to see whether internal factors, such as satiation, trade-off with external inhibitory cues from ants to determine the overall oviposition strategy. Such a trade-off with satiation has been found for inhibitory cues from conspecific larvae (Oliver *et al.*, 2006). The decision to lay eggs in a patch could be mediated by many different external cues interacting with internal motivation for oviposition.

In summary, ants that defend common resources such as Homoptera will frequently compete with predators such as ladybirds. Ant attacks have a negative impact on all ladybird developmental stages: eggs, larvae and adults, and significantly reduce individual fitness. Ladybirds unable to tolerate ant presence are likely to have adaptations to avoid patches with ants. In addition to avoiding foraging on resources guarded by ants, we show that ladybirds use information from ant chemical cues and adjust their oviposition strategy accordingly. Ant semiochemicals signal the extent of ant territories allowing ladybirds to effectively navigate a mosaic landscape of sub-optimal patches. Thus, the spatial distribution of ants that share mutualisms with homopteran pests is likely to also shape the distribution of coccinellid predators.

Chapter 4

Published as:

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Ant semiochemicals limit apterous aphid dispersal

Summary

Some organisms can manipulate the nervous systems of others or alter their physiology in order to obtain benefit. Ants are known to limit alate aphid dispersal by physically removing wings and also through chemical manipulation of the alate developmental pathway. This results in reduced dispersal and higher local densities of aphids which benefit ants in terms of increased honeydew and prey availability. Here we show that the walking movement of mutualistic apterous aphids is also reduced by ant semiochemicals. Aphids walk slower and their dispersal from an unsuitable patch is hampered by ants. If aphid walking dispersal has evolved as a means of natural enemy escape, then ant chemicals may act as a signal indicating protection; hence reduced dispersal could be adaptive for aphids. If, however, dispersal is primarily a means to reduce competition or to maintain persistent metapopulations, then manipulation by ants could be detrimental. Such manipulation strategies, common in host- parasite and predator- prey interactions, may be more common in mutualism than expected.

Introduction

Dispersal between hosts plays a key role in the outbreak of many pests and diseases (Peltonen *et al.*, 2002). Aphids (Hemiptera: Aphidoidea) are vectors of many plant diseases, in addition to causing considerable losses of agricultural yield themselves (Buckley, 1987). Aphid colonies tend to be short lived and transient (Dixon, 1998), thus frequent dispersal among host plants is important in maintaining persistent metapopulations. Factors that affect the persistence of aphid colonies or the founding of new colonies *via* dispersal are likely to alter the population dynamics of pest outbreaks. The presence of mutualistic ants can strongly affect both these parameters. Ants protect aphids from natural enemies (Way, 1963, Stadler and Dixon, 2005), so ant-attended aphid colonies tend to be more stable and persist for longer (Dixon, 1998). Ants are also known to limit aphid dispersal. This can occur through direct physical manipulation, e.g. ants may bite and remove the wings of alate aphids (Kunkel, 1973), or it may occur through chemical influence, e.g. the mandibular secretions of ants can inhibit alate development (Kleinjan and Mittler, 1975). These ant adaptations limit winged aphid dispersal and probably benefit ants by allowing unusually crowded aphid aggregations, producing more honeydew.

Winged dispersal is not the only means by which aphids colonise new plants. In response to crowded conditions, late instar apterous aphids will also leave colonies and wander to new locations on the same plant or along the ground to a new host (Hodgson, 1991). Indeed, this local wandering dispersal can be the primary means of aphid dispersal to neighbouring plants (e.g. Furuta and Aloo, 1994). Both aphid dispersal strategies are important in allowing aphids to efficiently exploit resources such as agricultural crops that are homogeneous at a local scale yet patchy at larger spatial scales (Lombaert et al., 2006). Banks & Nixon (1958) and El Ziady (1960) first noted that the presence of ants can produce a 'tranquillizing' effect on aphids, limiting their motor functions; although since then, to our knowledge no further reports or studies have been published on this issue. In the present study we investigate this phenomena and determine if it is attributable to direct contact with ants and whether it can be achieved through interspecific semiochemical communication.

Ants can actively lay semiochemical trails by touching exocrine glands onto a substrate surface. These actively laid chemical marks are often used to recruit nestmates to profitable food sources (Hölldobler and Wilson, 1990). Semiochemicals can also be applied to substrates passively through shedding of cuticular hydrocarbons (Yamoaka and Akino, 1994, Depickière et al., 2004). These cuticular hydrocarbons are important in colony nestmate recognition and, when transferred onto the ground, may also mark out home range territories (Devigne and Detrain, 2002). Other insects, including herbivores (Offenberg, 2004) and aphid predators (Oliver, in preparation), have recently been found to respond to chemical cues indicating ant presence. In this study we consider how the movement of apterous mutualistic aphids is affected by ant contact and passively laid ant semiochemicals.

Materials and Methods

Aphids and ants

Aphis fabae Scopoli and *Acyrthosiphon pisum* Harris collected from Silwood Park UK and kept in culture for two years were cultured on *Vicia faba* L. plants in netted cages. Aphids were allowed to reach high densities prompting the wandering behaviour of fifth instar apterae. For each experiment these aphids were taken directly from the plant using a fine paintbrush and used immediately. *Lasius niger* L. workers were from a queenless laboratory colony excavated from the field one month previously.

Video recording of aphid movement

Using a camera connected to a PC, aphid walking speeds were recorded under three different treatments: a) control, b) ant semiochemicals only, and c) immediately following direct contacts between aphids and ants; the hypothesis being that ant semiochemicals would reduce aphid walking speed. For control treatments, ten *A. fabae* were placed onto a filter paper in the lid of a 9cm Petri dish and the base, with Fluon® coated sides, was placed on top. After ten minutes to allow the aphids to settle, the Petri dish was placed onto the recording platform along with five identical

replicates. The recording platform consisted of two semi-transparent sanded glass sheets illuminated underneath by six green LED lights. A sensitive Watec 902 camera with HF9HA-1B Fujinon lens (ALRAD, UK) was suspended on a wooden frame 50 cm above the viewing platform (Fig. 1). Images were transmitted to a PC via Video-to-USB Converter (Imaging Source, Germany) and recorded with custom written software (available on request). Each record lasted for about five minutes with shots taken at 0.5s intervals. For the ant semiochemical records, filter papers were contaminated by keeping ten *L. niger* on the paper in the Petri dish with Fluon® coated sides for 4h previously. These *L. niger* semiochemicals are likely to be passively laid hydrocarbons rather than actively laid trail pheromones which are only laid when a food source is discovered (Beckers et al., 1992). Ants were then removed and ten aphids placed into the dish immediately. Recording commenced after ten minutes along with five identical replicates. To study the effect of previous direct contact between ants and aphids, ten *L. niger* ants were put together with the aphids in the Petri dish on clean filter paper. After 10-20 min ants were removed and the aphids recorded. In this way, there was no need to manually filter the ants' tracks from the records which could introduce human error.

The recording of six replicates of each the three treatments comprised one series. Five series were recorded altogether– two with 10 aphids per Petri dish and three with 20 aphids per Petri dish (n = 90). Series were recorded on three separate days. The first two days' recordings were complete with all factor levels (three paper contamination levels and two aphid densities). On the third date, however, only the higher density of aphids were recorded with the three paper contamination levels.

Aphid dispersal

As walking speed is not necessarily equivalent to dispersal (direction change can affect dispersal), an additional experiment was carried out to see if ant presence affected the time taken for *A. fabae* aphids to disperse outwards from an unsuitable patch. Also, *A. pisum*, a non-ant attended aphid species, was tested to compare for differences in the responses of mutualistic and non-mutualistic aphids to ants. A Petri dish with a 3cm diameter disc cut out of the centre had a *V. faba* leaf taped to the underside. Five aphids placed on the leaf dispersed outwards. The time taken for each aphid to reach the Petri dish edge was recorded for up to five minutes and the mean

time calculated. Another test was carried out with the same aphids on a similar sized leaf from the same plant, this time including 12 ants in the Petri dish. The experiment was replicated 20 times, each time alternating the order of control and ant treatment.

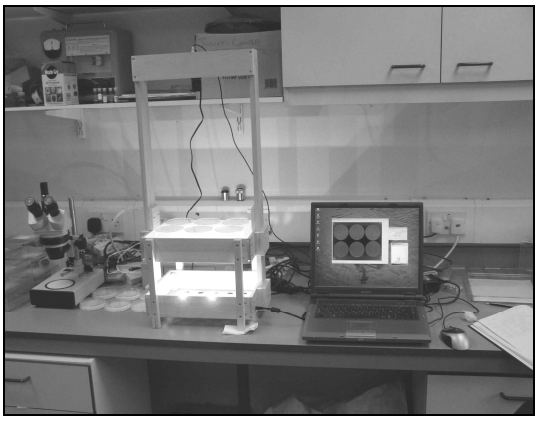


Fig. 1, Recording platform and PC

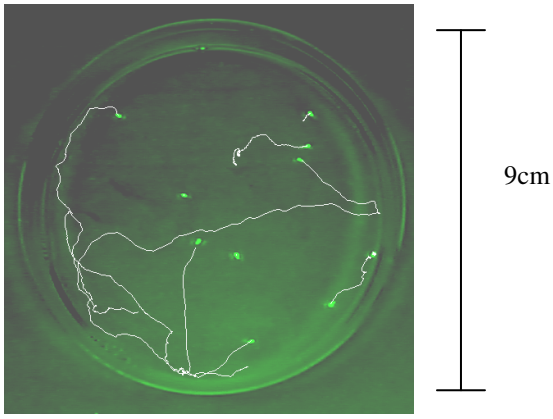


Fig. 2, Aphids and their tracks

STATISTICAL ANALYSIS

The records were analysed using free software (www.nimr.mrc.ac.uk/GMimPro) designed to track individual objects (Mashanov and Molloy, 2007). This software tracks the position of every positively identified object and stores the coordinates of the object at every time step (Fig. 2). When tracks cross, emerging tracks are estimated by by direction and speed of the tracks before crossing. From the coordinates of each individual track the distance moved in each one second interval (instant speeds) were calculated. On two occasions there were problems in the recording recognition due to Petri dish layout and the ten replicates affected were omitted, reducing the total sample size (n=80). Besides this, the mean value of the instant velocities of all the aphids in a Petri dish was used for each individual data point, thereby avoiding the problem of pseudoreplication. Because recordings were taken on different days and day had a significant effect on mean speed (one way ANOVA: $F_{2,77} = 36.39$, $p < 0.001$) , the analysis required a nested structure. Statistical tests were carried out using the program 'R' (Ihaka and Gentleman, 1996). A mixed effect model (lmer) was used

whereby the main effects and interaction between the two fixed effect factors (Contamination * Aphid density) were nested within Day. Non-significant terms were removed in a stepwise fashion to obtain the minumum adequate model for each analysis. Where relevant, the factor levels of the Contamination factor were collapsed allowing contrasts between different factor levels. Dispersal times, in the second experiment, were compared using a two-way ANOVA for each aphid species with treatment and treatment order as explanatory variables.

Results

Mean speed

Aphids moved slower in the presence of ant semiochemicals compared with the control. There was no interaction between filter paper treatment and aphid density on the mean speed of wandering aphids ($F_{2,74} = 0.33$, $P = 0.72$), thus a simple additive model can be used to describe the data. There was a significant effect of filter paper treatment on mean speed ($F_{2,77} = 13.20$, $P < 0.001$), but there was no difference between filter papers contaminated with only ant semiochemicals versus those on which the aphids had physically contacted ants previously ($\chi^2 = 0.191$, df = 1, p = 0.66). Therefore the two factor levels were combined (mean ±SE: 0.354 ± 0.0218 mm/s, n = 51). This ant treatment mean was significantly less that the control treatment (mean: 0.496 ± 0.0321 mm/s, n = 29; $\chi^2 = 22.93$, df = 1, $p < 0.001$) (Fig. 3). The main effect of aphid density was close to significance ($F_{1,76} = 3.541$, $P = 0.064$).

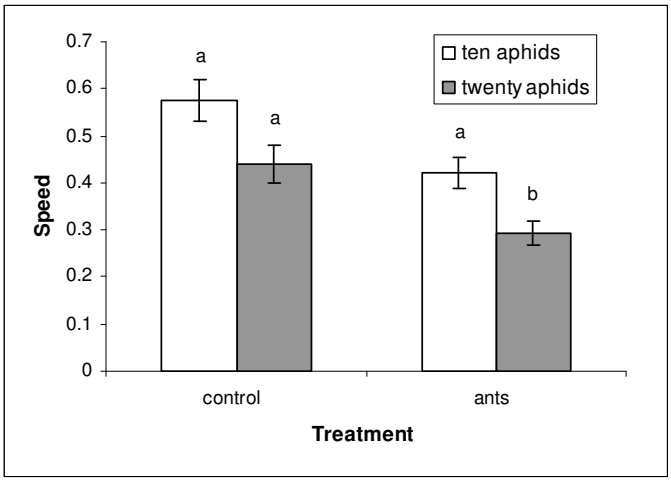


Fig. 3, Mean walking speed of aphids (mm/s) on different filter papers at two densities of aphids. Error bars represent standard error of means (n = 12,17,24,27, respectively). Different letters above the bars indicate significantly different means ($p<0.001$, Tukey HSD test).

Dispersal

The dispersal of *A. fabae* was significantly reduced in the presence of ants. Aphids reached the edge of the Petri dish after 88.3 ± 10.8 seconds in the control, but only after 117.5 ± 7.5s when ants were present in the dish ($F_{1,38} = 4.89$, $p = 0.033$). Order of treatment had no effect on mean speed ($F_{1,37} = 1.16$, $p = 0.29$). In contrast, ant presence had no effect on the dispersal times of the non-ant attended aphid *A. pisum* ($F_{1,37} = 0.37$, $p = 0.55$), yet treatment order was important ($F_{1,38} = 21.9$, $p <0.001$).

Discussion

Aphid walking speed was significantly reduced in the presence of ant semiochemicals. Previous direct contact with ants (this treatment includes the effects of ant semiochemicals) did not elicit any different effect on aphid mean speed compared with semiochemicals alone. Thus, ant presence is not required to obtain a 'tranquillizing' effect upon aphids (El Ziady, 1960, Way, 1963); instead, semiochemical cues are necessary and sufficient. The frequency of tactile contact between aphids has been shown to stimulate the formation of alate dispersers (Lees, 1967, Toba et al., 1967, Sutherland, 1969). Tactile contact frequency is likely to be

affected by the presence of boundaries (e.g. leaf margins) and the density and speed of individuals. If ant semiochemicals limit aphid walking speed, as demonstrated here, then tactile contact and hence alate development may also be affected. Thus, through limiting individual movement, ant semiochemicals may interfere with both local (apterous) and long distance (alate) dispersal of aphids. Indeed, in three out of four studies on aphid wing induction with different ant/ aphid species, ants were found to reduce alatae development, with the fourth study finding no effect either way (Müller et al., 2001). Chemicals, such as dendrolasin, produced by ant mandibular glands can limit alatae development (Kleinjan and Mittler, 1975). In addition to this, we propose that there may be indirect effects of ants on alate production, whereby ants limit aphid movement and thus reduce the frequency of tactile contact.

Aphid density had a marginal effect on aphid walking speed, with aphids at higher densities moving slower. Other studies have found apterous dispersal to be density independent (e.g. Lombaert *et al.*, 2006), although these are field studies where aphids are able to disperse off plants and are not confined within a Petri dish. Winged dispersal, in contrast, is often found to be positively density dependent (Müller *et al.*, 2001).

Ant presence reduced the dispersal of *A. fabae* aphids and it was observed that aphids often stopped moving when ants contacted them. In contrast, the dispersal of a non-attended aphid, *A. pisum*, was not affected by such ant contact. We have found that ant semiochemicals also reduce movement speed of mutualistic aphids. Given that ant semiochemicals are similarly effective on leaf surfaces, which is possible if they consist of relatively involatile hydrocarbons which are retained on the waxy surface of leaves (E.D. Morgan pers. comm.), then aphid colony dispersal from ant-attended plants will be limited, leading to local increases in aphid population density. Thus, we propose ant semiochemicals as an additional explanation for ant-tended aphid colonies found to be larger in size than untended colonies (Stadler and Dixon, 2005) . Previous explanations for this phenomena include ant-mediated protection from predators and direct increases in aphid feeding rate (Way, 1963, Stadler and Dixon, 1999).

The response of aphids to ant semiochemicals may be adaptive for aphids. By remaining within the foraging territory of mutualistic ants aphids derive benefits from protection from predators, reduced pathogen contamination *etc*. (see Stadler and Dixon, 2005 for a comprehensive review). In contrast to these benefits, however,

there are costs in the ant- aphid relationship such as increased parasitism by specialist parasitoids adapted to avoid ant attacks and occasional predation by ants themselves. In addition to these oft cited costs, it is possible that reduced disperal of aphids, maintaining them in close aggregations, leads to a decrease in host plant quality that is reflected in aphid fitness. Indeed, apterous (Johnson, 1965, Honek et al., 1998) and possibly alate dispersal (Müller *et al.*, 2001) is often in response to a decline in host plant quality. Additionally, by limiting aphid dispersal ants affect the ability of aphid clones to colonise new hosts and thus maintain a long-lived metapopulation. An ant-attended colony can be completely destroyed by specialist parasitoids which are immune to ant attack and tend to remain targeting the same aphid colony over multiple parasitoid generations until it is eliminated (Weisser and Völkl 1997, T.H. Oliver personal observation). Ants manipulate aphids chemically and physically to limit alate dispersal (Kleinjan and Mittler, 1975, Hölldobler and Wilson, 1990), so it should not be assumed that reduced apterous dispersal in response to ant semiochemicals is necessarily adaptive for aphids. Ants also delay the timing of dispersal of aphids (on average 0.5-2.5 weeks), and this may be an additional indirect cost of the interaction (Kindlmann et al., 2007).

In contrast, close aggregations of aphids probably benefit ant colonies. They provide dense, highly profitable patches of renewable carbohydrate and protein. Dispersing aphids could also move into territories of other ant colonies, thus benefitting competitors. This may be the reason why ants appear more likely to prey upon lone, rapidly moving aphids (Way, 1963, Cherix, 1981, Cherix, 1987, Rosengren and Sundström, 1991). Instead, the movement of aphids to new plants may be closely controlled. Ants transport aphids directly by carrying them to high quality host plants within the colonies foraging range (Collins and Leather, 2002).

To summarise, we show how the dispersal of a pest organism can be affected by interspecific chemical communication with a mutualist. The outbreak of such pests is likely to be highly dependent on these interspecific interactions. Whether aphids benefit from these effects may depend upon the environmental context (e.g. aphid density, plant quality, natural enemy abundance), and also whether apterous dispersal has evolved primarily through kin selection and to maintain persistent metapopulations or as a means of natural enemy escape. Ants can provide protection against natural enemies, making costly dispersal unnecessary for aphids. In contrast, however, ants may exacerbate intraspecific competition by causing crowded

conditions. Also, limited dispersal may result in an inability to form persistent metapopulations. In these cases, reduced dispersal by aphids would be costly and, thus, aphids are 'manipulated' by ants. Manipulation being a behavioural or physiological change, induced by another species, that benefits the second species yet is costly to the first. Ants manipulate aphids by physically removing alate wings and using allomones to inhibit alate development. Reduced dispersal by apterous aphids could be a similar behavioural change, costly to aphids, caused by ant semiochemicals. Manipulation is a common strategy in host- parasite interactions (e.g. viruses altering host behaviour to facilitate transmission) and predator- prey interactions (e.g. semiochemical 'lures' used by predatory insects), yet it also occurs in mutualisms. Even though the overall interaction is beneficial to both partners, manipulative expoitation by one partner allows derivation of greater benefits than would normally be possible.

Chapter 5

In press as:

Oliver, T.H., Leather, S.R., Pettitt, T., Cook, J.M., (in press) Numerical abundance of invasive ants and monopolisation of exudate producing resources- a chicken and egg situation. *Jounal of Insect Conservation and Diversity.*

Numerical abundance of invasive ants and monopolisation of exudate producing resources- a chicken and egg situation

Summary

Invasive ants commonly reach abnormally high abundances and have severe impacts on the ecosystems they invade. Current invasion theory recognises that not only negative interactions, such as natural enemy release, but positive interactions, such as facilitation, are important in causing this increased abundance. For invasive ants, facilitation can occur through mutualism with exudate-producing plants and insects. To obtain such partnerships, however, invaders must first displace native ants, whose communities are highly structured around such resources. By manipulating the abundance of an invasive ant relative to a native, we show that a minimum threshold abundance exists for invasive ants to monopolise exudate-producing resources. In addition, we show that behavioural dominance is context dependent and varies with spatial location and numerical abundance. Thus, we suggest a 'facilitation-threshold' hypothesis of ant invasion, whereby a minimum abundance of invasive ants is required before facilitation and behavioural dominance can drive abundance rapidly upwards through positive feedback.

Introduction

Invasive ants are a common problem worldwide and have serious negative impacts on ecosystems. Often reaching abnormally high abundances and functioning as predators, herbivores, competitors and ecosystem engineers, they disrupt native communites and may cause the loss of endemic biodiversity (for a comprehensive review see Holway *et al.*, 2002). There are several theories to explain how alien species reach such high densities compared with those in their native ranges. The most widely cited of these are based on negative interactions, such as release from the natural enemies and competitors that limit populations in the native range (e.g. Keane and Crawley, 2002). Positive species interactions, however, such as direct mutualisms and indirect cascade effects, are now also becoming increasingly implicated as important facilitators of biological invasion (Stachowicz, 2001, Bruno et al., 2003)

For ants, the importance of positive interactions with sugary-exudate-producing plants (e.g. extrafloral nectary bearing *Passiflora*) and insects (Homoptera and Lycaenidae) for determining community structure cannot be overestimated (Blüthgen et al., 2000, Wimp and Whitham, 2001, Blüthgen et al., 2004). Ecological dominance in ant communities is strongly associated with the ability to exclude competitors and monopolise exudate-producing resources (Fiedler 2001, Blüthgen *et al.*, 2004). Unsurprisingly, invasive ants are most likely to be able to infiltrate native communities by forging positive interactions with native or co-invading alien Homoptera (honeydew-producing insects) (Ness and Bronstein, 2004, Abbott and Green, 2007, Lach, 2007). Indeed, invasiveness in ant genera is associated strongly with trophobiosis with Homoptera (see Chapter 7). These homopteran mutualists probably provide the resources that allow ants to forage widely and achieve high abundances (Stadler and Dixon, 2005). Population growth is further facilitated by reduced intraspecific aggression between invaders and the formation of large unicolonial 'supercolonies' (Holway *et al.*, 2002). When abundant, ants can achieve numerical dominance improving both exploitative (resource acquisition) and encounter (interference) competitive ability (Holway, 1999, Human and Gordon, 1999, Morrison, 2000). Thus, native ants are often outcompeted and displaced by aliens, with the ensuing disruption of native communities (Holway *et al.*, 2002).

The above description of ant invasion seems logical, but biological invasions begin with the arrival of a small number of aliens, either as an isolated event, or as persistent propagule pressure (Hee et al., 2000, Mack et al., 2000). Initial invader abundances are thus likely to be very low. It is possible that in some cases, ants could forge associations with co-invading alien Homoptera, which natives are not adapted to tending, and increase in abundance this way (Simberloff and Von Holle, 1999, Helms and Vinson, 2003, Abbott and Green, 2007). In the many other cases where alien Homoptera are absent, however, there must be other reasons for their increase in abundance. Can alien ants outcompete natives straight away even at low abundances? Or, do invasive ants need to increase in abundance through some other means, such as natural enemy escape (e.g. Orr *et al.*, 1995), and only when abundant begin to displace natives from exudate producing resources (Le Breton et al., 2007)? If so, what is the threshold abundance of invaders for competitive exclusion of native ants? The answers to these questions will inform the planning of appropriate control measures in the event of invasions. Furthermore, such research also sheds light on a difficult question in ant community dynamics: do ants achieve numerical and ecological dominance primarily because they are able to exclude others from exudate-producing resources, or, does the initial use of these resources cause the increased colony size and activity, that facilitates dominance? Invasive ants provide a good means to solve this 'chicken and egg' dilemma because many are dominant in their native range, yet upon arrival in their new range they have no initial homopteran partners and native ant communities are often already highly structured around exudate producing resources (Blüthgen *et al.*, 2004).

In this experimental study, we manipulated the abundance of an invasive ant relative to a native ant and assess its ability to monopolise an exudate-producing resource. Initially, interactions between the two ant species were observed with abundances in the arena equal. We then increased the abundance of the invasive ant, so that numbers were twice that of the native. Small invasive ants, such as *Technomyrmex albipes*, very often reach large abundances in invaded ecosystems (Yamauchi et al., 1991, Holway et al., 2002). These two abundance ratios tested successfully demonstrated a lower threshold of invasive ant abundance required for resource capture, thus testing a lower abundance of the native ant was not necessary. Furthermore, we found an interaction between numerical and behavioural dominance. Behavioural strategies, which mediate interference competition, were shown to be context dependent, varying

with spatial location, time and conspecific abundance (Wilson, 1971, Sakata and Katayama, 2001, Katayama and Suzuki, 2005). We have chosen the white-footed house ant, *T. albipes,* to study because it is a highly invasive ant species (Holway *et al.*, 2002), but one that has received very little research attention. Also, invasion biology research needs to mature to the formulation of practical prophylactic and reactive protocols. *T. albipes*, probably originating from Indonesia (Warner, 2003), is a possible candidate for invasion of UK habitats given it has established in a similar temperate climate in New Zealand (Wetterer, 2002, Lester and Keall, 2005, Ward, 2005). It is also prevalent in the tropical biome of the Eden Project, Bodelva, UK; a group of biological conservatories containing plants from different biomes worldwide. Understanding interactions between *T. albipes*, native ants and Homoptera will help prepare effective control protocols for the UK and other susceptible countries.

Materials and Methods

Insect culture

Two colonies of *Technomyrmex albipes* Smith (Hymenoptera: Formicidae) workers were collected from the Tropical Biome of the Eden Project, Cornwall, UK. Ants were stored in sealed, ventilated 5 litre plastic containers with petroleum jelly lining the walls. Each container was placed in a larger plastic box part filled with water, so that water surrounded the container on all sides. Ants were fed an artificial diet every four days (recipe from Hölldobler and Wilson, 1990, pg. 632) and supplied with moist cotton wool to maintain a humid environment. The temperature was maintained at 20 ± 1˚C, with a photoperiod of LD 16:8h. *Lasius niger* L. (Hymenoptera: Formicidae) workers were collected from three colonies foraging around greenhouses at Silwood Park, Ascot, UK, and were stored similarly, except without the extra water barrier and with Fluon® (ICI, UK) lining the container walls instead of petroleum jelly. The ant colonies were maintained in culture for over ten weeks before the experimental period. For each experiment, ants were taken from these stock colonies, but, to avoid pseudoreplication, were not returned or used for further experimentation. *Aphis fabae* Scopoli (Hemiptera: Aphididae) were reared in cages on broad beans *Vicia faba* L., which also has extrafloral nectaries (as described by Oliver *et al.*, 2007a).

Behavioural observations

We tested whether *T. albipes* can outcompete native *L. niger* to control a homopteran resource and, by manipulating invader abundance, we considered whether competitive success is dependent on numerical advantage. *Technomyrmex albipes* (worker body length: 2-2.5mm) ants are smaller than *Lasius niger* (worker body length: 3-5mm) so may need larger numbers of ants to win resource competitions. We began the experiment with no ants commanding the resource, allowing us to compare the competitive ability of the invasive and native ant. Forty *T. albipes* and 40 *L. niger* workers were placed in one of two temporary 'nests' consisting of a 0.5l plastic box containing a smaller, foil covered 33cl plastic box with a damp piece of cotton wool inside, and left for 24h. Ants tended to aggregate in the dark, humid smaller box until the lid was removed from the larger box, at which point many ants streamed out to defend the nest or explore. Both nests were placed at either end of an open topped 5l plastic arena with sides lined with Fluon® and petroleum jelly (Fig. 1). A *V. faba* seedling, infested by several *A. fabae* colonies, was placed in the centre of the arena. The plant's roots were submerged in water to prevent ants nesting in a soil medium. To infest the plant, five late instar aphids had been placed onto a lower leaf seven days before observations began using a fine paintbrush. After a week, the aphids had formed several small colonies on the plant with approximately 100 individuals in total. After removing the lids of the ant colonies, ten minutes were allowed for ants to explore the arena. Experiments were run at room temperature (22-24°C) and humidity (although humidities in the boxes with saturated cotton wool were likely to be much higher).

Behavioural data on ant contests in different areas of the arena (Fig. 1) were then collected for 30 mins. Each area of the arena was observed for 10 s and any contests occurring within that time recorded, before observing the next area in turn, for a total duration of 30 mins. If ants made brief contact but then both ran away in opposite directions, this was recorded as 'mutual avoidance'. If ants made contact followed by conflict behaviour by one or both ants, such as biting or chemical spraying, then this was scored as a 'contest'. We also recorded whether contests resulted in one or both ants leaving rapidly, hereafter referred to as 'evasion'. After two hours, another identical 30 min period of behavioural observation was conducted. In addition, at 10

min intervals during the observation periods, and once more after 24h, the number of both species of ant on the aphid-infested plant were recorded. The next day, the entire experiment was then repeated using only 20 *L. niger* workers with 40 *T. albipes*. Over a period of 20 days, 10 replicates of each abundance treatment were tested. After each replicate, the plastic box used in the experiment was washed with boiling water to remove any residual semiochemicals.

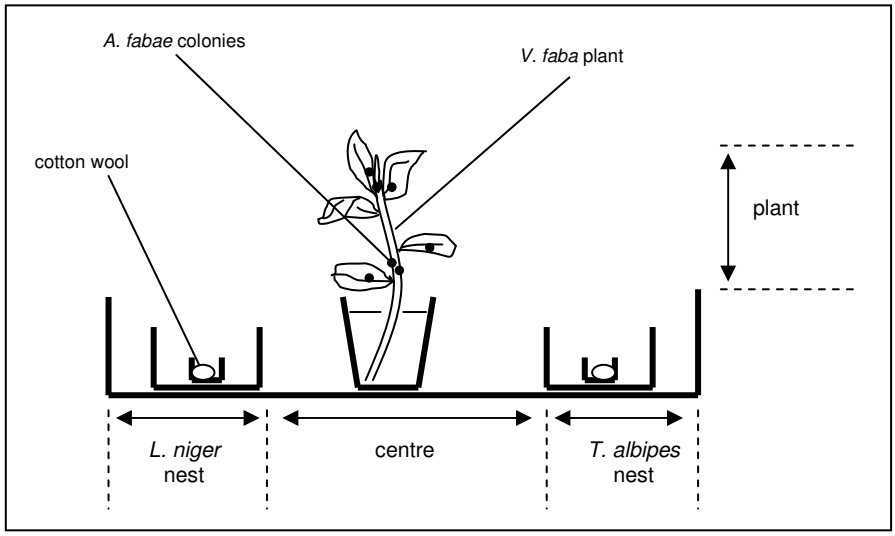


Fig. 1, Arena layout with area margins shown. Ants compete to monopolise the honeydew producing resources in the centre of the arena.

STATISTICAL ANALYSIS

Encounters between ants were analysed using the program 'R' (Ihaka and Gentleman, 1996). Firstly, the proportion of encounters in each replicate ending in contests rather than mutual avoidance were analysed with a mixed effects binomial regression, in which fixed explanatory effects were: spatial location in the arena (Fig. 1), sampling time (zero or two hours after release) and ratio of ant abundance (1:1 or 2:1). Errors for time were nested within replicate number (a random effect), which was nested within ant ratio. Next, the outcomes of contests were analysed in six separate mixed effects models. Explanatory variables were the same as above whilst binary response variables were: a) whether contests resulted in *L.niger* fleeing, b) in *T. albipes fleeing*, c) whether *L. niger* used biting in contests, d) whether *L. niger* used acid in contests,

and e) whether *T. albipes* used biting, or f) acid, in contests. Again, a mixed effects error structure was used to account for non-independence of contests within each replicate. There was little overdispersion in the models, so insignificant factors were removed in a stepwise fashion through chi-squared deletion tests ($p<0.05$) (Crawley, 2007). Factor levels of significant factors were collapsed where possible to obtain the minimum adequate model. For example, when considering the proportion of encounters resulting in contests, the different areas of the arena were grouped into statistically significantly different blocks as implied by model comparisons using Chi-squared tests.

The number of ants on the plant was analysed using an analysis of deviance. The mean ant count on the plant for each species was used for each of the two sampling periods from zero to 30 mins and 120-150 mins, whilst a single count was used after 24h. These ant counts comprised the response variable with ant species, time of sampling and ratio of ants as the explanatory variables. Fitting ant species as an explanatory variable allowed the two abundances of the two species to be compared in the same model. A significant interaction effect between ant species and abundance ratio indicates the effect of relative ant abundance on the species of ant dominating the resource. To test for competitive exclusion, we used a Pearson's correlation test for the abundance of each of the two species on the plant, using only one sampling period (after 24h) per replicate, to avoid pseudoreplication. A similar trend was found for the two sampling periods on the previous day (results not shown).

Results

Proportion of encounters resulting in contests

Overall, across all experiments 602 encounters were observed, 72% which ended in contest. Thus, there was a high level of aggression (propensity to engage in physical conflict) between the two ant species. All contests observed were one-on-one interactions, rather than group attacks by multiple ants on single individuals, as has been observed with some other invasive ant species (e.g. Rowles and O'Dowd, 2007). Spatial location in the arena and time of sampling were significant predictors of the proportion of encounters resulting in contests. Encounters ended in contests least

often in the area around the *L. niger* nest, compared with the rest of the arena (χ^2 = 7.04, d.f. = 1,5, p = 0.008). Similarly, there were proportionally more contests after two hours then in the period immediately following release (χ^2 = 9.23, d.f. = 1,7, p = 0.002) . The ratio of ant abundances had no effect on the probability of contest occurrence (χ^2 = 0.086, d.f. = 1,8, p = 0.769).

Contest outcome

Overall, 436 contests between the two ant species were observed, with an average of 2.72 ± 0.26 contests per replicate. On most occasions, *T. albipes* remained while *L. niger* was forced to flee (Dev = 50.7, d.f. = 1, p < 0.001) (Table 1). If the total ant abundances in the arena were equal and *L. niger* was not outnumbered, then evasion from contests was less likely. Indeed, *Lasius niger* lost 65% of the contests when outnumbered by *T. albipes* in the ratio 2:1, but only 27% when ant numbers in the arena were equal (χ^2 = 7.19, d.f. = 1,7, p = 0.007). Similarly, when the contest occurred close to the home nest or in the centre of the arena, *L. niger* was less likely to flee than if the conflict occurred on the plant (χ^2 = 4.163, d.f. = 1,6, p = 0.04). In contrast, *L. niger* was most likely to flee from contests that occurred in the area around the *T. albipes* nest (χ^2 = 17.94, d.f. = 1,5, p < 0.001). *Lasius niger* was more likely to use acid in its attack of *T. albipes* when close its own home nest (χ^2 = 10.23, d.f. = 3,4, p = 0.017). The use of acid was an infrequent strategy used on only 19 occasions. In contrast, biting was used on 130 occasions. Thus, *Lasius niger* was generally less aggressive in contests on the plant or close to *T. albipes*' nest compared with close to its own nest. Similar to acid attacks, biting was also used most frequently in contests close to the home nest or in the centre of the arena (χ^2 = 6.79, d.f. = 1,6, p = 0.009). Biting was used more in contests when *L. niger* was not outnumbered (χ^2 =5.10, d.f. = 1,5, p = 0.024), and in the first 30 mins (χ^2 = 10.96, d.f. = 1,5, p < 0.001).

Technomyrmex albipes ants were least likely to flee when they they outnumbered *L. niger* in the arena (χ^2 = 4.57, d.f. = 1,4, p = 0.033). In contrast, attack strategy was not affected by the ratio of *T. albipes* to *L. niger* ants (acid: χ^2 = 1.57, d.f. = 1,5, p = 0.210; biting: χ^2 = 0.04, d.f. = 1,8, p = 0.832). Acid was used in attacks most frequently (190 occasions), and was used more in the first 30 mins (χ^2 =18.32, d.f. = 1,4, p < 0.001). Biting was used less frequently (49 occasions), and was reserved for when defending

the home nest or when upon the exudate-producing plant (χ^2 = 19.99, d.f. = 1,4, p < 0.001).

Table 1. **Attack strategies and contest outcomes between two ant species.** The frequency of each behaviour is listed, with the percentage of total contests in parentheses. *Technomyrmex albipes*, although smaller, is more often able to cause *L. niger* to flee.

Behaviour	***L. niger***	***T. albipes***	**Both**
Biting attack	130 (29.8)	49 (9.6)	-
Acid attack	19 (4.4)	190 (43.6)	-
Flee from contest	183 (42.0)	85 (19.5)	23 (5.3)

Monopolisation of exudate producing resources

Lasius niger and *T. albipes* competed to occupy the plant containing honeydew-producing *A. fabae* and extrafloral nectaries. This is reflected in the negative correlation between ant abundances on the plant (Pearson's correlation test: t = 2.95, df = 18, p = 0.008). There was a marginal effect of sampling time on ant counts ($F_{2,114}$ = 2.72, p = 0.070), with counts for both species of intermediate value in the first 30 minutes, peak values after 2h, and lowest after 24h. Note that even after 24h, however, there was still a negative correlation between abundance of the two species. Ant ratio also had a significant effect on ant counts, and this was involved in a interaction with ant species ($F_{1,116}$ = 8.76, p = 0.004) (Fig. 2). This is because neither ant species was significantly more likely to dominate the plant when the ratio of abundances in the arena was 1:1 ($F_{1,56}$ = 0.55, p = 0.461). At this ratio, *T. albipes* dominated the plant on four out of ten occasions, with *L. niger* winning the remaining six. When *T. albipes* outnumbered *L. niger* by a ratio 2:1, however, then *T. albipes* counts were significantly higher ($F_{1,56}$ = 22.39, p < 0.001). In this case, *T. albipes* dominated the plant on eight occasions, *L. niger* on one occasion, with ant numbers equal on the remaining occasion.

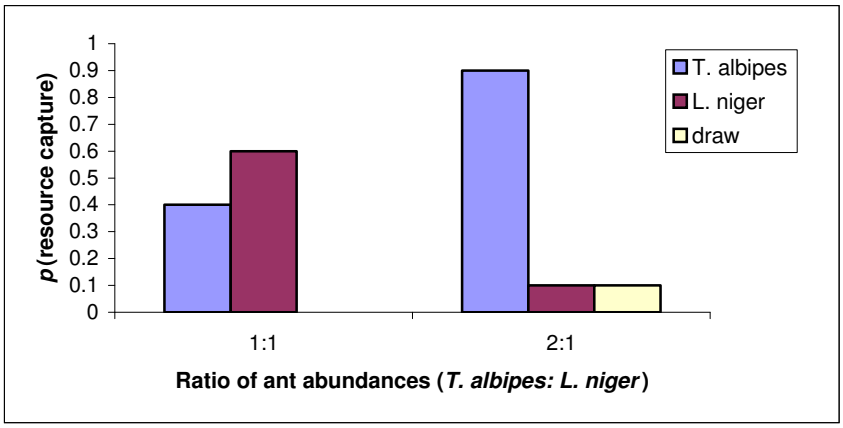


Fig. 2, The probability of capture and monopolisation of a honeydew-producing resource, at different relative abundances of an invasive and native ant (n=20).

Discussion

There was much aggression between the invasive and native ants used in this study and over 70% of encounters resulted in escalated contests. During contests, there were clear differences in strategy used by the two species. *Technomyrmex albipes* attacked primarily by spraying with acid and used biting attacks only close to the home nest or on the plant. Conversely, *L. niger* used biting attacks most frequently, using acid attacks only when close to the nest. Both ants were able to adjust their strategy depending on spatial location in the arena. This probably reflects certain strategies being more costly to the individual and only used when there is greater risk to the colony. Although there were fewer encounters escalating to contests in the first half an hour after release, when contests did occur they were more intense with biting and acid used more often. This may reflect the ants attempting to balance the costs of encounter in a previously unexplored area, with the benefits of territory acquisition. Both ants also adjusted their strategy depending on the ratio of conspecifics to competitors. Evasion from encounters was more likely when the abundance of competitors was higher. Ant behavioural strategies have long been known to be context dependent (e.g. Wilson, 1971), thus, it is not surprising that interference competition between ants is intrinsically linked to numerical abundance. Ant

aggression against herbivores when defending exudate-producing plants also increases with local abundance of conspecifics (Sakata and Katayama, 2001, Katayama and Suzuki, 2005). In this study, we found the two ant species showed different strategies around the exudate-producing resource. *Technomyrmex albipes* showed similar levels of aggression on the plant as at the home nest. In contrast, *L. niger* was much less aggressive on the plant compared to its home nest.

With regards to capturing exudate-producing resources such as aphids, there was clear competitive exclusion between the two ant species. Abundances of the two species on the host plant were negatively correlated. The invasive ant, *T. albipes*, was able to monopolise the aphid resource when it outnumbered native *L. niger* ants by a 2:1 ratio. *Technomyrmex albipes* was a superior competitor in individual contests, causing *L. niger* to flee on the majority of occasions. Such dominance in individual contests is not always common in invasive ants. For example, the Argentine ant *Linepithema humile* was not necessarily superior to native ants in individual contests and often relied on 'group attacks' (Rowles and O'Dowd, 2007). Indeed, many invasives rely on a large number of small ants to overpower competitors (McGlynn, 1999a, Morrison, 2000). *Technomyrmex albipes*, in addition to its fighting capacity, is also very small (2.5-3mm) and, given that colonies with smaller ants can grow faster (Bourke and Franks, 1995), it may be able to achieve the threshold abundances to displace *L. niger* relatively easily. Ideally, different spatial scales of competition also need to be investigated, however, as foraging, recruitment and aggregation responses will determine local abundance. Unfortunately, there are ethical limitations on the feasibility of these studies in uninvaded habitats.

Despite the aggressiveness of *T. albipes* and its capacity to win contests, we have clearly demonstrated that a threshold abundance for the displacement of native ants from exudate-producing resources exists. When the ratio of the *T. albipes* to the native *L. niger* was less than 2:1, the invasive ant was unable to successfully displace native ants from the resource. The population growth of invasive species often shows an extensive 'lag' phase before rapid growth (Mack *et al.*, 2000). A number of explanations have been suggested including: error from not detecting small populations (Crooks and Soule, 1996), spatial distance between invasion sites and dispersal limitations (Moody and Mack, 1988, Wangen and Webster, 2006), natural selection needing to occur before invaders can spread (Crooks and Soule, 1996), and stochastic effects disproportionately affecting small populations (Crawley, 1989).

What is clear, however, is that the best time to eliminate invaders is before the exponential growth phase (Mack *et al.*, 2000). For invasive ants, the temporary inability to wrestle exudate-producing resources from natives provides an additional explanation for a lag phase in population growth. Whilst all available exudate-producing resources are saturated by native ants, invasive ants may increase only slowly, if at all. When the threshold abundance allowing competitive displacement of natives is achieved, however, then populations of invasive species will increase rapidly (Mack et al., 2000, Le Breton et al., 2007), (Fig. 3). Positive feedback between ant and trophobiont partners can then drive both populations rapidly upwards (Oliver *et al.*, 2007a). Furthermore, the behavioural dominance of ants increases with abundance, as demonstrated here. Thus, there may be further positive feedbacks, where larger abundances allow increased aggression and additional resource capture.

To summarise, we have shown how invasive ants, although impressive competitors, require a minimum threshold abundance to monopolise exudate-producing resources. When this abundance is achieved, however, facilitation may lead to their rapid population growth. Additional feedbacks from the context dependent nature of ant aggression can lead to further capacity for resource capture. Control strategies to prevent invasive ants would profit from focusing on eliminating invaders while still in the susceptible lag phase (e.g careful monitoring of susceptible sites and use of baits (Warner, 2003)) , or by attempting to decouple the positive facilitation between ants and exudate producing resources such as Homoptera (e.g. biocontrol or pesticides targeted at Homoptera on host plants).

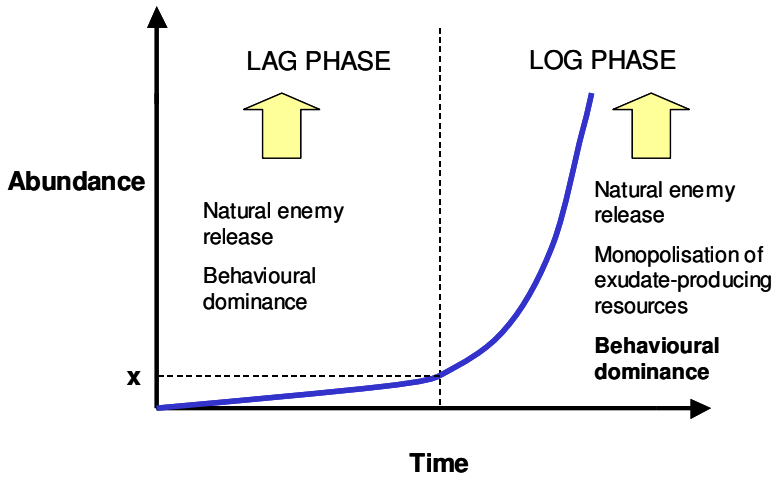


Fig. 3, The facilitation-threshold hypothesis of ant invasion. Natural enemy release has relatively little impact on population growth, but is important in achieving the threshold abundance (x) for the displacement of native ants from exudate-producing resources. After this lag phase, populations grow rapidly, fuelled by captured resources. Positive feedback between behavioural dominance and abundance allows further monopolisation of exudate-producing resources.

Chapter 6

The balance of mutualism: understanding what makes ant guardians prey upon their tended flock

Summary

Many species interactions are context dependent and can change in sign over space and time. Moreover, even overall mutualistic interactions are likely to contain antagonistic components, such as when ants tend and protect mutualistic aphids, but occasionally prey upon them. This experiment investigates factors that alter the fitness of aphid populations causing the balance of mutualism to shift. When ant larvae are present in the colony, aphid population growth rates are reduced. In addition, with larvae present, aphids feeding on higher quality host plants have reduced fitness compared with when when larvae are absent. Such changes in aphid fitness are likely to be mediated at least in part by changes in predation rates by ants. Ant behaviour towards aphids is determined by a complex interplay of internal and external cues, balancing colony demand with the cost of resource acquisition. Finally, we suggest that aphids too use a phenotypically plastic trait- the modification of honeydew, to minimise ant predation and maximise benefits from the relationship. Thus, past coevolution between these two groups of organisms may have been shaped by the interaction between flexible behavioural programs.

Introduction

Mutualisms are battlefields. Although they are defined as net positive interactions for both partners, they often comprise both positive and negative interaction components (van Baalen and Jansen, 2001, Bruno et al., 2003). Indeed, mutualisms are often best viewed as mutually exploitative relationships (Bronstein, 2001), where one partner uses another for some service or resource and in return offers goods that are often cheap to produce (e.g. 'by-product' goods *sensu* Connor, 1995). Costs and benefits inherent in the interaction can fluctuate over space and time, so interactions are said to be context dependent and may vary in magnitude and even sign (antagonism-commensalism- mutualism), depending on the particular biotic and abiotic environment (Thompson, 1982, Cushman and Addicott, 1989, Bronstein, 1994, Thompson, 1994). For ant-aphid relationships, a number of costs and benefits have been identified for both partners. Aphids benefit primarily in terms of protection from natural enemies (Way, 1963, Dixon, 1998, Fischer et al., 2001), shelter (Anderson and McShea, 2001), transport (Way, 1963, Collins and Leather, 2002) and hygienic services provided by ants (Sakata and Hashimoto, 2000). They also endure costs, however, including predation by ants (Way, 1954, Brian, 1977, Sakata, 1994, Offenberg, 2001) and the attraction of specialist parasitoids that preferentially target ant-attended aphid colonies (Völkl 1992, Dettner and Liepert, 1994). Ants benefit from the interaction by collecting sugary honeydew and preying upon aphids, but face the time and energy costs associated with collecting such resources and defending them from competitors and natural enemies (Stadler and Dixon, 2005).

The predation of aphids by ants is particularly interesting as it represents an instantaneous and direct way by which mutualistic interactions can switch to become antagonistic interactions. Ants often tend aphids and refrain from preying upon them, yet under certain conditions ant guardians can become predators, with up to eight times as many aphids being eaten than under normal conditions (Offenberg, 2001). The decision to prey upon, rather than tend aphids, appears to be complex and based on a number of factors. The quantity or quality of honeydew excreted by an aphid colony seems to be important. By banding plant stems and reducing phloem pressure, Edinger (1985) showed that ant predation rates increased. Other studies have shown that excess sugar in the vicinity of an aphid colony increases predation rates (Way,

1954, Cushman and Addicott, 1989, Sakata, 1999, Offenberg, 2001). Thus, the value of aphids to ants as sugar sources depends on the how productive aphids are, as well as the relative abundance of alternative sugar sources. This effect of honeydew attributes on ant behaviour led Cushman (1991) and Bristow (1991), to suggest that host plants may mediate the interaction between ants and aphids by exerting control over phloem, and hence honeydew chemistry. In this experiment, we create controlled variation in host plant quality by either stressing plants in de-ionised water, or growing them in rainwater. In addition to honeydew attributes, Pontin (1958) proposed that ants may monitor the availability of alternative prey and eat aphids when other prey are scarce, although a study by Offenberg (2001) has not supported this hypothesis. Other factors have been also been suggested to alter the value of aphids as honeydew providers, such as the distance between the aphid colony and the ants' nest, nutritional requirements of ant colonies, and age and size of ant colonies (Buckley, 1987, Cushman, 1991). Although, to the authors' knowledge, nobody has investigated the role of these factors using manipulative experiments.

In this study, we attempt to identify factors that affect aphid fitness and the balance of mutualism. The factors we test are: host plant quality, intraspecific variation between ant colonies in predation rates, presence of ant larvae and nutritional state of colonies (previous diet composition). In addition, we use a factorial experimental design to understand if the factors interact with one another to affect aphid population growth rates. We have selected three potential interaction effects to test, in order to avoid testing all possible combinations of factors, which could result in false significance from the large number of tests (data mining). Our hypotheses are: (a) a possible interaction between diet and host plant, because ants previously fed a protein diet may respond differently to aphids feeding on different qualities of host plant; (b) an interaction between larval presence and host plant quality, as the presence of ant larvae may cause increased predation of aphids growing on low quality plants, effectively 'saving' the more productive aphids on high quality plants for honeydew production; and (c) an interaction between previous diet and larval presence, as there may be non-additive effects between these factors in determining the internal physiological state of ants.

Materials and methods

Insect culture

The effects of ants on the fitness of mutualistic aphids feeding on plants of different host quality was assessed using ants fed on different diets and kept with larvae present or absent. Three *Lasius niger* colonies were excavated from the grounds of Silwood Park, Ascot, UK in October 2006, and stored in 5l plastic containers with soil, moist cotton wool and a test tube containing sucrose solution with a cotton wool bung. Colonies were overwintered by storing in a refrigerated room at 5°C. Before bringing back to room temperature in February or April, colonies were kept at 10°C for one week (Wardlaw et al., 1998). Ants were then placed into thirty 20x10cm plastic containers with 20 workers per box. Each box contained an upturned plastic plant saucer for ants to gather beneath, a test tube with sucrose solution and two small open plastic boxes containing water and moist cotton wool.

Ant treatments were as follows: Colony origin (three different colonies), Larval presence (20 larvae present, or larvae absent), Diet (protein and sugar, or sugar only). To mimic different developmental stages, the experimental colonies, which were all queenless, were either kept with larvae during the winter and throughout the experiment, or larvae were absent with workers only present. For the different dietary regimes, some ants were fed sugar and, twice a week, protein in the form of chopped *Calliphora* larvae (Diptera: Calliphoridae) (Crowthorne Fishing Supplies, Berkshire, UK), while the remaining ants subsisted on sugar alone. Each combination of factor levels was tested with the exception that no larvae were found in the third colony excavated, so only worker treatments were tested for this colony (Fig. 1). This amounted to ten treatment combinations, and with six replicates of each, to 60 boxes in total. In addition, 36 replicates of a control treatment, with aphids and plants but no ants present, were conducted.

Due to space and time constraints, only 16 boxes were tested per week over six weeks. To achieve a fair test, these 16 boxes comprised the complete combination of factor levels (ten boxes) plus six control boxes, and time was included as a blocking factor in the analysis. Of the ant treatments, thirty boxes were initially set up on diets in February and testing with aphids began in March. Thus, ants were kept on diet for four, five or six weeks before experimentation. The second set of thirty boxes was

removed from refrigeration and set up in March. Tests began again four, five or six weeks later.

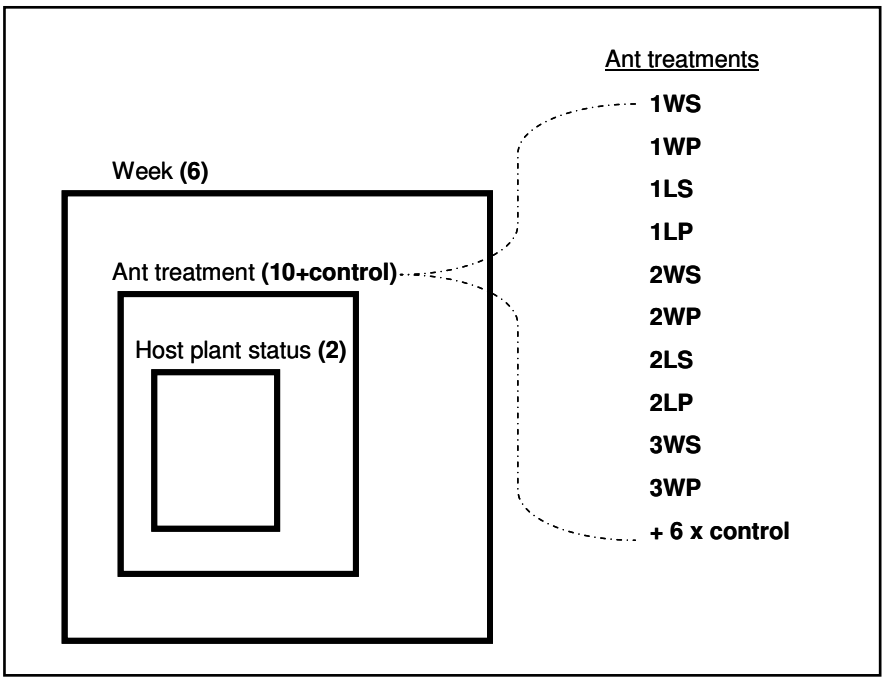


Fig. 1, Diagram illustrating the nested-factorial experimental design. Each box had two host plants (potted in de-ionised water or rainwater) and there were ten different ant treatments comprising combinations of three factors (colony number: 1, 2 or 3; larval presence: workers only (W) or workers and larvae (L); diet: sugar only (S) or sugar and protein (P)). In addition, there were six control replicates with no ants present, also tested every week. The experiment ran for six weeks and was completely replicated each week.

Aphid predation and ant counts

In previous studies, *L. niger* has been observed to prey upon *Aphis fabae* Scopoli aphids, occasionally picking up and carrying them back to the nest, rather than tending them (Offenberg, 2001, Oliver, unpublished). We investigated the tendency of ants from our different treatments to prey upon aphids, by comparing aphid counts on plants after seven days. For each test, the ant colonies were allowed access to two *Vicia faba* L. bean plants, upon which five adult *Aphis fabae* Scopoli aphids were placed at the start of the experiment (Fig. 2). The plants had been removed from soil a week beforehand and the roots washed. To consider the effects of varying host quality,

similar sized plants were paired together, and one plant from each treatment was kept with its roots submerged in de-ionised water before, and during, the experiment. The second plant was kept in rainwater collected from a plastic water butt. Water was used instead of soil, so that the lack of ions in the plant growth medium could be carefully controlled. De-ionised water is likely to result in lower quality host plants due to severe osmotic stress. The numbers of ants and aphids on each plant were counted at midday on seven consecutive days. In addition, each day the overall number of ants foraging in each box was recorded. This was the sum count of ants on each plant and ants foraging in the base of the box outside the covered 'nest'.

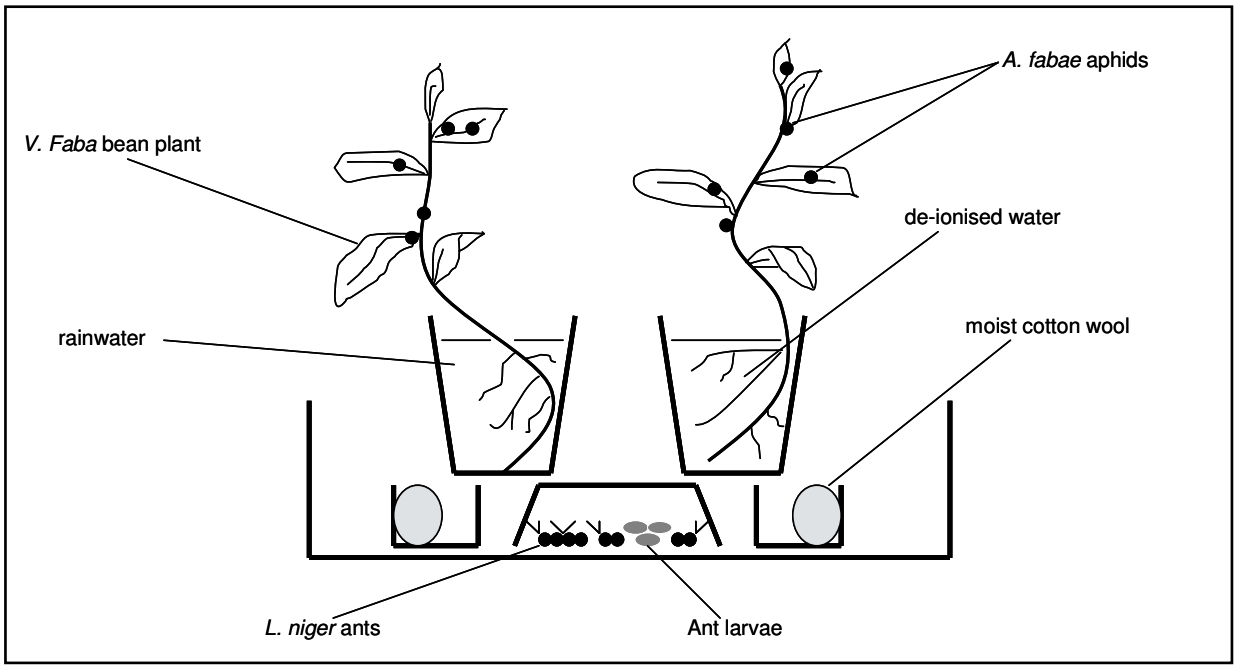


Fig. 2, Experimental set up. Ants are free to collect honeydew or prey upon aphids from either plant.

STATISTICAL ANALYSIS

Data were analysed using linear mixed effects models (lme) in the program R (Ihaka and Gentleman, 1996). Error variance for Host Plant Quality was nested within the ant colony treatments (Colony, Diet and Larval Presence), which in turn were nested within Week. During the experiment, a few plants died for no apparent reason, severely reducing aphid counts. If this occurred, the results from both plants in the affected box were excluded from analysis. In total, seven boxes were excluded, resulting in a final total sample size of 53 ant treatment boxes (106 plants) with 36 control boxes (72 plants). Initially, the simple effect of ant presence or absence on aphid numbers was considered by comparing ant treatments with the controls without ants. It was established that there was significant predation by ants and so, to understand drivers of this predation, a second model with controls excluded allowed comparison of differences between the ant treatments i.e. Colony, Diet and Larval presence. Finally, to test for interaction effects between factors that had been predicted by *a priori* hypotheses (see Introduction), a full additive model was fitted, plus the interaction effect to be tested. This was repeated sequentially with all three interaction terms. With regards to the effects of host plant quality, we would naturally expect aphids to reproduce more slowly on poor quality plants and this effect is apparent by considering the effect of host plant quality in the control treatments without ants. Difference in this trend of host plant quality with aphid numbers in the ant treatments indicates that ant predation varies depending on host plant quality. This is apparent in the statistical interaction term between larval presence and host plant quality.

Ant counts on plants were analysed using a similar nested approach. To avoid temporal pseudoreplication, ant counts on each plant were averaged over the seven days and the mean value used as the response variable. In a separate analysis, the mean number of ants foraging in each box was used as the response variable. The lowest hierarchy of nesting (host plant) was absent in this analysis, as replication was at the whole box-level.

Results

Aphid counts

During the experiment, we directly observed ants preying upon aphids and, from analysis of counts of surviving aphids, colony development and host plant nutrition were found to be significant predictors of this predation. After seven days, the ant treatment plants had significantly fewer aphids than control treatment plants (ant treatment: 64.52 ± 3.07 aphids; control: 75.57 ± 4.43 aphids ($t = 2.85$, $df = 135$, $p = 0.0051$). In the control treatments without ants, aphids survived and reproduced better on plants rooted in rainwater than in de-ionised water, indicating an effect of plant quality on aphid reproduction rates, (rainwater: 79.28 ± 4.59 aphids; de-ionised water: 71.67 ± 7.62; $t = 2.183$, $df = 29$, $p = 0.037$) (fig. 3).

Comparisons between ant treatment plants, excluding controls, allowed assessment of the importance of the different treatments on the fitness of aphids. The presence of ant larvae in the colony was an important predictor variable; when larvae were present in the experimental colonies, there were significantly fewer aphids on plants at the end of the experiment (Table 1). In addition, there was significant variation between colonies in their effects upon aphids. This significance is not simply because colony three contained worker only treatments, as there was also a significant difference in aphid counts between colonies one and two, which had similar treatment combinations ($t = 7.50$, $df = 45$, $p = 0.042$). Previous diet was not a significant predictor of aphid predation (Table 1). After excluding controls, host plant nutrition as a main effect was only a marginally significant predictor of aphid predation, although it was involved in a significant interaction term (see below).

Next, certain interaction terms were included successively into the full model, following *a priori* hypotheses (see Introduction). Of these, there was no interaction between diet and water ($t = 0.95$, $df = 51$, $p = 0.348$), nor previous diet and larval presence ($t = 0.10$, $df = 42$, $p = 0.924$). There was, however, a significant interaction between larval presence and host plant nutrition ($t = 2.26$, $df = 51$, $p = 0.029$). Significantly fewer aphids remained on plants grown in rainwater when larvae were present in the ant colonies (Fig. 3).

Table 1, Explanatory power of different drivers of the predation of aphids by ants. Variation was compared between different levels of the ant treatment factors, with controls excluded. The control mean is listed as a reference for aphid counts in the absence of any predation. Significant p-values ($p<0.05$) are highlighted in bold.

Explanatory variable	*n*	Mean aphids	SE	*t*	df	*p*
Water	106			1.84	52	0.071
De-ionised	53	60.81	3.54			
Rainwater	53	68.27	5.01			
Diet	106			0.12	43	0.905
Sugar and protein	54	64.09	4.69			
Sugar only	52	64.96	3.99			
Larval presence	106			2.80	44	**0.008**
Larvae and workers	42	59.26	3.34			
Workers only	64	67.97	4.57			
Colony	106			1.44	44	**0.015**
one	40	69.93	4.39			
two	44	62.50	5.18			
three	22	58.73	6.91			
Control	36	75.47	4.43			

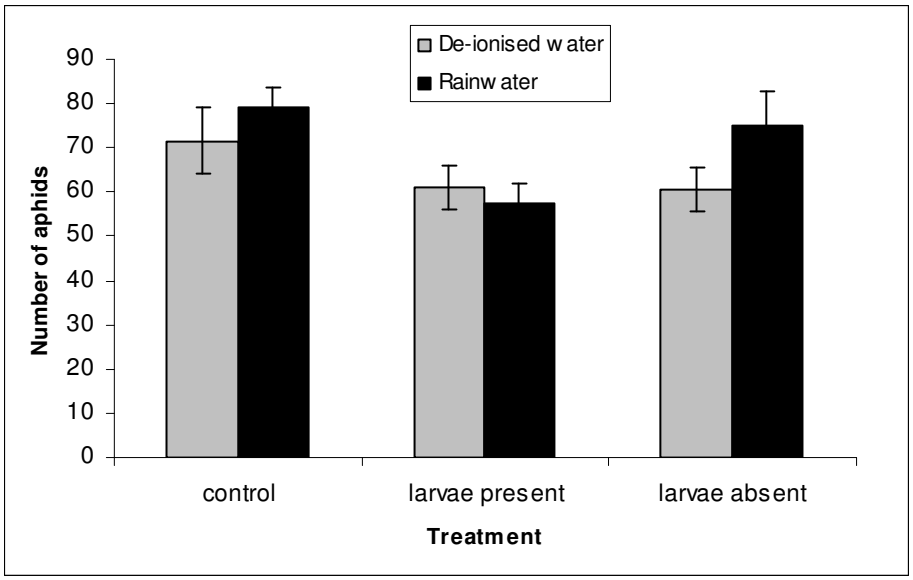


Fig.3, The interaction between larval presence and host plant nutrition. When ants are absent aphids are fewer on stressed plants grown in de-ionised water. A similar trend occurs when ant workers are

present but without larvae in the colonies. This trend is changed, however, when ant larvae are present; there remain fewer aphids on the higher quality plants than expected. Error bars represent standard errors of the means.

Ant counts

None of the factors tested significantly predicted the number of ants on each plant, although there was significant variation between colonies in the total number of ants observed foraging in boxes (Table 2). On average, there were more than twice as many ants visiting plants growing in rainwater compared with de-ionised water, although due to the large within treatment variation this difference was not statistically significant.

Table 2, Number of ants on plants or foraging in boxes. Comparisons are between the factor levels of the treatments. The host plant quality treatment (water) was nested within each box, hence there are no values for mean ants per box for this factor. Significant p-values ($p<0.05$) are highlighted in bold.

Explanatory variable	Mean ants/plant	SE	*n*	*t*	df	*p*	Mean ants/box	SE	*n*	*t*	df	*p*
Water				1.595	52	0.167						
De-ionised	0.133	0.029	53									
Rainwater	0.291	0.103	53									
Diet				0.121	45	0.903				0.184	45	0.855
Sugar and protein	0.218	0.075	54				0.758	0.191	27			
Sugar only	0.206	0.076	52				0.789	0.217	26			
Larval presence				0.625	46	0.535				0.108	44	0.914
Larvae and workers	0.168	0.073	42				0.625	0.158	21			
Workers only	0.241	0.076	64				0.871	0.213	31			
Colony				1.16	44.00	0.252				2.478	46	**0.017**
one	0.117	0.076	40				0.344	0.157	20			
two	0.308	0.112	22				0.936	0.242	22			
three	0.250	0.095	44				1.227	0.347	11			

Discussion

Our study has shown that aphid fitness in this mutualism can be affected by a number of factors. Aphid growth rates were significantly affected by host plant nutrition and larval presence, and there was also significant variation between colonies. In contrast, the previous diet of ant colonies was not a significant predictor of aphid population growth. Thus, nutritional state of ant colonies may be less important in determining the balance of mutualism.

Reductions in aphid population growth rates in this experiment could be mediated through two mechanisms: increased death rates (i.e. ant predation), or though decreased birth rates (i.e. aphids re-direct resources from reproduction in order to increase the quality of honeydew). Although we did occasionally observe ants preying upon aphids, we cannot rule out that some fitness loss of aphid populations could also be caused through reduced birth rates. Thus, further work could investigate the relative importance of these two mechanisms on ant induced changes in aphid fitness. One possible study could involve a 'cafeteria' type experiment where ants can feed on amino acids or sucrose from separate microcapilliary tubes. If ants truly increase predation rates to obtain more protein under certain conditions (e.g. in the presence of larvae), then we would expect ants in these conditions to feed more at the microcapilliaries containing amino acid rich solutions.

In this experiment, the previous diet of ants had no significant effects on aphid fitness. Different protein diets were fed to ants up to the start of the experiment, after which, no alternative foods besides aphids were available. This supports Offenberg's (2001) results that showed *L. niger* aphid predation rates were not affected by the presence of alternative prey (i.e. absolute increases in the availability of protein), but only by the availability of other carbohydrate sources (i.e. absolute increases in the availability of carbohydrates). In our study, however, ants were confined to boxes where the small aphid colonies represented simulataneous sources of protein or carbohydrate. This could be representative of situations early in the year when aphid colonies are a limited resource. In this case, despite different levels of protein fed to

the ant colonies there was no significant difference in the predation of aphids. Thus, if carbohydrate supplies are below some threshold to support the colony, perhaps ants save aphid colonies as renewable carbohydrate sources rather than to use as a protein source.

Even if ants do not adjust predation in response to protein supply, they certainly do seem to respond to nutritional demand from larvae within the nest. When larvae were present aphid population growth rates were much lower. Given that larval development places a greater requirement on the colony for protein-based food (Carroll and Janzen, 1973), lower aphid fitness in the presence of larvae could reflect either increased predation of aphids by ants (increasing aphid death rates), or costly upregulation of amino acid production in the honeydew of aphids (with consequent reductions in birth rates). Moreover, there was an interaction between presence of larvae and host plant quality on aphid fitness. With larvae present, the fitness of aphids growing on rainwater-grown host plants was lower than expected (Fig. 3). In control treatments where ants were absent, stressed host plants had fewer aphids than plants grown in rainwater, presumably due to slower reproduction on low quality plants. In ant treatments where ant larvae were absent this trend was also evident. When larvae were present in the ant colonies, however, the numbers of aphids on both plants were much more even (fig. 3).

If this reduction in aphid fitness on plants grown in rainwater is due to increased ant predation by ants then this result is contrary to our hypothesis that, due to the increased demand for protein, workers of ant colonies with larvae present would prey more upon aphids on lower quality plants. Perhaps aphids grown on higher quality plants could provide a better source of protein? The presence of ant larvae in colonies may provide a cue to workers that protein is in increased demand and workers then respond to this demand by increasing predation on high quality aphids. In this way, ants could manage the flow of protein or sugar resources derived from aphids in response to colony demand. An alternative hypothesis is that aphids grown on rainwater plants have more resources available than those grown on de-ionised plants and they can therefore afford to produce higher quality honeydew. Such production of higher quality honeydew may lead to decreased birth rates. There are two possible explanations for why aphids would suffer a cost to produce better honeydew for ants: a) aphid colonies compete with other populations for the attention of mutualists and, although costly, attracting mutualists is, overall, beneficial, (Yao and Akimoto, 2002),

or, b) increased attention by ants signals an increased threat of predation and aphids upregulate honeydew production to avoid predation.

Whichever explanation is correct, this effect of host plant on aphid predation supports the hypothesis that host plants can mediate the ant-herbivore interaction (Cushman, 1991). Host plant characteristics are well known to determine the sugar composition of honeydew (Hendrix et al., 1992, Fischer and Shingleton, 2001) and this can affect levels of ant attendance (Völkl *et al.*, 1999). In this study, ant attendance on plants rooted in rainwater was more than twice that on plants in de-ionised water, although the difference was not significant due to large variation in ant activity, which may have been a result of the small colony sizes used in this experiment.

The variation in population growth rates of aphids exposed to ant colonies of different origin could be explained by a number of reasons. Age, size and nutritional state of ant colonies have all been suggested to mediate the value of aphids to ants (Buckley, 1987, Cushman, 1991). In this study, however, all ants were overwintered for at least four months before being placed on a controlled diet treatment for four to six weeks. Thus, we believe the nutritional state of the colonies should have been standardised. Similarly, all colonies were kept with 20 workers present, with or without an equal number of larvae. If ants can retain a memory of 'colony state', e.g. of past colony size or age, from the previous year, then such long-term phenotypic plasticity could potentially mediate differential predation of aphids between colonies. Alternately, there may simply be considerable intraspecific genetic variation in the tendency to prey upon aphids. Indeed, if we presume that the decision to prey upon or tend aphids is a trait that has evolved through natural selection to maximise benefit to ant colonies, then we automatically assume the previous existence of such intraspecific variation. Whatever the primary cause, it is clear that the design of future studies should take into account such variation and, if possible, test a number of different colonies to obtain reliable results. There are also a number of other potential cues that have not yet been tested which could mediate aphid predation. For example, the distance between aphid colony and ant nest (Sudd, 1983). In addition, there may be polyethism within ant colonies, where younger workers tend aphids while older workers act as predators and attack aphids not close to, or marked with pheromones by, their more pastoral sisters (Weir, 1958a, Weir, 1958b, Way, 1963, Sakata, 1994). Such chemical marking of aphids mediating predation by ants may be responsible for

the negative density dependence of benefits often found in ant aphid interactions (Sakata, 1995, Sakata, 1999).

The balance of mutualism in ant-aphid relationships appears to be strongly controlled by the ants, i.e. there is asymmetry in control over the relationship. The tendency to prey upon rather than tend aphids appears to be determined by a trade-off of cues relating to supply of potential resources (e.g. quality and quantity of honeydew offered by aphids or their value as a protein source) and demand by the ant colony (e.g. the presence of larvae demanding more protein). Ants are able to manage the flow of two separate resources from aphids, protein and sugar, by balancing their availability, relative to external sources, with demand for each resource by the ant colony. Because ants manage resources in such a way, however, aphids may have some opportunity to alter ant behaviour towards them by altering their value as sugar producers relative to other sources. In this way aphids wield some control, albeit minor, over the mutualism-antagonism balance. Aphids producing good quality honeydew are more likely to be tended and protected, and predation by ants is diverted to competing neighbouring aphid colonies (Addicott, 1978, Cushman and Addicott, 1989, Fischer et al., 2001). Indeed, aphids are able to modify the composition (e.g. the proportions of different amino acids and sugars) and the volume and frequency of their honeydew excretion, to maximise the benefits of ant-attendance (Fischer and Shingleton, 2001, Yao and Akimoto, 2001, Yao and Akimoto, 2002). This plasticity of honeydew production has, in the past, been viewed as a means to maximise the number of attending ants, given that ants are a limiting resource shared between competing mutualists (Addicott, 1978, Cushman and Addicott, 1989, Fischer et al., 2001). In this chapter we have suggested an alternative explanation that, rather than as a means to mediate competition between mutualists, the plasticity in aphid honeydew production has evolved to prevent predation by ants. By increasing their value as honeydew producers, aphids may be able to appease ants and divert predatory- into mutualistic behaviour.

To conclude, we are beginning to understand how the demand for and supply of resources, and the costs and benefits of obtaining those resources, can lead to dynamic shifts in the balance between mutualism and antagonism. The majority of interactions between ants and aphids are facultative (Bristow, 1991, Delabie, 2001), and associations between both groups have been lost and gained multiple times over their

evolutionary history (aphids: Shingleton and Stern, 2003, ants: Oliver, Chapter 7). Dynamic flux in interaction strength and sign is, therefore, something that both partners are likely to have evolved to deal with. Ant behaviour towards aphids is determined by a complex interplay of internal and external cues balancing colony demand with the cost of resource acquisition. Similarly, aphids can respond to the presence and absence of ants with phenotypic plasticity in honeydew attributes such as the quality, quantity and rate of excretion. Natural selection has worked like a clever economist to achieve plastic traits that are 'fine-tuned' to dynamic changes in the interaction sign and strength. Our task now, is to understand how these flexible behavioural programs have interacted with each other over the evolutionary history of these two groups of organisms, characterising the coevolution of facultative mutualistic and antagonistic interactions.

Chapter 7

In press as:

Oliver, T.H., Leather, S.R., Cook, J.M., (in press) Macroevolutionary patterns in the origin of mutualisms involving ants. *Journal of Evolutionary Biology*

Macroevolutionary patterns in the origin of mutualisms involving ants

Summary

Ants are a diverse and abundant insect group that form mutualistic associations with a number of different organisms from fungi to insects and plants. Here we use a phylogenetic approach to identify ecological factors that explain macroevolutionary trends in the mutualism between ants and honeydew-producing Homoptera. We also consider association between ant-Homoptera, ant-fungi and ant-plant mutualisms. Homoptera-tending ants are more likely to be forest dwelling, polygynous, ecologically dominant and arboreal nesting with large colonies of 10^4 – 10^5 individuals. Mutualistic ants (including those that garden fungi and inhabit ant-plants) are constrained to under half of formicid subfamilies. At the genus level, however, there is a negative association between ant-Homoptera and ant-fungi mutualisms, while there is a positive association between ant-Homoptera and myrmecophytic plant mutualisms. Perhaps species can only specialise in multiple mutualisms simultaneously when there is no trade-off in requirements from the different partners and no redundancy of rewards.

Introduction

Mutualisms are common, fundamental interactions shaping species' communities and, through coevolutionary adaptation, even changing species themselves (Herre et al., 1999). Within a family that contains mixtures of mutualistic and non-mutualistic species we can identify ecological factors that are associated with mutualism. These factors may be preadaptations that predispose a lineage to mutualism or they may be the selective consequences of mutualistic coevolution. Mutualisms are not always stable over evolutionary time and may be lost and gained repeatedly (Sachs and Simms, 2006). If a clade is involved in several different types of mutualism, these mutualisms may be positively or negatively associated or show no association at all.

The ants (Family: Formicidae) engage in a broad range of mutualistic interactions with many different organisms including plants, insects and fungi (Hölldobler and Wilson, 1990). This diversity of mutualistic interaction may reflect the widespread abundance and ecological dominance of ants. Alternately, perhaps they are ideal mutualistic partners because, compared with solitary living species, ant colonies are able to provide a more efficient, non-localised defence for vulnerable species. Not all ants are involved in mutualisms, however. By comparing the ecology of mutualistic versus non-mutualistic ant taxa, we are able to characterise ecological traits that are associated with mutualism. This allows insight into the origin of these mutualisms and can also lead to speculation over how mutualisms can shape species. In this study we focus primarily on the mutualism between ants and Homoptera (aphids, scales *etc*.). We consider how various ecological traits correlate with the occurrence of this mutualism in ant genera. In addition, we compare how the ant: Homoptera mutualism is associated with mutualisms between ants and other organisms.

Many ants are well known to tend Homoptera for their sugary 'honeydew'; a process known as *trophobiosis*. In addition, ants also visit lycaenid caterpillar larvae and the extrafloral nectaries of some plants to collect sugary secretions. These excretions/ secretions contain a variety of amino acids and carbohydrates which ant colonies use to fuel foraging activity and feed developing brood. In return ants provide a wide range of benefits to their partners including protection from predators and parasites (Jones, 1929, Stadler and Dixon, 2005), shelter (Way, 1963), transport (Collins and Leather, 2002) and reduction of pathogen contamination (Way, 1954,

Fokkema et al., 1983). The mutualism between ants and Homoptera is economically important because Homoptera are major agricultural pests worldwide and ants are ecologically dominant organisms having widespread impacts on the community structure (Wimp and Whitham, 2001) and nutrient cycling of ecosystems (Folgarait, 1998). There is broad diversity in the degree of association between aphids and ants. For example, one aphid species may often be tended by ants while a congener is not (Stadler and Dixon, 2005). Also, different aphids sharing the same host plant may differ in their degree of trophobiosis with ants (Bristow, 1991). A wide range of explanations has been suggested for the variation in trophobiosis in ant-aphid interactions; however, it is useful to distinguish between factors that promote variation in trophobiosis *within* species (i.e. across populations) and those that cause differences between species. Within a single aphid species there may be spatial and temporal variation in the degree of ant tending at any given time. Similarly, there will be differences between ant colonies in their investment in Homoptera. This variation across populations is characteristic of facultative mutualisms where both partners can survive alone and do not exclusively require interspecific association. Obligate mutualisms, in contrast, always find the two partner species in association. The majority of ant-aphid and ant-lycaenid relationships are thought to be facultative (Bristow, 1991, Pierce et al., 2002).

Population level variation in trophobiosis between ants and aphids can be satisfactorily explained by two hypotheses. One is that, although relationships may be mutually beneficial, ants and aphids are limited in their ability to encounter one another. Ants are not omnipresent and their colony territories do not encompass the entire landscape. Similarly, there is no evidence that alate aphid dispersal is directed to patches with ants, even though apterous aphid dispersal may be limited by ant semiochemicals (Oliver et al., 2007b). The second explanation is context dependence, whereby the costs and benefits of an interspecific association depend on a multitude of biotic and abiotic factors which vary in time and space (Cushman and Whitham, 1989). Thus the net effect of an interaction may change in magnitude and even sign (Bronstein, 1994, Thompson, 1994). For example, in cases where costs exceed benefits, aphids will profit from leaving the association with ants. This can be achieved through selection for traits that prevent ant tending e.g. sequestering plant toxins in honeydew (Buckley, 1987). If the interaction frequently fluctuates between mutualism and parasitism then natural selection can optimise benefits by evolving

phenotypically plastic traits that facilitate association only when conditions are favourable e.g. increasing honeydew quality in the presence of ants (Yao and Akimoto, 2002). A wide variety of ecological factors have been proposed to account for population level variation in ant-aphid interactions including local species composition and abundance of ants (Horvitz and Douglas, 1984, Oliver et al., 2007a) or aphids (including competing mutualistic species) (Addicott, 1978); seasonal change in ant nutritional requirements (Sudd and Sudd, 1983); presence of alternative sugar sources (Offenberg, 2001) and host plant quality (Auclair, 1963, Cushman, 1991). Variation in trophobiosis between ant and aphid *species*, however, can encompass additional explanations. For example, morphological differences between ant species in the development of the proventriculus can affect their ability to gather large quantities of honeydew in the crop and thus affect suitability for trophobiosis (Davidson et al., 2004). Factors, such as morphological change, operate on evolutionary timescales and can shape species level variation. Evolution of third-party antagonists can also disrupt mutualisms (Sachs and Simms, 2006). For example, specialised parasitoids may arise which are able to subvert ant defences and because of the enemy free space they find there, develop specially to exploit ant attended aphids (Völkl 1992). Furthermore, mutualistic aphids may find themselves in competition with other aphids which are attractive to ants (Addicott, 1978, Cushman and Addicott, 1989, Cushman and Whitham, 1989). Dynamic indirect coevolution (mediated through the ant mutualist) may ensue, in order to 'win' the attention of ants.

Generally, past attention has focused on aphids, with attempts to predict factors that explain their asscociation with ants. Reduced alternative defences, such as cornicles releasing waxy secretions and saltatorial legs for escape, have been hypothesised to characterise ant attended aphid species, although evidence is equivocal (Bristow, 1991). Bottom-up effects of the type of host plant may go some way towards explaining variation, with certain plant families (e.g Anacardaceae, 100%) supporting considerably higher proportions of ant-tended aphids than others (e.g. Fagaceae, 2%) (Bristow, 1991). Association with ants could also potentially be limited by aphid phylogenetic contraints; however this does not appear to be the case (Bristow, 1991). Indeed, there is evidence that association with ants is a highly evolutionary labile trait that has evolved and been lost multiple times in aphids (Shingleton and Stern, 2003). Feeding position has been shown to predict some variation in ant attendance (Shingleton et al., 2005). The most comprehensive analysis to date identifies feeding

on woody plant parts, non- mobile aphids, non- alate adults and aggregated colonies as traits that are significantly associated with ant attendance (Stadler et al., 2003).

In comparison, there has been little attention given to factors that explain variation between ant species in their association with aphids. Indeed, it is not even known roughly what percentage of ants practice aphid tending. Only one study to date (ant proventriculus development (Davidson et al., 2004)), links an ant trait to interspecific association with trophobionts. The current study addresses this paucity of knowledge by identifying several ecological factors that are characteristic of homopteran-tending ants. We specifically test three *a priori* hypotheses, two of which have been suggested by previous researchers:

a) Dominant and polygynous ants are more likely to monopolise resources and thus form close associations with sugar-producing insects (Fiedler, 1991, Davidson, 1998, Eastwood and Fraser, 1999, Blüthgen et al., 2004).
b) Ant- Homoptera, ant- lycaenid and ant- extrafloral nectary plant mutualisms will be positively associated. Ants species that visit Homoptera and lycaenids appear to be a subset of those that visit extrafloral nectaries (Fiedler, 2001, Blüthgen et al., 2004). Liquid-feeding adaptations in ants may create opportunities to engage in all three mutualisms simultaneously.
c) Fungus gardening will be negatively correlated with trophobiosis because there is a trade-off preventing specialisation in two very different types of mutualism. In contrast, there will be a positive association with domatia bearing plants as these have similar requirements as Homoptera (protection), yet provide different rewards.

To test the hypotheses a database was collated of 139 ant genera and several of their ecological traits (Table 1). It is likely that there will be non-independence between the traits of closely related genera, thus requiring autocorrelation from phylogenetic relationship to be taken into account. For this we used generalised estimating equations (GEE), which are similar to the GLS (generalised least squares) approach (Grafen, 1989), except that non-normal errors in response variables can be(Paradis and Claude, 2002). They are suitable for data with discrete response variables, through the specification of binomial, rather then Gaussian, error structures, and also

allow the exploration of interaction effects between explanatory variables (Paradis and Claude, 2002, Paradis, 2006).A phylogeny is required to produce a correlation matrix derived from the distances between species in a tree, which is then used to weight variances in a generalised modelling framework. We have used a recent phylogeny of ants resolved to the genus level by Moreau *et al.* (2006). The number of species in the genera and sampling effort were included as additional blocking explanatory variables. Selected pairwise interaction effects between some ecological factors were also considered, in order to generate functional explanations for homopteran tending (for hypotheses see Table 2).

In addition to the GEE analysis, we also carry out an additional binary logistic regression analysis that does not account for relatedness between taxa. This analysis indicates association between ant traits, independent of shared evolutionary history between taxa, *i.e.* it simply asks: which traits best describe ant genera that tend aphids? If we then want to ask whether this association implies some kind of causal linkage between the traits rather than simply association through shared evolutionary history, we can then refer to the GEE analysis that takes phylogenetic non-independence of taxa into account.

Usually, phylogenetic comparative methods are conducted with species-level data, although genus-level data has also been used (e.g. Koh et al., 2004). In this case, the very large number of species in the Formicidae (~10,000) (Hölldobler and Wilson, 1990) and limited knowledge of many of these species makes any large scale analysis at the species level impossible. Instead, we score traits at the genus level and use genus as the tips of the hypothesised ant phylogeny. A caveat is that there may exist polymorphism of traits within a genus. Such polymorphism at the tips of a phylogenetic tree, however, is a problem for any comparative analysis; species are assumed to have a certain trait yet in reality there may be intraspecific variation within the trait. Thus the question is not necessarily whether data is collected at genus level or species level, but rather are the proposed traits truly representive enough of the taxa? Moreover, even if there is polymorphism of a trait within taxa, it can be argued that these taxa are still qualitatively different from monomorphic taxa in which no members of the taxa show the trait. Trait polymorphism within phylogenetic tips may present problems in the reconstruction of ancestral nodes and related comparative methods that use such an approach e.g. phylogenetically independent contrasts

(Felsenstein, 1985). Comparative methods that do not rely on ancestral state reconstruction, however, but rather use a correlation matrix to weight non-independence should be suitable to model such traits, as long as it is kept in mind that exactly what is being modelled could be presence of polymorphism of a trait, rather than the simple presence or absence of the trait. Despite this, for the majority of traits we have tested if a trait is scored as a positive for a genera then it is likely that most species share the trait. The two traits most likely to have higher degrees of polymorphism within genera are polygyny are invasiveness.

Materials and methods

Data collection and analysis

Data on the ecological traits of 139 ant genera were collated from primary literature sources. The genera selected (139 of a possible 288 extant described genera (Moreau et al., 2006)) were those used by Moreau *et al* (2006) in a large scale molecular phylogeny of the Formicidae. Binary ecological traits (see Table 1) were scored as positive if at least one species in the genus showed the trait. A similar approach has been used by Koh (2004) to model extinction risk in tropical butterflies. An obvious caveat with this approach is that, by chance, large genera are more likely to contain species with the trait. The sampling effort devoted to a genera may also have a similar effect, with species with the trait in question more likely to be found for well studied genera. To overcome this, we have included the number of species in each genus and the sampling effort (both logged) were included as explanatory variables to account for variation caused by these effects. Potential interaction effects were chosen only with *a priori* hypotheses. This prevented obtaining spurious significance effects from analysing a very large number of potential interactions (~100). Generalised estimating equations were carried out using the 'compar.gee' function in the 'ape' package (Paradis et al., 2004) of the program 'R 2.6.1' (Ihaka and Gentleman, 1996, R.Development.Core.Team, 2007). The binary response variable was the occurrence of homopteran tending (trophobiosis) in the genus and thus binomial, rather than Gaussian, errors were specified with either logit or complementary log-log links, depending on whichever minimised residual variance. Computational iterations often diverged when both species count and sampling effort were included as blocking

explanatory variables as these two are highly correlated (Pearson's product moment correlation: $t = 8.61$, d.f. = 145, $p < 0.001$). Therefore, only sampling effort was included. After testing each potential trait (Table 1) singly for an association with trophobiosis, pairwise interactions were tested (Table 2), again with sampling effort as a blocking factor.

For the non-phylogenetic comparative analysis, binary logistic regressions specified with binomial errors were carried out with sampling effort and species count as additional explanatory variables. The comparison between small and large dominant ants of the proportions of genera with certain traits (Table 5) was carried out using a proportion test calculating Pearson's chi-squared statistic, again using the program 'R'.

Table 1. Methods used for scoring ecological traits.

Ecological trait	Description	Factor type
Dominance	One or more species in the genus reported to have attained dominance in their native community	Binary
Polygyny	One or more species reported as polygynous at least 6 months after colony founding	Binary
Polymorphic workers	One or more species have polymorphic worker castes	Binary
Invasiveness	One or more species is an 'invasive' or 'tramp' species (McGlynn, 1999b)	Binary
Arboreal nesting	One or more species of the genus nests in trees	Binary
Nomadism	One or more species have distinct nomadic phases with frequent daily colony movement	Binary
Trophobiosis with Lycaenidae	Species of ants in the genus also tend lycaenid caterpillars	Binary
Feed at extrafloral nectaries	Species of ants in the genus also feed at extrafloral nectaries	Binary
Latitude	The majority of species in the genus occur in temperate regions, tropical or both	Categorical
Colony size	Average colony size of the genus is small (0-200 workers), medium (200-10,000), large (10,000-100,000) or very large (100,000+)	Categorical
Body size	Mean body size of ants in the genus	Continuous
Habitat types	The number of different habitat types species of the genera occupy from a list of: forest, grassland, scrub, arid, wetland, disturbed	Continuous
Geographical range	The number of different biogeographic realms species of the genus occur in from a list of: Nearctic, Neotropic, Palearctic, Afrotropic, Oriental, Indomalay, Australasia	Continuous
Fungus gardening	One or more species grows fungus in gardens within the nest	Binary
Ant-plant mutualism	One or more species live in specialised plant domatia (Hölldobler and Wilson, 1990)	Binary
Blocking factor		
Subfamily	Subfamily of the genus (Moreau *et al.*, 2006)	Categorical
Species number	The number of species in the genus, obtained from the Species 2000 database	Continuous
Sampling effort	The number of hits for the genus on ISI Web of Science (ISI) search engine	Continuous

Table 2. Hypotheses for pairwise interaction effects of ant ecological factors on the occurrence of trophobiosis with Homoptera

Interaction effect	Hypothesis
Arboreal nesting and latitude	Both temperate and tropical ants tend Homoptera, yet arboreal nesting species are generally found only in the tropics.
Polygyny and colony size	Polygyny may allow dispersed nests (polydomy), facilitating homopteran tending. Colony size is likely to covary with polygyny, but very small ant colonies are unlikely to able to defend homopteran resources, even if polygynous.
Dominance and body size	Ants may need to attain a certain body size before becoming dominant enough to monopolise Homoptera.

Results

Taxonomic distribution

Forty one percent of ant genera were found to contain species that engage in trophobiosis with Homoptera. The taxonomic distribution was uneven with certain subfamilies more likely to contain mutualistic ant species (deviance = 52.195, d.f. = 18,119, $p < 0.001$) (Fig. 1). At the subfamily level there is a positive association between ant-trophobiont, ant fungi and ant-plant mutualisms (trophobiont-fungi: dev = 5.99, d.f. = 1,17, p = 0.014; trophobiont-plant: dev. = 4.11, d.f. = 1,17, p = 0.043; plant-fungi: dev. = 9.84, d.f. = 1,17, p = 0.002). Ant-fungi mutualisms always occur in subfamilies that also have mutualisms with plants and trophobiotic insects. For fungi, however, this positive association does not carry down to genus level and the trend becomes reversed (see next section). Over half of ant subfamilies do not engage in any type of mutualism with Homoptera, fungi or domatia bearing plants.

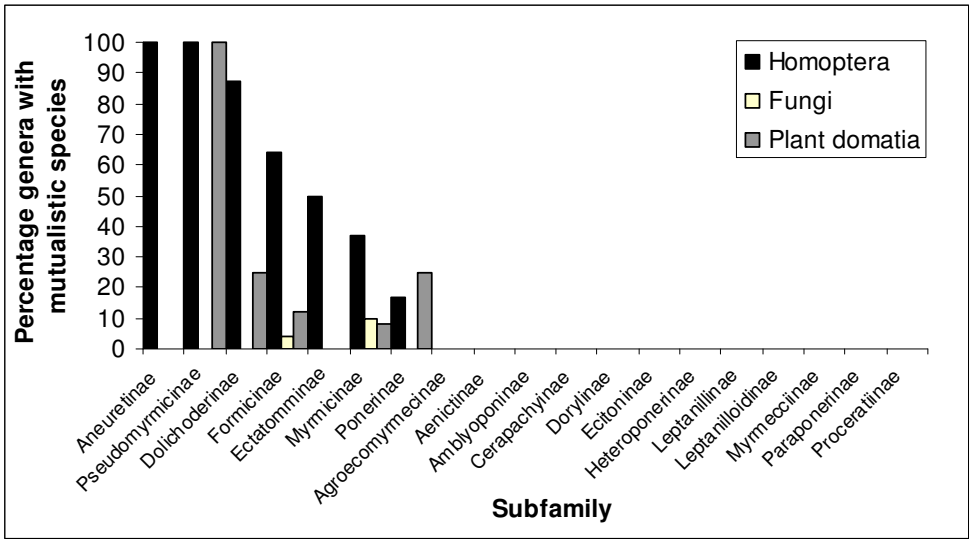


Fig. 1, Ant subfamilies engaging in different mutualisms. The percentage of ant genera that contain mutualistic species in each subfamily. Subfamilies are in order of occurrence of trophobiosis with Homoptera, but also included are percentages engaging in mutualisms with plants and fungi.

Ecological correlates of trophobiosis with Homoptera

Without controlling for phylogenetic non-independence there were a number of ecological traits significantly associated with trophobiosis (Table 3). We show that homopteran-tending ants are more likely to be dominant, polygynous, arboreal nesters with large colonies of 10^4-10^5 workers. Furthermore, invasive ants and ants that are found in disturbed habitats are more likely to be trophobiotic. There was a positive association between trophobiosis mutualisms and mutualisms between ants and domatia-bearing plants. Ninety four percent of the ant genera with species that live in plant domatia also contain trophobiotic species. In addition, ant-trophobiont and ant-plant mutualisms also co-occur at species level. For example most *Crematogaster* species that inhabit domatia of the ant-plant *Macaranga* keep scale insects (Homoptera) inside the plant chambers (Heckroth et al., 1998).

Table 3. Association between ecological traits or habitat types on the occurrence of trophobiosis with Homoptera. The method involved binary logistic regressions with species number in genus and sampling effort as blocking factors. Asterisks are significance codes for each ecological trait: *0.01<p<0.05, **0.001<p<0.01, ***p<0.001.

Ecological trait	Dev	df	P	Association	
Dominance	8.82	1,133	0.003	positive	**
Polygyny	7.02	1,93	0.008	positive	**
Polymorphic workers	1.01	1,114	0.315	none	
Invasiveness	4.28	1,136	0.039	positive	*
Arboreal nesting	10.34	1,130	0.001	positive	**
Nomadism	3.19	1,131	0.074	none	
Trophobiosis with Lycaenidae	85.22	2,135	<0.001	positive	***
Feeding at extrafloral nectaries	74.88	2,135	<0.001	positive	***
Latitude	3.02	2,134	0.221	none	
Colony size	4.84	1,92	0.027	large colonies	*
Body size	8.83	1,135	0.003	negative	
# Habitat types	4.92	1,131	0.027	positive	*
# Geographical Realms	0.14	1,135	0.709	none	
Fungus gardening	4.90	1,135	0.027	negative	*
Ant-plant mutualism	12.46	1,135	<0.001	positive	***
Habitat	**Dev**	**d.f.**	**P**	**Association**	
Forest	0.67	1,130	0.413	none	
Grassland	0.01	1,130	0.972	none	
Scrub	1.45	1,130	0.228	none	
Disturbed	5.45	1,130	0.020	positive	*
Wetland/ Heath	2.9	1,130	0.088	none	
Arid	0.56	1,130	0.453	none	

Using the GEE method to account for phylogenetic non-independence, many of these significant associations drop out (Table 4). This suggests a shared evolutionary history is responsible for the association in many of the cases. In contrast, those traits that are still significant after the GEE analysis are likely to have some causal relationship with trophobiosis because across the whole phylogeny they are repeatedly associated on many occasions. These associated traits include the other 'trophobiosis-like' mutualisms: feeding at extrafloral nectaries and the tending of lycaenid larvae, and also fungus gardening (a negative association). Six ant genera contain ants that

engage in symbiotic mutualism with fungi. Five of these six genera are attine ants (tribe Attini) that grow nutritional fungi in gardens and do not engage in trophobiotic mutualisms with Homoptera. The sixth genus, *Lasius* (subfamily Formicinae) is somewhat of an exception as only one species shares a mutualism with fungi. *Lasius fuliginosus* associates with an ascomycete fungi which grows in the walls of its carton nests and strengthens them structurally (Hölldobler and Wilson, 1990). Thus, in this trophobiotic genus there is an isolated occurrence of an ant-fungi mutualism, but it is not one in which ants derive nutrition from fungi.

There was a positive association between dominance of ant genera and trophobiosis. Sixty seven percent of ant genera with ecologically dominant species contain homoptera-tending species, compared with only 23% of genera without ecological dominants. Interestingly, a significant association between nomadism and trophobiosis also emerged from the GEE analysis, whilst the effect was only close to significance in the binary logistic regression. Nomadic ants are less likely to tend Homoptera that non-nomadic genera. As with all comparative analyses, only association between can be inferred, not the direction of causality; thus, whether these traits are a cause or consequence of trophobiosis is unknown. In the absence of a species level phylogeny onto which the origin of traits could be more accurately mapped, one crude method to give a suggestion of the direction of causality is to consider the proportions of genera (e.g. nomadic or trophobiotic) in each group (e.g. trophobionts or nomadic ants, respectively). For example, if becoming nomadic tends to cause ants to relinquish trophobiotic relationships then we would expect the majority of nomadic ants to be non-trophobionts (lack of trophobiosis is necessary for nomadism). In contrast, if not tending Homoptera caused ants to live a nomadic lifestyle then we would expect most non-trophobiotic ants to become nomadic (nomadism is necessary for non-trophobiosis). Considering the data, we find that a significantly higher proportion of nomadic ants do not tend Homoptera (10/13, 77%), compared with only a small minority of non-trophobiotic ants that happen to be nomadic (10/84, 12%).

This suggests that nomadism is a precursor to the loss of the trophobiosis mutualism, and, thus, that relinquishing trophobiosis is a consequence of being nomadic (but with many exceptions to this rule, or reversions from trophobiosis after nomadism has developed, because only 77% rather than a 100% of nomadic ant genera do not tend Homoptera). A similar comparison for dominance and trophobiosis traits is less

conclusive. Sixty two percent of trophobiotic ant genera are dominant, compared with 76% of dominant ants that are trophobiotic, the difference being non-significant ($\chi 2$ = 1.69, d.f. = 1, p = 0.193).

Table 4. Association between ecological traits or habitat types on the occurrence of trophobiosis with Homoptera, controlling for shared evolutionary history. The method involved phylogenetic generalised estimating equations with sampling effort as a blocking factor. Asterisks are significance codes for each ecological trait: *0.01<p<0.05, **0.001<p<0.01, ***p<0.001.

Ecological trait	**t**	**dfP**	**P**	**Association**	
Dominance	2.10	27.00	<0.001	positive	***
Polygyny	1.64	17.54	0.123	none	
Polymorphic workers	0.25	19.91	0.800	none	
Invasiveness	1.76	27.11	0.089	none	
Arboreal nesting	-	-	-	-	
Nomadism	2.11	21	0.049	negative	*
Trophobiosis with Lycaenidae	3.43	21.61	<0.001	positive	***
Feeding at extrafloral nectaries	3.57	21.61	<0.001	positive	***
Latitude	0.28	21.61	0.783	none	
Colony size	0.67	17.47	0.516	large colonies	
Body size	0.04	20.84	0.967	none	
# Habitat types	0.58	22.12	0.56	none	
# Geographical Realms	1.73	21.61	0.101	none	
Fungus gardening	2.74	21.61	0.014	negative	*
Ant-plant mutualism	1.46	21.61	0.161	none	
Habitat	**t**	**dfP**	**P**	**Association**	
Forest	0.62	26.42	0.389	none	
Grassland	0.13	26.42	0.896	none	
Scrub	0.12	26.42	0.909	none	
Disturbed	1.35	26.42	0.191	none	
Wetland/ Heath	0.4	26.42	0.695	none	
Arid	0.91	26.42	0.373	none	

Finally, of the proposed interaction effects between ecological factors (Table 2), there was a significant interaction between ecological dominance and mean ant body size on the occurrence of trophobiosis with Homoptera (Binary log. reg: Dev = 6.01, d.f. = 1,128, p = 0.014; GEE: t = 4.29, d.f. = 27.0, p < 0.001). Size had a significant

effect on the occurrence of trophobiosis only in dominant ant genera. The direction of effect was opposite to that predicted, however: dominant ants tending Homoptera are on average much smaller than dominants that do not (mean size trophobionts(± SE) = 4.53± 0.14mm, mean size non-trophobionts = 8.20 ± 1.40mm; Dev = 6.213, d.f. = 1,35, p = 0.013) (Fig. 2). Thus dominant ants fall into two distinct groups, those that tend Homoptera and those that do not, and several other ecological factors also differ markedly between these two types of ant. Small, dominant ants that tend Homoptera are more likely to be polygynous, invasive and thrive in disturbed habitats. Furthermore, they are less likely to be nomadic or have polymorphic workers (Table 5). There was no significant interaction between polygyny and colony size on trophobiosis (Binary log. reg: Dev = 1.817, d.f. = 3, 69, p = 0.61; GEE: t =1.163, d.f. = 15.7, p = 0.284). Finally, there were no arboreal nesting genera that occurred only in temperate regions, thus consideration of an interaction effect with latitude was not applicable. For ants found solely in the tropics, 72 % of genera (18 out of 25) that tended Homoptera nested in trees. For temperate zone-only ants 0% of genera that tended Homoptera nested in trees (zero out of nine).

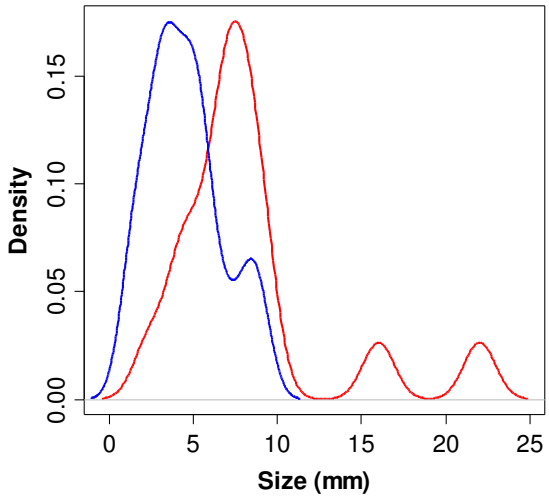


Fig. 2, Probability density function describing the size of two groups of dominant ants (small, trophobiotic genera, n = 33; large non-trophobiotic genera, n = 16)

Table 5. Characteristics of small dominant and large dominant ants. Percentages given are of genera scoring positive for each trait. Asterisks are significance codes for each ecological trait: *0.01<p<0.05, **0.001<p<0.01,***p<0.001.

Characteristic	Small dominants	Large dominants	
Mean body size:	4.53mm	8.20mm	**
Tend Homoptera?:	Y	N	*
Polygynous	86%	36%	**
Invasive:	48%	0%	**
Disturbed habitats:	48%	12.5%	*
Polymorphic workers:	39%	80%	*
Nomadic:	6%	38%	*

Phylogeny

The presence or absence of trophobiosis with Homoptera can be mapped onto a phylogeny of the Formicidae (Fig. 3 in Appendix). With data collated at the genus level however, ancestral state reconstructions can be inaccurate if there is considerable polymorphism for the trait at the species level. Thus Figure 3 is an accurate reflection of the evolution of trophobiosis given the assumption that, if trophobiosis is scored as positive for a genera, then the majority of the species in the genus are trophobionts. If this assumption is violated then there may be differences in the evolutionary history of trophobiosis than shown here. For the trophobiosis trait, we believe that this assumption is reasonable. For example, in the recently resolved genus *Linepithema,* Homoptera tending is ubiquitous across all 19 species (Wild, 2007). Also, we have refrained from estimating the relative rates of transition between trophobiosis states, although it is reasonable to use the phylogeny to calculate the minimum number of state changes. Using the program PAUP (Swofford, 2000) the most parsimonious number of changes is an impressive 31. This figure is likely to be highly conservative as the phylogeny is resolved only to genus level and there may be many transitions at the level between genus and species. There may also be under-reporting in the literature which could lead to trophobiosis being incorrectly assumed to be absent in poorly sampled genera. This may slightly inflate or decrease the number of changes depending on whether it occurs in mutualistic or autonomous

clades. Regardless, trophobiosis in ants appears to be a highly evolutionarily labile trait.

Discussion

The tending of homopteran and lycaenid insects by ants occurs on a broad taxonomic scale, with 45% of ant genera containing trophobiotic species. The taxonomic distribution is uneven, however, with certain subfamilies specialising in trophobiosis (e.g. the Aenictinae, Aneuritinae and Pseudomyrmicinae and of the larger subfamilies: the Dolichoderinae and Formicinae), while trophobiosis is entirely absent in others. We have identified a number of ecological traits that are correlated with the occurrence of trophobiosis in ant genera. Thus, species most likely to tend Homoptera are ants that are polygynous, dominant in their community, arboreal nesting, with large colonies of $10^4 – 10^5$ individuals, and those that can occupy disturbed habitats. Several ecological factors, such as latitude and worker polymorphism, did not correlate with trophobiosis. This could indicate these factors have little influence on the evolution of trophobiosis. It is also possible however, that they are important, but are simply not detected in a genus level analysis.

The association between dominance, polygyny and and trophobiosis has been noted by a number of researchers (Fiedler, 1991, Davidson, 1998, Eastwood and Fraser, 1999, Fiedler, 2001, Blüthgen et al., 2004). Dominant ant species can monopolise honeydew producing resources by excluding subdominant species. Subdominant and specialised ants tend to have smaller colonies and as such may be confined to peripheral ecological niches where resources are more scarce. Thus a few key Homopteran species colonising certain host plants can contribute to shaping the ant community of an ecosystem (Blüthgen et al., 2004). At this point, however, we are unable to determine which trait, dominance or trophobiosis, precedes which. Dominant ants may be more able to outcompete others to obtain resources, but the converse is also possible whereby the evolution of adaptations to exploit new resources allows ants to become dominant (Davidson, 1998). Honeydew is a profitable, renewable source of carbohydrates as well as essential amino acids (Völkl et al., 1999, Fischer and Shingleton, 2001). With access to such food, ant colonies may be able to forage wider areas (Stadler and Dixon, 2005) and support larger

populations of vigorous workers able to outcompete other ants (Davidson, 1997). Modification of the proventriculus is one such adaptation that facilitates the trophobiont-ant relationship and may allow ants to achieve dominance (Davidson et al., 2004). There may also be covariance in some of the ecological traits important in trophobiosis. For example, polygynous ant colonies tend large and polydomous (Hölldobler and Wilson, 1990); attributes which no doubt help them to achieve ecological dominance and exclude competitors from sugar producing resources (Davidson, 1998). Polygyny also breaks down within-colony relatedness and so may also facilitate the formation of 'unicoloniality', very large cooperative entities of ants that exclude competing species (Davidson, 1998). Alternatively, Rosengren *et al*. (Rosengren et al., 1993) have instead suggested that polygyny may result *from* trophobiosis as the relative benefit of long distance dispersal by the female reproductive class declines compared with remaining with the native colony in competion-free, resource rich areas. Our study shows a positive association between polygyny and trophobiosis with Homoptera, but this relationship disappears after accounting for phylogenetic non-independence using the GEE method.

A novel finding in this paper is that dominant ants appear to fall in to two categories: small dominant ants that tend Homoptera and large dominant ants that do not. A number of other ecological traits also differ significantly between the two groups (Table 5), supporting this grouping of dominant ants on the basis of homopteran tending. Small ants may be more able to match worker allocation to resource value and allow a continuous, steady flow of resources to the nest. In addition they may provide a more effective, non-localised defence force through increased sensitivity (greater number of- and more widely dispersed 'receptors'), followed by pheromone-mediated mass recruitment towards targeted threats. Polygyny and polydomy can further aid this resource monopolisation (Davidson, 1998) and invasiveness of ants may be an emergent trait facilitated by a combination of small size, polygyny and homopteran tending (Ness and Bronstein, 2004). Large dominant ants, in contrast, include ants such as the Ponerines which tend to have large body size and adopt a more solitary mode of foraging. Also, on average, large dominants have a greater degree of worker polymorphism which is also associated with nomadic lifestyles.

It is noteworthy that trophobiosis was positively associated with invasiveness of an ant genera. This could be due to traits that covary with trophobiosis, for example dominance. Regardless, it highlights that the trophobiotic association with Homoptera

is very flexible. Invading ants can easily adopt new partners in a novel habitat. It may be that general exaptations (preadaptations) of the ant-Homoptera mutualism allow easy transfer to new partners (Mondor and Addicott, 2007) and a dynamic flux in mutualist identity. Partner switching may occur not just with different congeneric species, but across large taxonomic divides, such as the exploitation, by a single ant colony, of many different Homoptera, lycaenid and extrafloral nectar-bearing plant species. This may be especially useful if a partner's range and abundance is limited and also in novel habitats where a previous partner is absent. Such partner switching may give facultative mutualisms an advantage over obligate pairwise mutualisms. Although interactions between ants and domatia-bearing plants (Beattie, 1985) or fungi (Mueller et al., 2005) are often obligate and specialised, those between ants and sugar-producing trophobionts are most often not (Bristow, 1991, Pierce et al., 2002).

Finally, it may be intuitive to expect that close coevolution with a mutualistic partner limits the extent to which a species can participate in other coevolutionary relationships. Strong directional or balancing selection imposed by one coevolutionary partner may limit the degree to which a trait can be selected by another partner. We find, however, that trophobiotic ants are able to maintain several mutualisms simultaneously. Species that tend Homoptera are also highly likely to be those which tend lycaenids and feed at extrafloral nectaries. Ants provide similar benefits to each of these groups (primarily defence against enemies), and receive similar rewards (sugary secretions). Thus, general adaptations may facilitate all three mutualisms. For example, effective resource defence abilities, group recruitment and a modified proventriculus allow exploitation of these similar resources. In addition to maintaining mutualisms with trophobionts, ants can also simultaneously engage in mutualisms with domatia-bearing plants. A similar pattern of association has been found for plants with extrafloral nectaries: there is a positive association between extrafloral nectary bearing plants and ant-tended aphids (Offenberg, 2000). Ants that inhabit plant domatia often bring their Homopteran mutualists with them and use plant shelters to farm their herds. Similar to trophobionts, domatia-bearing plants require an effective defence force. By moving mutualistic partners together, ants can achieve this and reap the rewards from both (shelter from plants and food from Homoptera).

When requirements from mutualistic partners diverge, however, it appears ants may be required to specialise in one type of mutualism. Ants that garden fungi, for example, do not engage in trophobiotic interactions with Homoptera. Fungi require the gathering of growth substrates and maintenance of ideal conditions, Homoptera require a patrolling defence. Hoeksema *et al.* (2000) suggest that there may be allocation trade-offs in mutualisms, where limiting resources are allocated to traits associated with different partners. We suggest that foraging time and energy of ants may also be viewed as such a limiting resource, which trades off between potentially mutualistic partners such as fungi and Homoptera. Over evolutionary time this partner choice becomes cemented by the evolution of specialised traits that maximise benefits from a specific partner type, but reduce the ability to exploit other partners. For example, both partners offer a different type of food as a reward to ants, that may require different physiological adapations to consume and digest.Where fungi can offer non-food rewards however, (*e.g*. as a structural building tool in *L. fuliginosus* nests), then ants seem to be able to adopt both Homopera and fungi as mutualistic partners. Thus, from these observations we propose as a general hypothesis that *species can specialise in multiple types of mutualism simultaneously only when there is no redundancy of rewards and no trade-off in requirements* (Fig. 4). In cases such as the ant mutualisms with Homoptera, lycaenids and extrafloral nectaries there appears to be redundancy of rewards (all offer sugary secretions) but no trade off in requirements (all require protection), a somewhat 'halfway house' satisfaction of the above criteria. In these cases we predict that the species can maintain multiple *unspecialised faculative* mutualisms but specialised obligate relationships will not develop. Conversely, for the other halfway house possibility, when there is no redundancy of rewards but there is a trade-off in requirements, we predict that there will be no multiple mutualisms maintained whatsoever. Thus, requirement trade-off can be viewed as the most important criteria in determining multiple mutualisms, with redundancy of rewards secondarily determining whether the mutualisms are facultative or specialised obligate relationships.

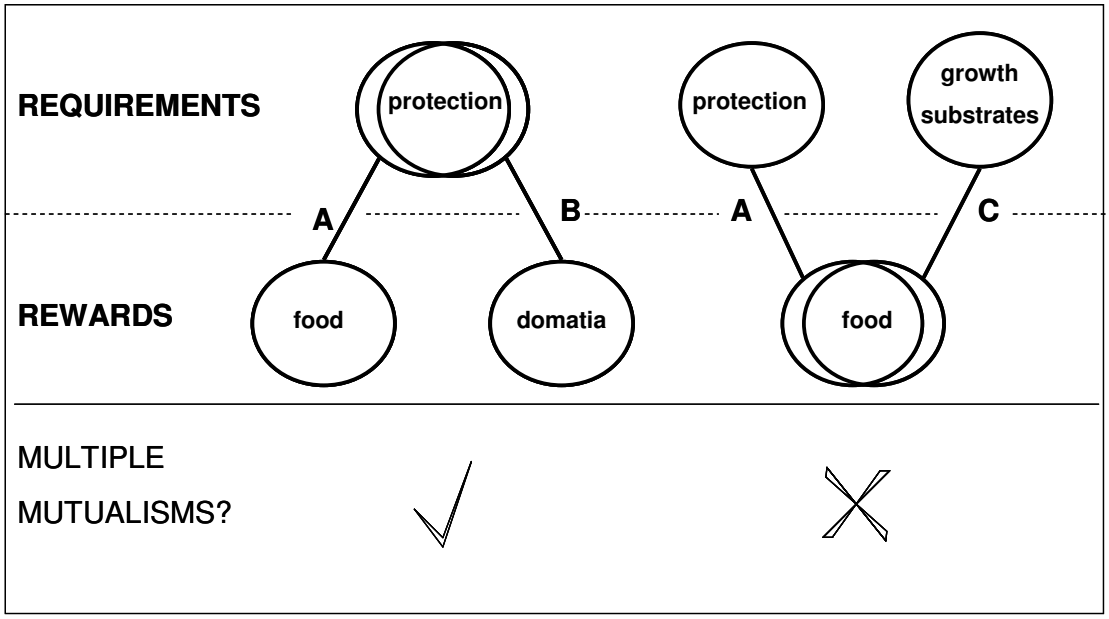


Fig. 4, Multiple mutualisms cannot occur if there is a trade-off in requirements and redundancy of rewards

Ants simultaneously maintain specialised mutualisms with Homoptera (A) and domatia bearing plants (B). In contrast, there is a negative correlation between ant- Homoptera (A) and ant –fungi (C) mutualisms.

Conclusion

To summarise, we have identified a number of ecological traits that characterise homopteran tending ants. Trophobiotic ants are more likely to be forest dwelling, polygynous, ecologically dominant and arboreal nesting with large colonies of 10^4 – 10^5 individuals. Some of these associations remain even after accounting for phylogenetic non-independence, suggesting causal relationships. For example, it is highly likely that ecological dominance in ants is either responsible for- or a consequence of- the maintenance of trophobiotic mutualism. We are not yet in a position, however, to predict the direction of causality. We have also found that dominant ants fall into two distinct groups depending on whether they tend

Homoptera, and these groups also differ in several related ecological traits, such as invasiveness, polygyny and polymorphism. Finally, we observe that ants appear to specialise in multiple mutualisms only when partners offer different types of reward and there is no trade-off in requirements from the ants. We propose this as a general rule that may govern the formation of multiple mutualisms by any species.

Chapter 8

Theories of mutualism in ant-aphid interactions

Mutualisms are cooperative interactions between species where each individual partner yields a net fitness benefit. Because partners are unrelated, however, explanations from cooperation theory depending on kin selection (e.g. Axelrod and Hamilton, 1981) are not applicable. Instead, the main theories for explaining mutualisms are a) by-product benefits, b) partner fidelity, c) partner choice and d) host sanctions or punishment. In this chapter, we explain each of these mechanisms and explore how they may apply to ant- aphid interactions.

a) By- product benefits

Reciprocal positive fitness effects in mutualistic interactions are achieved through the trading of some kind of goods (e.g. honeydew) or services (e.g. protection). The goods produced may be costly to the supplier or, alternatively, they may simply be waste products. Similarly, services to a partner may simply result from selfish acts that increase actor fitness but also incidentally benefit partners. Mutualistic interactions where all products traded have zero cost to the suppliers are termed *by-product mutualisms*. In contrast, interactions where goods or services are costly for suppliers to produce are called *investment mutualisms* (Connor, 1995).

In some ant-aphid interactions honeydew can be viewed as by-product goods that are utilised by ants. Honeydew is a waste product generated because aphids obtain an excess of carbohydrates from their diet of plant sap (Dixon, 1998). It is produced by all aphid species, whether tended by ants or not. Many non-tended aphids flick honeydew away from the plant to prevent infection by pathogens (Morales, 2000). In contrast, ant- tended aphids retain honeydew on the abdomen in the presence of ants (Way, 1963). This is a phenotypically plastic adaptation that allows aphids to yield benefit from the waste product because ant attendance is increased around productive aphids thereby increasing protection. In other aphid species, aphids are even more well-adapted to promote the interaction with ants. These aphids modify the composition of their honeydew to increase the amount of amino acids that are especially valued by tending ants (Banks and Nixon, 1958, Takeda et al., 1982, Del-Claro and Oliveira, 1993). In addition, rates of honeydew production can be increased

when ants are present (Yao et al., 2000, Fischer and Shingleton, 2001, Yao and Akimoto, 2002). These goods offered to ants can be viewed as investments rather than simply by-products as they are costly for aphids to produce (Fischer and Shingleton, 2001, Yao and Akimoto, 2001). Aphids invest fitness in producing a honeydew product that is more attractive to ants and that fitness is recouped by the benefits gained from increased levels of ant attendance. This bears similarity to the sugary secretions offered by lycaenid caterpillars to ants. These secretions are not waste products and produced only by caterpillars associated with ants.

With regards to the services carried out by ants in the ant- aphid interaction (e.g. protection, sanitation services; see literature review in Chapter 1), these could arguably be viewed primarily simply as by-product services. Collection of honeydew by ants from aphid abdomens and from plant surfaces can be viewed as a purely selfish act of foraging by ants. Any benefits to aphids of reduced contamination by pathogens is likely to be purely incidental. It could be similarly argued that the protection of aphids from natural enemies is simply a by-product of ants selfishly protecting their food source. Ants show particular aggression towards aphids predators, and although this aggression is costly (in terms of time and energy) it yields direct benefits to the ant colony.

b) Partner fidelity

Partner fidelity occurs where cooperating lineages are coupled through an external mechanism such as vertical transmission, which allows cooperator genotypes to maintain persistent links over time (Bull and Rice, 1991, Margulis and Fester, 1991, Herre, 1993, Herre et al., 1999, Bot et al., 2001, Thompson, 2005). Although partner fidelity may be important in many symbiotic mutualisms (e.g. between aphids and *Buchnera*- an intracellular gut bacteria aiding food digestion), in ant-aphid interactions there is little evidence for vertical transmission of mutualists. Aphid sexual forms are often winged and can disperse large distances away from ant colonies. Furthermore, aphid populations are very transient and long term persistence is maintained through metapopulation structure (Dixon, 1998). There is some evidence that ant- attended aphid colonies are more persistent in a location due to increased protection from natural enemies that destroy aphid colonies (Dixon, 1998) and through reduced dispersal when ants are present (Oliver et al., 2007b). It is highly

unlikely however that such mechanisms would allow ant and aphid lineages to be coupled together over sufficient evolutionary time for mutualistic coevolution to occur. Therefore in the case of ant- aphid interactions we discard this hypothesis as an explanation for the origin of mutualism.

c) Partner choice

Partner choice or 'filtering' occurs when independently dispersing cooperator genotypes selectively recognise and direct benefits to cooperator genotypes in the other species (Nilson, 1988, Bull and Rice, 1991, Broughton et al., 2000, Brouat et al., 2001). Such a mechanism could go some way to explaining mutualisms between ants and aphids. Although there is no evidence that aphids can recognise the levels of protection offered by ants, it is clear that ants recognise more productive aphids and offer them greater levels of protection. The recruitment of ant foragers is positively correlated with the value of a food source (Bonser et al., 1998, Katayama and Suzuki, 2003, Mailleux et al., 2003, Portha et al., 2004), and high quality honeydew-producing aphids attract more ants per aphid (Fischer et al., 2001, Fischer et al., 2005). This greater attention by ants to more productive aphids appears to have led to the evolution of phenotypically plastic honeydew production whereby aphid honeydew quantity (Banks and Nixon, 1958, Takeda et al., 1982, Del-Claro and Oliveira, 1993), and quality (Yao et al., 2000, Fischer and Shingleton, 2001, Yao and Akimoto, 2002), is actively increased in the presence of ants.

d) Host sanctions/ punishment

The term 'host sanctions' refers to when the usual services or goods traded within mutualisms are withheld if cheat genotypes are encountered. Alternatively, 'host punishment' refers to when cheat genotypes may be physically penalised so that they face net costs from the interaction (Trivers, 1971, Axelrod and Hamilton, 1981, Pellmyr and Huth, 1994, Johnstone and Bshary, 2002, Hoeksema and Kummel, 2003, Kiers et al., 2003, Bshary and Grutter, 2005, Edwards et al., 2006). With regards to the ant and aphid interaction, there is some evidence that sanctions and punishment may be used by ants to maintain productive relationships with aphids. As mentioned

earlier, ant attention is directed towards more productive aphids. Thus, aphids that cheat and produce less honeydew will receive fewer benefits of ant protection (sanctions). Furthermore, there is anecdotal evidence that ants are more likely to prey upon rather than tend poorly producing aphids. (Edinger, 1985, Sakata, 1995, Sakata, 1999, Fischer et al., 2001). This can be viewed as a form of punishment against cheats.

Conclusion

Of the existing theories of cooperation, the mutualism between ants and aphids seems to be best explained by the fact that goods and services traded are mainly by-products and also that ants can withdraw protection from or punish aphids that are not productive enough. There seems to be little role for partner fidelity or choice within these interactions and that may well explain why the majority of ant- aphid interactions are facultative and non-specific. Dynamic aphids metapopulations make reciprocal interactions between the same ant-aphid genotypes less persistent, thereby reducing partner fidelity. Instead, general by-product goods and services may facilitate easy partner switching. Indeed such partner switching can be beneficial as it allows new environments with previously unencountered, potential mutualists to be exploited.

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Appendix

Chapter 7, Fig. 3, Cladogram of the Formicidae showing trophobiosis with Homoptera. Trophobiosis occuring within a genus is shown in black. The six bottom-most genera are vespoid and sphecoid outgroups.

-Please see overpage

Eutetramorium
Colobostruma
Colliemovia
Wasmannia
Myrmica
Myrmicaria
Megalomyrmex
Tetramorium
Carebara
Crematogaster
Melissotarsus
Rhopalomastix
Aenictomyrmex
Dilobocondyla
Formicoxenus
Leptothorax
Temnothorax
Mayriella
Solenopsis
Carebarella
Oligomyrmex
Carebara
Pheidologeton
Atopomyrmex
Veromessor
Pogonomyrmex
Aphaenogaster
Metapone
Poldomyrma
Goniomma
Oxyopomyrmex
Messor
Pogonomyrmex
Cataulacus
Cyrtomyrmex
Strumigenys
Eurhopalothrix
Cyphomyrmex
Atta
Trachymyrmex
Sericomyrmex
Paratrachycerus
Pheidole
Tranopelta
Cataglyphis
Proformica
Formica
Oecophylla
Stigmacros
Opisthopsis
Colobopsis
Camponotus
Myrmoteras
Polyrhachis
Euprenolepis
Acropyga
Myrmecorhynchus
Melagnorus
Notoncus
Plagiolepis
Pseudolasius
Prenolepis
Myrmecocystus
Acanthomyops
Lasius
Myrmelachista
Brachymyrmex
Ctatomma
Gnamptogenys
Typhlomyrmex
Heteroponera
Bothriomyrmex
Bolichoderus
Paparius
Anonychomyrma
Anillus
Ochetellus
Iridomyrmex
Froggattella
Inopopthema
Dorymyrmex
Orectia
Azteca
Leptomyrmex
Technomyrmex
Tapinoma
Lometopum
Aneuretus
Paratrechina
Terataner
Myrmecia
Aenictus
Dorylus
Nomamyrmex
Eciton
Neivamyrmex
Yunodorylus
Sphinctomyrmex
Cerapachys
Leptanilloides
Phaulomyrma
Cryptopone
Pachycondyla
Diacamma
Hypoponera
Mesoponera
Ectomomyrmex
Odontomachus
Anochetus
Odontomachus
Odontoponera
Centromyrmex
Paraulacus
Platythyrea
Adlerzia
Amblyopone
Concoctio
Onychomyrmex
Myopopone
Amblyopone
Adetomyrma
Mystrium
Discothyrea
Proceratium
Probolomyrmex
Protanilla
Cryptopone
Evagetes
Odontophotopsis
Vespula
Philanthus
Apis

PAUP 1