RESOLVING ANT-PLANT CONFLICTS : MECHANISMS AND FUNCTIONS OF FLORAL ANT-REPELLENCE

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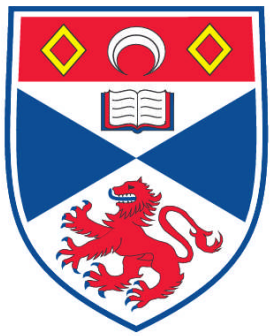

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Resolving ant-plant conflicts: mechanisms and functions of floral ant-repellence

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I, Jonathan Pattrick, hereby certify that this thesis, which is approximately 18600 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in September, 2009 and as a candidate for the degree of M.Phil. in January 2011; the higher study for which this is a record was carried out in the University of St Andrews between 2009 and 2011.

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Contents

Abstract

Although ants have numerous, often beneficial interactions with plants, as pollinators they are poor. Potential reasons for this include restrictions resulting from their morphology and specific foraging behaviours, and detrimental effects of their surface secretions on pollen. This, coupled with other possible negative effects of ants on floral structures, puts pressure on plants to exclude ants from flowers. One common strategy to achieve this is via behaviour-modifying repellent floral volatiles; however, few studies have identified the volatiles concerned. Here I considered two aspects of this interaction.

Firstly, I assessed seven temperate angiosperm species for floral repellence to *Formica aquilonia* ants using a simple two-way olfactometer. In agreement with previous studies showing that floral ant-repellence is common, significant repellent effects were found in 3/7 species. I also analysed the floral bouquet of *Petasites fragrans*, a plant previously shown to possess ant-repellent floral volatiles. The most prominent volatile was identified as 4-methoxybenzaldehyde and olfactometer testing of a pure sample of this confirmed it as the likely source of floral repellence in *P. fragrans*. Although the natural interactions between *P. fragrans* and ants are unknown, intriguingly 4-methoxybenzaldehyde has been detected from floral volatiles of two further species with frequent ant interactions.

A second study compared effects of ants and bees on pollen function to explore the supposed significance of ant-induced reductions in pollen viability. Lily pollen was exposed to either *F. aquilonia*, *Apis mellifera*, or *Bombus pascuorum*, germinated in vitro and assessed for viability. Small, marginally significant reductions in viability were identified for all three insects compared to a control, but with no differences in the reduction between each insect. Although this indicates that the pollen inviability hypothesis for the scarcity of ant pollination may be incorrect, a comparative study with several pollinator and plant species is needed to substantiate this conclusion.

Chapter 1: Introduction: The mechanisms and functions of floral ant-repellence

1.1. Ants and Ant-plant evolution

Ants are some of the most successful and numerous organisms in the world, found in nearly all ecosystems and often forming a significant proportion of the total biomass in each ecosystem (Wilson & Holldobler 2005). Owing to their abundance and numbers of interactions with other species, they are often key players in the locations where they are found (Hölldobler & Wilson 1990).

The high numbers stem from the fact they are eusocial, living in colonies. The size of a mature colony can vary massively, ranging from just tens up to millions of workers depending on the species. The number of queens per colony and the degree of polymorphism between queens, males and workers and within worker castes also varies across different species (Hölldobler & Wilson 1990). Within a colony, queens are specialised for the task of producing workers (which are all female), males and new queens. Queens in particular are generally much larger than workers and both queens and males have wings for at least part of their life whereas workers are wingless. Males have the sole role of mating and tend to have a short life span (Hölldobler & Wilson 1990). Workers are specialised for carrying out non-reproductive tasks and although completely sterile in some species, in others they may be able to reproduce to some degree (Bourke 1999). Division of labour within the worker castes can just be reflected in behavioural differences in some species; however, this task specialisation (or polyethism) can extend to extreme polymorphisms with workers highly specialised for particular roles such as defence. The degree to which castes specialise across different species is probably influenced by many factors, particularly their life histories, mating/breeding system and colony size, (Hölldobler & Wilson 1990; Bourke 1999; Fjerdingstad & Crozier 2006). Colonies can live in one nest or can be polydomous, distributed across several nests with high degrees of relatedness between each. A single colony can hence potentially cover and forage over substantial areas (Debout *et al.* 2007).

In accordance with their diversity, the range of food types utilised by ants is also considerable. They are found as predators and scavengers, herders/tenders of sap sucking, honeydew secreting homopterans and lepidopteran larvae, dispersers of seeds of a wide range of plants, farmers of fungi and as guards of plants which provide food in return for protection (Beattie 1985; Hölldobler & Wilson 1990; Blüthgen & Feldhaar 2010).

Foraging for food typically occurs out from a central nest (Buhl *et al.* 2009) but the strategies used can vary considerably. If hunting for small dispersed, unpredictable prey, ants may forage individually, or, when the food items are larger, they can forage in groups with recruitment to a discovered food source. Foraging can be more complex though, for example the use of networks of trails (often pheromone based) connecting nest to food sources, often associated with long-lived, stable resources such as plants (Dornhaus & Powell 2010).

Many of these foraging interactions involve plants to some degree, and particularly when considering the angiosperms, ant-plant interactions are very common indeed. Correspondingly, throughout their evolutionary history the success of the ants has been closely linked with that of the angiosperms.

Ants arose during the Cretaceous (Wilson & Holldobler 2005), but it was not until the late Cretaceous to early Eocene when they started appearing in large numbers, undergoing a rapid diversification seemingly triggered by the coincident diversification of the angiosperms (Soltis & Soltis 2004; Wilson & Holldobler 2005; Moreau *et al.* 2006; Bell *et al.* 2010). Ants were initially predators and scavengers on the forest floor; however, the progression from primarily gymnosperm to angiosperm forests improved the soil litter as well as leading to an explosion in the number of other insects, facilitating a change in lifestyles. It is probable that ants began moving upwards into the canopy at this time, switching to the more herbivorous mode of feeding by collecting honeydew secreted by the increasing numbers of homopterans and lepidopterans. The new abundance of seeds and elaiosomes also permitted a move into more xeric environments out and away from the forests (Wilson & Holldobler 2005; Moreau *et al.* 2006; Perrichot *et al.* 2008).

This has led to the modern day abundance of ant-plant interactions, a plethora of associations ranging from simple facultative events to complex mutualisms. Although some of these are antagonistic relationships, many are not and can benefit the plants concerned.

1.2. Ant benefits to plants

Perhaps the most common modality through which ants benefit plants is the phenomenon of ant-guarding, where ants protect the plants against the actions of herbivores. In some cases this protection is gained through highly specific mutualisms where the plants provide nest sites (domatia) and food in the form of extrafloral nectaries (EFNs) and/or food bodies for obligate ants that defend the plant, first described in acacia species (Janzen 1966), and also found in many others (e.g. Heil & McKey 2003; Grangier *et al.* 2008). But protection is not always so specific, and the presence of EFNs on plants can also attract facultative ant visitors that then confer herbivore protection.

Another very common interaction of ants and plants is the tending of honeydew-secreting hemipterans. While this may sometimes be detrimental to the plant concerned (Oliver *et al.* 2007), in many cases the presence of ants tending specific hemipterans affects the plant insect community in such a way that actually improves plant fitness, typically by an overall reduction in herbivory (Whittaker & Warrington 1985; Mahdi & Whittaker 1993). Beneficial effects from aphid-tending ants may not always be obvious, for example although Puntilla *et al.* (2004) found that aphid tending *Formica aquilonia* ants seemed to cause reduced growth rates in birch trees, they could also provide protection against outbreaks of *Epirrita autumnata* moths, which often kill trees. A meta-analysis of these ant-aphid interactions by Strysky and Eubanks (2007) found the plant benefited in 73% of a total of 30 studies.

The presence of ant-guarding mutualists can also benefit the host plant by weeding out encroaching vegetation e.g. (Federle *et al.* 2002; Frederickson *et al.* 2005), or by actively protecting against fungal infection (Heil *et al.* 1999; Heil *et al.* 2000). Plants can even gain additional nutrients from ants living in domatia by absorbing the nitrogenous waste and carbon dioxide they produce (e.g. Treseder *et al.* 1995). Simply being situated next to ant nests can also benefit the plant as a result of higher soil nutrient content (Wagner 1997; Wagner & Nicklen 2010).

It should also be mentioned that ants can be of huge benefit to plants in the role of seed dispersers. General interactions where ants collect and disperse seeds from the ground (e.g. Ness *et al.* 2009) can have substantial impacts (Lengyel *et al.* 2009) but there are also the more specific mutualisms of ant-gardens where ants actively disperse and germinate the seeds of plants they subsequently live in close association with (Davidson 1988; Youngsteadt *et al.* 2008).

Although there are many potentially beneficial effects of ants on plants, there is one interaction that is conspicuously absent, that of ant pollination. A few examples do exist (e.g. Jones *et al.* 2010) and see Rostás and Tautz (2011) for a review, but generally this is rare. This is particularly surprising given the frequency with which ants are encountered on plants, their diversity and abundance and their long shared evolutionary history (Wilson & Holldobler 2005; Moreau *et al.* 2006).

1.3. Absence of ant pollination

The apparent paucity of ant pollination has been commented on for many years with multiple suggestions given as to why ants could be poor pollinators, some warranting more attention than others.

Firstly, due to their small size, ants are often a poor physical fit for flowers (Willmer *et al.* 2009), not contacting the anthers during flower visits. It has also been argued that because of their smooth integument, pollen does not adhere to them very well (Beattie *et al.* 1984; Willmer *et al.* 2009). However, these morphological arguments do not apply in all cases; not all ant species are hairless and pollen is definitely able to stick to the surface of at least some ants (Wyatt 1981; Beattie *et al.* 1984; Rostás & Tautz 2011). Also, the small size of ants will not be a hindrance in all cases; when the flowers are also small, this is not an issue (Gómez & Zamora 1992).

Additionally, and perhaps more obviously, the workers of ants are wingless and so they are likely to only transport pollen between flowers on the same plant. More important than this, ants show ortstreue (literally site-fidelity), with individuals focusing on foraging in a specific

area so although together a colony will forage over a large area, individuals will not (Hölldobler & Wilson 1990). Therefore, ants are likely to be poor providers of outcrossing, only effecting geitonogamous self pollinating at best (de Vega *et al.* 2009), which can be of significant detriment to plants.

Hickman (1974) suggested that ant-pollinated plants would fall into a pollination syndrome with floral features - including short morphologies and self-compatibility - that would negate these effects; and, in agreement with this, many subsequent examples of ant pollination involve plants possessing these attributes (Ramsey 1995; Sugiura *et al.* 2006; de Vega *et al.* 2009). This supports the suggestion that the specific nature of ant foraging behaviour is potentially a significant barrier to the evolution of ant pollination.

One of the most frequently quoted reasons as to why ants are poor pollinators, and potentially as important as that of being poor outcrossers, is the negative impacts that ant surface secretions have on pollen viability (Beattie *et al.* 1985; Galen & Butchart 2003), which is a particular focus of this study.

1.4. Ant-induced inhibition of pollen function

Investigation into the role of ant surface secretions on pollen started with the discovery that the metapleural gland secretions of leafcutter ants had antimicrobial properties (Maschwitz *et al.* 1970) and the subsequent identification of myrmicacin as at least one of the chemicals responsible for this effect (Schildknecht & Koob 1971). Further tests exposing myrmicacin to pollen of five different plant species led to the important finding that myrmicacin also has negative effects on pollen (Iwanami & Iwadare 1978; Nakamura *et al.* 1982), including reducing percentage pollen germination, pollen tube mitosis and pollen tube elongation as well as specific visible changes in golgi movement and cytoplasmic streaming.

Beattie *et al.* (1984) took this one step further, demonstrating that directly exposing pollen to ants was enough to produce these effects on its viability. In a series of assays bringing the pollen of one or more of four species, *Prunus avium*, *Rhododendron arboreum*, *Lycopersicon peruvianum*, and *Acacia retinodes* into contact with 12 ant species, they observed significant

reductions in pollen viability, percentage germination and pollen tube length in all combinations tested. Additionally, after exposing *L. peruvianum* pollen to an *Aphaenogaster* sp. and then using this to hand pollinate flowers, a significant reduction in seed set was found.

Beattie *et al.* (1984) suggested that ants possess these antimicrobial secretions due to their specific requirement to keep their nests free of potentially harmful microorganisms, and that the negative effects on pollen are a by-product. To explain why this effect is not important in other pollinating Hymenoptera, Beattie *et al.* (1984) argued that this requirement for a clean nest is greater in ants because firstly, ants often nest in the soil rather that creating their own nest materials, and secondly, their larvae are not contained in specialised brood cells.

With regard to the nature of the pollen inhibiting chemicals, Beattie *et al.* (1984) and Beattie (1985) stressed that from these initial studies, it was impossible to say if myrmicacin or other metapleural gland secretions were the only cause of this effect. Further investigations have aimed to resolve the significance of the metapleural glands but no clear picture has emerged; metapleural gland secretions appear to be responsible for negative effects on pollen viability in some situations but not in others.

In a more precise study by Beattie *et al.* (1985) the viability of ant-exposed *Brassica campestris* pollen decreased with increasing proximity of exposed pollen to the metapleural gland of *Myrmecia nigriscapa*, indicating its role as a source of the effect. Not all ant species possess metapleural glands (Hölldobler & Engel-Siegel 1984; Yek & Mueller 2010), yet species without them may still have negative effects on pollen. For example decreased pollen viability was also observed after exposure to a *Camponotus* sp. lacking metapleural openings (Beattie *et al.* 1985). Therefore, while the metapleural gland has a significant role to play, the overall phenomenon is possibly common to many ant secretions.

More recent evidence suggests that many chemicals could be responsible for the detrimental effects. Following analysis of metapleural gland secretions of another leafcutter ant species, a whole host of compounds have been identified with a seemingly general antimicrobial function (Ortius-Lechner *et al.* 2000; Yek & Mueller 2010) and there is also evidence that ants do actively use these to deal with potential infections (Fernandez-Marin *et al.* 2006). Furthermore, antimicrobial compounds on ants may come from sources other than

themselves; in some cases mutualistic microorganisms living on the integument of ants produce antifungal chemicals (Currie *et al.* 1999; Haeder *et al.* 2009), though the specific effects of these on pollen are unknown.

It now appears that reduced pollen viability following contact with ants is fairly common, and it has been described for many ant and plant species (Hull & Beattie 1988; Peakall *et al.* 1990; Gómez & Zamora 1992; Ramsey 1995; Wagner 2000; Galen & Butchart 2003; de Vega *et al.* 2009). These include species that frequently occur together in natural interactions (e.g. Gómez & Zamora 1992; de Vega *et al.* 2009), with, in a few cases, clear indications of fitness costs to the plant concerned (Wagner 2000; Galen & Butchart 2003; Nicklen & Wagner 2006), answering the criticisms of the earlier studies which examined many species that do not normally interact.

However, contrary to the findings of initial studies, reduced pollen viability has not been found in all species tested, with several examples to the contrary (Peakall & Beattie 1989; Sugiura *et al.* 2006; de Vega *et al.* 2009). Again, the indications are that focussing on the metapleural glands may be misleading as their presence does not consistently predict negative effects on pollen. As a good illustration of this, of the four ant species exposed to the pollen of *Cytinus hypocistis*, the two that had no negative effects on pollen viability both possessed metapleural glands whereas one of the ants producing negative effects did not possess metapleural glands (de Vega *et al.* 2009). Overall therefore, it is not clear why negative effects on pollen exist in some cases but not in others.

So far, this gives a fairly confused picture of the whole phenomenon. To elucidate this it is helpful to consider those situations in which ants are pollinators and see what fitness consequences exist.

In the case of *Iridomyrmex gracilis* as pollinators of the orchid *Microtis parviflora*, pollination is effected by the workers transporting stalked pollinia (Peakall & Beattie 1989). The workers do have metapleural glands and while it was initially hypothesised that the stalked pollinia protect the pollen by holding it away from the potentially damaging effects of surface secretions, in tests exposing the pollen directly to the surface of the ants, there is no drop in pollen viability. It may be that the pollen is resistant in some way, or that the metapleural gland activity of the

ants is not strong or subject to seasonal variation (Peakall & Beattie 1989); such variation is known in other ant species (Yek & Mueller 2010). Alternatively, this may simply be an indication that gland secretions do not always have negative effects. If this were the case for the majority of examples of ant pollination, but not in other ant species with frequent plant interactions, this would be strong evidence that pollen degradation is a significant factor explaining the lack of ant pollination. However, as previously mentioned there are many examples which do not support this hypothesis.

In a second example of ant pollinated orchids, pollination of *Leporella fimbriata* is effected by the males of *Myrmecia urens* through pseudocopulation (Peakall *et al.* 1987). As with several other ant species, the males of *M. urens* do not possess metapleural glands yet despite experimental assays showing reductions in pollen viability when pollen is exposed directly to the ant integument, there is no corresponding reduction in seed set following ant pollination compared with hand-pollinated controls (Peakall *et al.* 1990). In this case, it may be that the pollen is protected from these negative effects, perhaps either by the morphological feature of the stigmatic secretions attaching the pollinia to ants acting as a barrier to ant secretions or, because *L. fimbriata* pollinia are formed of many thousands of pollen grains, the effect of a decrease in viability is diluted by the sheer number of grains so that the overall effect is negligible (Peakall *et al.* 1990).

This dilution effect also seems to be significant in other non-pollinia examples. *Hormathophylla spinosa* is pollinated by *Proformica longiseta* ants and although ant contact with pollen decreases pollen viability, ants are still significant contributors to pollination (Gómez & Zamora 1992). They suggest that this is because the quantity of pollen transferred outweighs the effects of reduced quality. In a further example de Vega *et al.* (2009) found that despite two out of four of the most prominent ant pollinators of *Cytinus hypocistis* having negative effects on pollen viability, ants are still effective pollinators here too.

This suggests that, at least in some cases, ant pollination is not restricted by their effects on pollen viability. It may be that in plants where the other potential problems with ant pollination (such as reduced out-crossing) are not significant factors, pollen viability reductions are never as important as the quantity of pollen transferred. Additionally, many studies may have overestimated the effect of ant secretions through unrealistically long exposure times

(e.g. Beattie *et al.* 1984) or high concentrations of secretions used (e.g. Beattie *et al.* 1985), so that although negative effects are present, in natural interactions where multiple pollen grains are being transferred the effects on seed set would not be significant. This pattern has been found in some studies, for example Ramsey (1995) identified a significant ($P < 0.001$) reduction in *Blandfordia grandiflora* pollen viability following thoracic contact with pollinating *Iridomyrmex* sp. ants; however, the reduction was only 6% and did not translate into any significant reduction in seed set. There is also evidence that the effects of ant secretions are reversible (Iwanami & Iwadare 1978; Nakamura *et al.* 1982), again a potential source of overestimation of the effect in the wild.

Furthermore, common pollinators, for example honeybees (Iwanami *et al.* 1979), are known to produce substances that can have negative effects on pollen and consequently it may even be that many different groups of pollinators have negative effects on pollen viability and that this is a common standard cost of pollination. Under this hypothesis, negative effects of pollen exposed to ants are no different from the effects of other pollinators, and consequently the pollen inviability phenomenon would not explain the scarcity of ant pollination. A test directly exposing pollen to a variety of potential pollinators and ant species and comparing the any effects on viability would give strong evidence supporting or rejecting this hypothesis, however to my knowledge, no such tests have been carried out.

Harriss and Beattie (1991) did examine the effects of two bee species: *Apis mellifera* and *Trigona carbonaria*, and the wasp *Vespula germanica* on the pollen of *Brassica rapa*; however, although no reductions in pollen quality were found with *A. mellifera* and *V. germanica* and only minor reductions with *T. carbonaria*, this plant species has not been tested with ants.

In summary, evidence that pollen viability effects consistently preclude ant pollination is not strong and, as suggested by others (e.g. Yek & Mueller 2010), it is likely that the infrequency of ant pollination may be better explained by both their lack of wings and unique foraging behaviours.

1.5. General detrimental effects of ants on flowers

As ants are unlikely to be contributing to pollination, flower visits that incur costs to the plant will not be balanced by the provision of pollination services and will therefore have an overall detrimental effect on plant fitness. In addition to the possible impacts on pollen viability, there are several other ways in which these costs can arise, and together these can be categorised into four types: nectar and (occasionally) pollen theft, deterrence of legitimate pollinators, mechanical damage to floral parts, and (as reviewed above) detrimental effects on pollen viability.

Nectar theft is perhaps the most common of these, with multiple examples of ants taking nectar without effecting pollination (e.g.Herrera *et al.* 1984; Galen & Butchart 2003). Nectar is expensive to produce, so its removal incurs a direct cost to the plant as well as indirect costs through leaving a less rewarding flower for legitimate pollinators. Theft can additionally occur in the form of pollen removal without subsequent transfer to other flowers (Byk & Del-Claro 2010).

Ants can also deter pollinators from visiting flowers through aggression (Tsuji *et al.* 2004; Ness 2006; Hansen & Mueller 2009). This is often a particular problem in ant-guarded plants where the plants benefit from having aggressive ants deterring herbivores from vegetative structures. For example, in the interaction between the EFN-bearing *Ferocactus wislizeni* and four associated ant species, the most aggressive ants also deterred more pollinators and resulted in reduced seed set (Ness 2006). Resource depletion and pollinator deterrence by ants is not necessarily detrimental though. In some cases, it can increase pollinator movement thus promoting outcrossing and increased pollen transfer (e.g. Altshuler 1999).

Flower visiting ants can cause more direct damage of floral structures, for example foraging *Formica neorufibarbis gelida* ants frequently chew through the ovary of *Polemonium viscosum* (Galen 1983), and on the flower coronal ring of *Eritrichum aretioides* (Puterbaugh 1998). In some ant-plants, parasitic resident ants can damage flowers even more specifically, attacking and castrating flowers to stimulate domatia production. This is found with an *Allomerus* sp. and its host plant *Cordia nodosa*, and results in drastically lowered fruit production for the plant (Yu & Pierce 1998).

As a result of these detrimental effects, many plants stand to benefit by excluding ants from flowers. Many examples exist, and can these be grouped into three different modalities (Willmer *et al.* 2009). These are, "Architectural Filters", "Decoys/bribes", and "Chemical Deterrents". The majority of work has been carried out with chemical deterrents, which is the other focus of this thesis; however, I will first review the other two.

1.6. Architectural Filters

Floral structures with the apparent purpose of excluding ants and other potentially damaging visitors have been known about, and commented on, since the 19th century. Kerner (1878) observed a plethora of different floral structures fitting this description, which have more recently been summarised by Guerrant and Fiedler (1981). These include sticky mucilage, bracts, or hairs around the flower stem or base, water jackets or moats blocking flower access, and narrow corollas. More recent work has provided many examples of these restricting ants from flowers.

In a study of 75 Spanish angiosperms, Herrera (1984) found that plants with narrower, more closed corollas had reduced numbers of nectar-feeding ants compared with plants with more open corollas. This implication of a direct benefit of narrow floral form restricting ants is supported by other examples (Junker *et al.* 2011). However the effects of narrow corollas are not always beneficial, as they can also restrict pollinators. In *Polemonium viscosum*, although flowers with narrow corollas experienced less ant damage (Galen 1999), they were also visited less by pollinating bees (Galen & Newport 1987).

Barriers that do not also exclude pollinators are likely to be more beneficial. Water moats around flowers are one method of achieving this, and have been reported in a number of cases (Feinsinger & Swarm 1978; Wootton & Sun 1990). Although they have not been shown to exclude ants from flowers, slippery, waxy stems (Federle *et al.* 1997) and trichomes (Davidson *et al.* 1989) have been shown to exclude ants in other situations and so could do this with flowers. Faegri and van der Pijl (1979) describe the sticky stems of *Viscaria vulgaris* as an assumed adaptation for excluding ants, and sticky glandular hairs with a similar function were described in *Plumbago zeylanica* by Junker et al (2011). These authors also reported fine

dense hairs around the calyx of *Abutilon eremitopetalum*. The hairs always excluded ants except when part of the floral structure above the barrier was touching unprotected non-floral structures. This simple bypass of barriers is potentially a significant problem with architectural filters.

Architectural filters are also potentially problematic as the initial investment in specific structures may be quite large, and once in place they are not easily modified making them less flexible than other mechanisms for deterring ants from flowers. This may be particularly significant in ant-guarded plants where in addition to stopping ants visiting flowers, a morphological barrier could unnecessarily keep ants away from the developing buds and fruits, thus losing any potential benefits of protection (Altshuler 1999).

1.7. Decoys/bribes

Although for many years there were suggestions that an alternative function of EFNs is to distract unwanted visitors from flowers, this has only recently been verified. Wagner and Kay (2002) demonstrated through experiments with artificial "replica plants" and nectaries, that the addition of further nectaries reduced ant visits to the primary nectar sources. More conclusively, in a field study with the senita cactus *Pachycereus schottii*, when EFNs were experimentally removed, ant visits to flowers increased (Chamberlain & Holland 2008).

An alternative way in which decoys could work was suggested by Galen (2005). Although experimentally adding EFNs actually increased ant visitation to *Polemonium viscosum* flowers, plants with a larger overall floral display (and hence greater nectar provision), had a reduction in ant effects on individual flowers, in line with the hypothesis of the ants being satiated by the extra nectar.

The use of EFNs as decoys may be less common than other methods of deterring ant visits to flowers because the extra nectar production is more costly. Additionally, despite being more flexible than architectural features, EFNs as decoys also have the same issue in that they require a large initial investment (Willmer *et al.* 2009).

1.8. Chemical deterrents

Chemicals for deterring ants can be found in a variety of different locations in and around floral structures, including in the nectar and as volatiles present as part of the floral bouquet.

Repellent nectar

The presence of repellent nectar for deterring ants was originally suggested by Janzen (1977) and he hypothesised this is the main reason why ants do not visit flowers. This stimulated much research (Baker & Baker 1978; Feinsinger & Swarm 1978; Guerrant & Fiedler 1981; Haber *et al.* 1981; Stephenson 1981), with the general finding that while ants find floral nectar from some angiosperms unacceptable, generally this is not a common occurrence. More recent work supports this view, with ants readily accepting nectar from 64 out of a wide range of 72 angiosperms (Junker & Bluthgen 2008) with only one of these being specifically rejected.

Although secondary compounds are frequently found in nectars (Adler 2000), these do not necessarily have adverse effects on ants (Haber *et al.* 1981) and potentially toxic chemicals may simply be present in nectar as a side effect of their occurrence systemically throughout the plant as a general herbivore deterrent (Adler 2000). Also whilst toxic or repellent nectar can be effective against nectar-thieving ants (Junker *et al.* 2011), it will not specifically repel ants from flowers and as such would provide little benefit when the negative effects of ants are due to reasons other than nectar theft. Furthermore, repellent nectar has the significant drawback that it can also repel pollinators (Adler & Irwin 2005; Gegear *et al.* 2007) and so reduce the pollination success of the plant.

Altogether, there is little evidence for repellent nectar as a common method for deterring ants and any overall effect on plant fitness may be negligible (Adler 2000; Adler & Irwin 2005).

1.9. Repellent floral chemicals

Unlike repellent nectar, ant-repellent chemicals present in floral tissue, or released as volatiles from floral structures are particularly common. Again, it was Kerner (1878) who suggested that floral tissues may contain repellent compounds. Evidence to support this was found by

Guerrant and Fiedler (1981) in some tropical angiosperms from Costa Rica with further hints of a volatile chemical deterrent identified by Galen (1983) in the North American *Polemonium viscosum*. She found that ants visited "sweet" smelling morphs rather than "skunky" morphs, an effect also apparent in lab-based choice tests.

Willmer and Stone (1997) were the first to present conclusive evidence of a chemical ant-deterrent, found in flowers of the ant-plant *Acacia zanzibarica*. This repellent effect was specifically found in newly dehisced flowers and was transferable to otherwise non-repellent older flowers by wiping them with repellent flowers, confirming the presence of a chemical as the likely source of the repellent effect.

Floral ant-repellence has since been identified as a common phenomenon, present in around a third of tested species (Willmer *et al.* 2009), with examples from numerous plant families across most continents (Ghazoul 2001; Raine *et al.* 2002; Ness 2006; Nicklen & Wagner 2006; Junker *et al.* 2007; Agarwal & Rastogi 2008; Junker & Bluthgen 2008; Willmer *et al.* 2009; Ballantyne 2011; Galen *et al.* 2011; Junker *et al.* 2011; Ballantyne & Willmer 2011 (in press)).

These studies present a range of methodologies for determining the presence of ant-repellent chemicals. The flower wiping procedure of Willmer and Stone (1997) clearly indicates that a chemical - almost certainly a volatile - is responsible for the repellent effect and other studies have made use of this technique (e.g. Raine *et al.* 2002). Evidence of floral repellents also comes from methods looking at the response of ants to contact with floral parts; this can additionally detect tactile repellence in floral structures (Nicklen & Wagner 2006; Junker *et al.* 2007).

More recently, studies concentrating specifically on floral scents have demonstrated that ant-repellent effects can be elicited by volatiles alone. The first of these was by Junker and Bluthgen (2008), who used a four-way olfactometer to test the responses of four species of ant to floral volatiles from a selection of 30 plants. Significant repellent effects were identified in flowers from 20/30 plants tested with *Camponotus floridanus* and 8/26 plants tested with *Lasius fuliginosus*. Additionally, the presence/absence of repellent floral volatiles (as determined by olfactometer trials) correlated with the pattern of *Formica rufibarbis* ant visits to four plant species they commonly interact with in the field. *F. rufibarbis* were not repelled

by floral volatiles of two species where they regularly visited flowers whereas they were repelled by the volatiles of flowers from the two species they do not normally visit.

Using the elicitation of aggressive behaviours after exposure to floral volatiles as a proxy for floral repellence, Willmer *et al.* (2009) presented evidence of repellent volatiles in a variety of temperate angiosperms using the ant species *Formica aquilonia* and *Lasius niger* as well as with the African ant-plant *Acacia seyal fistula* and several additional *Acacia* species.

Although the general indications are that volatiles are causing ant-repellence, this may not be the case in all situations. Ballantyne (2011) found that while contact repellence from floral structures of Costa Rican plants was relatively frequent, occurring in at least 14/33 Costa Rican angiosperms tested with *Camponotus novograndensis*, only one of these was found to elicit significant behavioural changes during experiments exposing the ants to the floral volatiles in isolation, suggesting that volatile repellents may not be common in some locations. It is possible that the high temperatures of some ecosystems affects the suitability of volatile repellents and they may be either absent, short lived or only function at very close range (Ballantyne 2011).

Many studies have sought to identify the actual source of the repellent volatiles. There is limited evidence that petals (Ballantyne & Willmer 2011 (in press)) and buds (Junker *et al.* 2007) can sometimes be repellent, but most analyses demonstrate that the appearance of a repellent is synchronised with floral dehiscence and therefore may be produced from the anthers or even the pollen itself (Willmer & Stone 1997; Raine *et al.* 2002).

Pollen can absorb volatiles from other parts of flowers (Dobson & Bergstrom 2000) and although this may account for the observed repellent effects of pollen in some studies (Nicklen & Wagner 2006; Ballantyne 2011) this was ruled out in several detailed assays of the repellent effects of pollen and anthers by Willmer *et al.* (2009). Dehiscing anthers of three species produced considerable repellence-related behaviours in *Formica aquilonia* ants even when detached from the flower and tested in isolation, whereas old anthers and flowers with anthers removed did not elicit a repellent response. Additionally, a greater standing crop of pollen on flowers of *Acacia seyal fistula* corresponded to greater ant-repellence, and when polyads were experimentally retained on *A. etbaica* inflorescences, repellent effects were still

present despite loss of anther glands, the other potential source of the repellent (Willmer *et al.* 2009). Pollen odour is known to be an important component of the floral bouquet of many plant species (Jurgens & Dotterl 2004) and having it as a source of ant-repellent volatiles neatly facilitates the transient appearance of ant-repellence when it will be most effective i.e. when pollinator visits are likely to be highest and ant interference with anthers is likely to be most costly. Additionally, the repellence stops as soon as the pollen has all been removed.

1.10. Adaptiveness of repellent floral traits

As expected given the many possible negative effects of ants on flowers, there is increasing evidence that ant-repellent floral volatiles are adaptive and provide fitness benefits for the plant. This is perhaps clearest in the cases of ant-guarded species. Plants benefit from reduced herbivory when occupied by aggressive ants, but the same ants are also likely to be particularly effective at deterring pollinators (Willmer & Stone 1997; Ness 2006). As exemplified by *Acacia zanzibarica*, a temporary ant-repellent effect provided by floral volatiles only when flowers are newly dehisced will be effective at reducing the potential costs of pollinator deterrence while still allowing ants to patrol buds and developing seeds where their protection will be particularly useful. Indeed, the presence of ants significantly enhances seed set for *A. zanzibarica* (Willmer & Stone 1997).

There is also evidence that by preventing ant visits to flowers, repellent floral chemicals can specifically help in avoiding ant induced reductions in pollen viability. *Acacia constricta* plants benefit from being located near ant nests (Wagner 1997; Wagner & Nicklen 2010); however, reductions in pollen viability following ant contact with flowers is potentially a significant cost of the interaction to plants (Wagner 2000). Ant visits to the flowers are, however, infrequent in the field, an effect produced by repellent flowers (Nicklen & Wagner 2006).

Most recently Ballantyne and Willmer (2011 (in press)) have presented substantial evidence that one adaptive function of ant-repellent floral compounds is to reduce nectar theft. They found a significant positive correlation between flower nectar volume and magnitude of floral repellence against *Camponotus novograndensis* workers across 49 plant species in Costa Rica.

Ecosystems that have evolved without ants make a good location for further investigating the adaptiveness of ant-repellent floral volatiles. In a comparative study between native and introduced plants in Hawaii, a location where ants traditionally were absent, there was a conspicuous absence of ant-deterring traits - both chemical repellents and architectural filters - in native angiosperms compared with introduced species (Junker *et al.* 2011). Several introduced ant species are now common in Hawaii and (corresponding with their lack of protection) native plant species were more commonly exploited by these ants. This absence of traits where historically they were not needed suggests these traits are costly, and adaptive.

It also appears that where one modality of restricting ant access to flowers is not present, others take its place and that these different traits trade-off against each other. Evidence for such a trade-off between chemical and a number of morphological traits was found by Willmer *et al.* (2009) across a wide range of temperate angiosperms and was also apparent in the introduced species tested by Junker *et al.* (2011) in Hawaii.

Ballantyne and Willmer (2011 (in press)) found no consistent evidence of a trade-off in Costa Rica; however, this may be because a trade-off here is of a more complicated multivariate nature (see Agrawal 2007), with multiple factors influencing the susceptibility of flowers to ant damage and the presence of ant-deterring traits.

This raises the question of why deterrence is not present in all plant species negatively affected by ants, and why ant-repellent floral compounds do not repel all ant species (Junker *et al.* 2007; Ballantyne & Willmer 2011 (in press)). Presumably some ants are not affected by, or can overcome the repellent effect (Junker *et al.* 2007). The degree to which this can occur may depend on the potential value of floral resources to ants and the potential costs to the plants of ant visits to flowers.

In the sponge gourd, *Luffa cylindrica*, Agarwal and Rastogi (2008) tested for floral repellence with six of the most frequent EFN visiting ant species, finding five of these were significantly deterred by a chemical repellent. The one undeterred species, the “Tiny Ant”, *Tapinoma melanocephalum*, was a regular flower visitor exploiting floral nectar, but appeared to be too small to contact anthers (and thus potentially affect pollen viability) and had no significant

deterrent effect on pollinators. The value of the floral nectar to the ant may explain its resistance/insensitivity to the repellent chemicals.

Junker *et al.* (2010) found that facultative flower visitors are more likely to be repelled by floral volatiles than obligate ones. Obligate visitors will experience greater pressure to tolerate floral chemicals that are primarily there for defence, or even to use them as an attractant. In agreement with this, it has been suggested that floral volatile attractants have evolved from compounds with a primarily defensive role (Thien *et al.* 2000). For facultative flower visitors, being unable to exploit a floral resource will be of less consequence as they will have the option of alternative resources.

However, this dual role of floral volatiles as both attractant and repellent is not evident in all situations. In *Polemonium viscosum*, repellent floral volatiles reduce ant damage to flowers; but, when the same volatiles are present at high concentrations in flowers they also deter pollinating bumblebees (Galen *et al.* 2011).

1.11. Identity of repellent compounds

So far, little is known about the identity of ant-repellent floral compounds with no obvious overall patterns emerging. Willmer *et al.* (2009) identified E,E-α-farnesene as a likely component of the repellent effect of *Acacia seyal fistula* as this was released from the flowers in greater quantities at peak dehiscence and coincident with maximum avoidance by ants. E,E-α-farnesene is particularly interesting as a contender for the repellent effect as it is a known component of insect alarm pheromones and has also been identified from the floral bouquets of other plant species (Dotterl *et al.* 2005) including the ant-repellent *A. collinsii* from Costa Rica (Willmer *et al.* 2009).

Another specific component responsible for ant-repellence was reported by Galen *et al.* (2011) who identified 2-phenylethanol as the source in *Polemonium viscosum*. Again, this is a compound that is found in many floral bouquets (Knudsen *et al.* 2006).

Contrasting with these specific volatiles identified as ant-repellents, Junker and Bluthgen (2008) found ant-repellent effects for several pure samples of very common volatiles found in

floral bouquets across the angiosperms. Linalool was particularly repellent, deterring all three ant species tested and other compounds including geraniol, limonene and α-pinene also had observable repellent effects. Interestingly these authors also found that a combination of five other floral volatile compounds repelled ants despite none of the individual chemicals being repellent by themselves.

In an attempt to identify any patterns of compounds present in ant-repellent flowers, Junker *et al.* (2011) analysed 29 floral scent bouquets of flowers also assayed for ant-repellence but found no specific chemicals or combinations thereof standing out as being repellent, giving the general indication that chemical composition is not a specific predictor of defensive function.

Overall therefore, the evidence presents a somewhat mixed picture with both very specific and very common floral volatiles potentially producing repellent effects. As only a few ant-repellent compounds have been specifically identified, there is clearly great potential for work in this area.

More broadly, although there is evidence that the presence of ant-repellent compounds is adaptive across a wide range of species, there is still a need for more comparative studies such as Junker *et al.* (2011) and Ballantyne and Willmer (2011 (in press)). This will allow further resolution of the general importance of ant-repellent volatiles in structuring ant-plant interactions.

1.12. Aims and objectives

In this thesis, I investigated two aspects of ant-flower interactions:

1. Behavioural testing and chemical analysis of ant-repellent floral volatiles

Here, I aimed to quantify the relative attraction or repellence of floral volatiles from seven native or naturalised British plant species to *Formica aquilonia* ants using a simple two-way olfactometer. Plant species of interest were chosen for a variety of reasons, including observed interactions with ants in the field, inflorescence and plant morphologies suggesting possible ant-plant conflicts and also their abundance.

To help quantify the response of ants to volatiles in the olfactometer and better understand their behaviour, *Formica aquilonia* were also tested using three concentrations of their alarm and defence compound formic acid. Löfqvist (1976) found that ants from the closely related species *F. rufa* responded to relatively high concentrations of formic acid by aggressively approaching the source and therefore a significant behavioural response was expected. However, as to my knowledge there have been no tests of individual *Formica* sp. ants to formic acid in an olfactometer setting, it was not known what the nature (repellence or attraction) of the response was likely to be here.

To investigate the chemical basis of repellence in a plant with ant-repellent floral volatiles, I analysed, using SPME GC-MS, the floral bouquet of *Petasites fragrans*, a plant previously identified by Willmer *et al.* (2009) (and named incorrectly there as *P. hybridus*) as eliciting particularly strong repellence-related behaviours in *F. aquilonia*. After identifying one prominent chemical from the floral volatile samples, a pure sample of this was tested in isolation with *F. aquilonia* to test for repellence or attraction.

2. Flower visitors and their effects on pollen viability: comparisons between ants and bees.

It is not known whether the potential reductions in pollen quality experienced following contact with some ant species is an effect also present in other pollinators. To test this hypothesis and compare any differences in the effect between ants and bees, I investigated

the impacts of one species of ant and two species of bee on pollen viability of a *Lilium* sp. by directly exposing the pollen to the insects. Whether ants do, or do not have a greater reduction on pollen viability than bees will provide support or contradict (respectively) the pollen inviability hypothesis of why ant pollination is rare.

The *Lilium* sp. was selected for the large amounts of easily accessible pollen produced by the anthers, facilitating both a simple procedure for exposing the pollen to the insect and allowing different samples of pollen from the same anther to be subject to different conditions. The ant species used was the Scottish wood ant *Formica aquilonia*. Wood ants forage almost exclusively on plants, particularly trees (Domisch *et al.* 2009) and so have the potential to come in contact with flowers. The two bee species chosen for comparison were the very common pollinators, *Apis mellifera* and *Bombus pascuorum*.

Chapter 2: Behavioural testing and chemical analysis of ant-repellent floral volatiles

2.1. Methods and Materials

2.1.1. Olfactometer testing of floral volatiles

Olfactometer setup

The olfactometer consisted of a 230 mm length, 19 mm diameter acrylic (Perspex) tube with a central opening (diameter 17 mm). Both ends of the tube were sealed with a tapered silicone bung/stopper (19-24 mm (min-max) diameter) with a central hole through which a short section of polythene piping (2.8 mm I.D. 4.10 mm O.D.) had been fitted protruding 15 mm from the outside of the stopper. The stoppers were covered with fine synthetic gauze on the inside to prevent ants accessing the piping. Whilst in use, the central opening was also sealed with a third silicone stopper (17-20 mm diameter) with a similar polythene piping fitting for the airflow output. Introducing air flows of two different volatiles (one at either end of the olfactometer) with equal flow rates allowed the maintenance of a distinct airspace in either half of the olfactometer. Floral volatiles and test chemicals were incubated, concentrated and contained using custom-made flower incubation/volatilisation chambers. During trials, a chamber containing the test floral volatiles/chemicals was connected to one end of the olfactometer and control chamber connected to the other using a 220 mm length of PTFE piping (3 mm I.D.).

Flower incubation / volatilisation chambers

The flower incubation chambers were constructed from acrylic and were sealed at either end using hand turned, circular acrylic lids with a fitted rubber seal (Figure 2.1). Two connectors, (I.D. 2 mm) smoothly tapered at one end and with a tapered screw thread at the other, were fitted to opposite sides of the container, one 15 mm from the lowest point of the lid and the other 15 mm from the uppermost point of the base so the smooth tapered side protruded

from the outer surface of the chamber. The base had a small recess cut to securely hold a small vase if required.

Figure 2.1. Large floral incubation chamber with *Linaria purpurea*.

Two sizes of incubation chamber were constructed:

Small: Outer dimensions -- diameter 40 mm, height 108 mm with lid diameter 50 mm. Inside/incubation airspace dimensions -- diameter 35 mm, height 85 mm with an incubation volume of ~82 cm^3. These held glass vases of size 40 x 13 mm (height x diameter).

Large: Outer dimensions -- diameter 76 mm, height 180 mm with lid diameter 90 mm. Inside/incubation airspace dimensions -- diameter 70 mm, height 140 mm; incubation volume ~540 cm^3. These held plastic vases of size 40 x 13 mm.

For each treatment, the incubation chambers were prepared in accordance with the flower species/chemicals being tested. During testing, once the incubation chambers had been sealed, they were immediately connected to the olfactometer and the behavioural recording protocol started.

Airflow setup

Unfiltered air was introduced through the apparatus using a pump connected to approximately (1.5 m) of silicone piping (3.2 mm ID) subsequently split twice using Y-dividers. One split of the first Y-divider connected to a release valve to allow the airflow to be regulated. The other split continued to a second Y-divider from which two 22 mm lengths of silicone piping led to the lower connector of each incubation chamber. The PTFE piping connecting the chambers to the olfactometer was run from the upper connector of each incubation chamber. The airflow was adjusted so the output from each incubation chamber was at a flow rate of 100 cm^3 min^{-1}, measured and adjusted pre-testing using two flow meters (Influx 5-100 cm^3 min^{-1}, Caché Instrumentation Ltd) connected in line after the chambers.

Collection and care of ants

Formica aquilonia ants were collected from the RSPB National Nature Reserve at Abernethy Forest near Boat of Garten, Inverness-shire, OS Grid reference: NH 966 178. Around 200 ants were collected from the surface of one nest and transported back to the laboratory in sealed plastic containers and then transferred to a glass formicarium, of approximate size 200 x 450 x 200 mm.

The formicarium was prepared with pine forest soil and needles and kept humid by regularly adding water to the soil. Water was provided by filling a glass vial and plugging the end with cotton wool, thus keeping the cotton wool saturated without allowing water to leak. Ants were fed on a diet of honey, supplemented regularly with dead insects.

For use in trials, ants were collected individually from the surface of the formicarium in small glass vials and kept under low light conditions until use. Ants were left for at least 20 minutes from collection until use.

Behavioural recording protocol

The olfactometer was illuminated using natural light and orientated parallel to the windows in the laboratory but with screens to give shading from direct sunlight. The olfactometer was

positioned in front of a white background scaled with lines dividing the central olfactometer chamber into four equally sized quadrants (Figure 2.2).

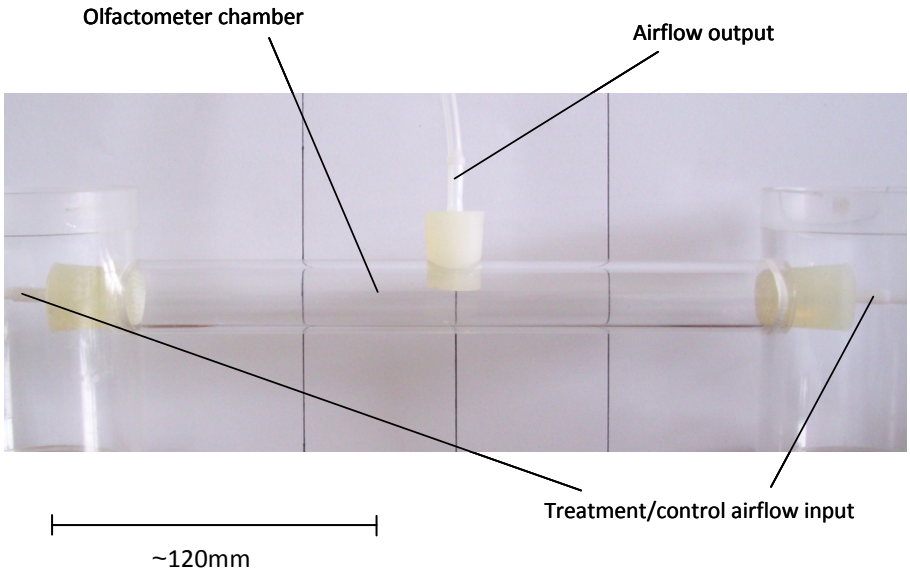


Figure 2.2. Olfactometer chamber with airflow in/outputs and scaled background.

A *Formica aquilonia* ant was introduced into the central opening of the olfactometer which was then sealed using the silicone stopper. The pump was switched on to create an airflow through the chambers and into the olfactometer, and the location of the ant was recorded for five minutes using a web cam in line with the olfactometer and scaled background. 16 replicates were carried out for most treatments, 8 with the test volatiles introduced at one side of the olfactometer and 8 with the test volatiles introduced at the other side. The order in which the treatments were rotated was pre-selected randomly. For the few treatments where it was not possible to take 16 readings, the pre-selected order of 16 treatments was followed but stopped early. The ambient temperature was recorded regularly throughout the trials using an alcohol thermometer.

Nitrile gloves were worn at all times throughout the course of the experiment.

Equipment cleaning and re-use

Before each replicate, to remove possible chemical trail markers left by the ants (Junker & Bluthgen 2008; Morgan 2009), the inside of the olfactometer was cleaned using a pipe cleaner, cloth and distilled water, and immersed and then rinsed in distilled water. The inside faces of all three stoppers were also scrubbed using the soft brush on the end of the pipe cleaner. The olfactometer tube, stoppers and gauze were all dried before further use. The build-up of static was a potential problem following repeated successive test runs and cleaning so care was taken during the cleaning process to minimise this.

The PTFE piping connecting the incubation chambers to the olfactometer and the polythene connectors within the two side stoppers were flushed through with air after each trial. At the end of each day of recording and between different treatments, the PTFE piping, polythene connectors and all three stoppers and gauze were cleaned more thoroughly by running through/rinsing with methanol, isohexane, distilled water and then drying.

The incubation chambers were thoroughly cleaned between each trial by first soaking in water and then washing with water and cloth, and drying.

The incubation chambers and connected piping were not rotated between replicates, so that a chamber and piping was either used for the treatment or for the control for all replicates for one treatment.

Equipment bias testing

Following the initial setup, a run of 16 replicates was carried out with only distilled water in vases in both incubation chambers, so that blank air was introduced at both sides of the olfactometer to test for inherent equipment side bias. These trials were carried out using the small incubation chambers. As a significant side bias was present with this initial setup (see Results), the olfactometer was recalibrated and re-setup in a more constant environment. This was achieved by moving the olfactometer to a different location, taking greater care to ensure the degree of illumination was equal on both sides of the olfactometer, improving the shielding from direct sunlight, ensuring the chamber was as horizontal as possible using a spirit

level, and further reducing any possible vibration effects from the air pump by locating it further away.

Following a second equipment control with a run of 17 replicates, there was negligible side bias (see Results), and so this setup was used for all subsequent trials. The floral trial with *Hesperis matronalis* was carried out using this initial equipment setup so although the statistical analyses were set up to compensate for potential side bias, extra care was required in the analysis of this dataset.

Formic acid repellence testing

To investigate the suitability of the olfactometer for attraction/repellence testing and the response of *F. aquilonia* to one of their own pheromones, the defence compound and alarm pheromone, formic acid, was tested at three different concentrations. The amounts used for testing were chosen based on the concentration and common quantities of formic acid that ants are likely to come into contact with. The quantity of formic acid stored by an individual wood ant worker has been estimated at ~1-10 µl, at a concentration of 63% (Löfqvist 1976, 1977).

Formic acid was diluted to 63% by adding 3.6 ml of distilled water to 6.4 ml of 98% formic acid (Acros Organics 14793-2500). Two trials were carried out using different volumes of 63% formic acid; the third trial was set up using 8% formic acid (prepared using 800 µl 98% formic acid with 9.2 ml distilled water). The treatments were prepared as follows.

1. 5 µl, 63% formic acid.
2. 2 µl, 63% formic acid (2/5 of the quantity in trial 1).
3. 2 µl, 8% formic acid (1/20 of the quantity in trial 1).

With a micropipette, the required amount of formic acid was released onto an ~1.8 cm^2 segment of filter paper (Whatman) and placed, using forceps, at the bottom of one of the small incubation chambers. For the control, the same volume of distilled water was released onto a second piece of filter paper and placed in the control chamber.

Floral testing

Flowers selected for testing were chosen following informal preliminary fieldwork. Multiple short excursions were made in the weeks before and during the olfactometer testing to local gardens, paths, parks, wasteland and the surrounding countryside of St Andrews, (Fife, UK). This amounted to a total time of roughly 12 hours. Flowers were chosen for a number of reasons; specifically, observed interactions with ants, their abundance, morphologies that are possible predictors of ant-plant conflicts (such as narrow corollas and small plant size) and prominent floral scents. For the two species tested where interactions with ants of *Lasius* sp. were recorded (*Sambucus nigra* and *Digitalis purpurea*), this was observed in several individual plants with some time being spent in each case observing the nature of the interaction. *Buddleja davidii* was chosen as it is invasive and very abundant in some locations (Rose *et al.* 2006).

When testing with flowers, flower incubation chambers were chosen appropriate to the size of inflorescence cutting. For the large chambers, to ensure a seal between the lid/base of the chambers and the chamber itself, a square of Nescofilm (Azwell Inc. Osaka, Japan) was stretched over the lid and base before fitting.

Cuttings of the flowers selected for testing were taken from the plant at the beginning of the morning or afternoon on which they were tested. The cuttings were kept with their ends immersed in water throughout testing and were selected so that the presence of vegetative structures was minimised. Features such as the number of flowers and size of cutting used in trials varied for the different species tested, but were selected with the aim of balancing a suitably representative number of flowers with ease of testing; additionally, where possible, inflorescences were assessed for dehiscence to ensure there were some flowers with newly dehisced anthers on each cutting (Table 2.1). In preparation for testing, the stem with inflorescence was placed with the cut end underwater in a vase of suitable size for the chamber chosen (Table 2.1). A vase with an equal amount of water but no inflorescence was placed in the control chamber.

A new inflorescence, each from a different individual plant, was used for each replicate, except in a few cases where additional inflorescences from a previously used plant were tested. This

reusing of new inflorescences from the same plant occurred with *B. davidii* (9 replicates from 2 plants), *H. matronalis* (1 replicate from a previously used plant) and *A. maritima* (7 replicates using a second inflorescence from a plant previously used once).

Table 2.1. Plant species tested with number of replicates, date(s) of testing, nature of inflorescence tested with a description of the condition of the inflorescence, and the size of incubation chamber used.

Plant species	N	Date(s) [# reps]	Inflorescence form used	Inflorescence condition / description	Incubation chamber size
Armeria maritima (Thrift)	16	13/7 [16]	One inflorescence with rounded flower head of several flowers	Mix of newly dehisced anthers, old anthers, some buds.	Small
Hesperis matronalis (Dame's Violet)	13	17/6 [6] 24/6 [7]	One inflorescence with 6-15 open flowers	Mix of new, dehisced and old anthers, some buds. White, pink, violet flowers used	Large
Digitalis purpurea (Foxglove)	15	6/7 [8] 9/7 [7]	A single flower from raceme	Mix of new, dehisced and old anthers White, pink, purple flowers used	Small
Linaria purpurea (Purple Toadflax)	16	20/7 [15] 26/7 [1]	One raceme	Mix of open flowers with buds and old flowers.	Large
Buddleja davidii	9	19/7 [9]	Inflorescence section with ~15 flowers	New flowers only	Small
Sambucus nigra (Elder)	16	2/7 [7] 5/7 [9]	Secondary umbel with many flowers	Mix of new, dehisced and old anthers, some buds.	Large
Centranthus ruber (Red Valerian)	16	12/7 [16]	One inflorescence with many flowers	Mix of new and older flowers	Large

Statistical Methods and Data Analysis

The time spent by an ant in the treatment airspace varied considerably for each replicate, and it was not uncommon for an individual ant to spend the whole 5 minutes in just one side of the olfactometer. However, over multiple replicates, for no significant effect of treatment, the expectation is that the distribution of these times would be centred around 50% of time spent in the treatment airspace. The fixation tendency of some ants to spend the entire recording time at one side created non-normal distributions and thus the data were analysed by designing a Randomisation Test (Manly 2007) to account for this.

The Randomisation test compared a test T^2 statistic, calculated from the observed data, with a null distribution of 10,000 T^2 values created from the observed data. This null distribution was formed by taking the same time values and number of treatments per side as the observed data and then randomising the sides the recorded times were assigned to, to create 10,000 randomised T^2 values. Calculating the proportion of randomised T^2 values greater than the observed T^2 test statistic gives an effective probability (or p-value) that the observed data were solely due to random effects i.e. there was no significant departure from equal time spent by the ants in the treatment and control airspaces. Hence, the magnitude of the p-value indicates if there is a significant departure from equal time spent in both the treatment and control airspace due to some repellent or attractant effect.

The creation of a null distribution for analysing each assay provides a much more efficient statistical test than would be possible through analysis with standard distributions. When determining significance, the design of the test takes into account the magnitude to which individual ants are repelled, but places more weighting on the consistency of repellence across replicates. Hence, several ants showing a small but consistent repellent response will be more significant than a few showing a large response, so that if two or three ants all show fixation to one side or the other by chance, this does not bias the results.

Although the final olfactometer setup was found to be bias-free, the calculation of the T^2 statistic was designed to compensate for the effects of any possible equipment side bias (so compensating for the side bias present with *H. matronalis* in the initial olfactometer setup). Additionally, although the majority of trials had an even number of replicates, for the trials with an odd number of replicates where the treatment airflow was assigned to one side more than the other, the effects of a side bias would be more noticeable.

The calculation of the test T^2 value for each assay was as follows. First, the mean proportion of time spent in the left (S_L) and right (S_R) sides/halves of the olfactometer was calculated. The proportion of time spent in the treatment airspace (P^T) was calculated for each replicate and subtracted from the mean proportions for the respective side of treatment (P^T-S_L or P^T-S_R depending on treatment side), giving a bias corrected proportion (P^B) for each replicate. The value of P^B indicates therefore, the departure from the expected proportion of time spent in

the treatment side if the treatment has no effect. The mean of all the P^B values was then calculated, giving a bias corrected mean difference from 0.5. In reality, for the majority of assays, this was very similar to the original mean proportion of time spent in the treatment airspace. A T^2 value was calculated from this bias corrected mean by dividing by the standard error and squaring the product. The randomisation test was carried out using Microsoft® Excel 2003.

A temperature variation of 9.5°C was present in the olfactometer trials between different treatments (Table 2.2). As this could potentially influence both the volatilisation of chemicals and ant activity, the data were further modelled using a GLM to attempt to account for any effects this could cause.

This compared between treatment differences in time spent in the treatment airspace, with temperature as a covariate in the model. Several treatments were excluded from the model, one as sufficient temperature data were not available (2µl 63% formic acid), one as the data had a highly skewed distribution (4-methoxybenzaldehyde (see Methods section 2.1.2)), the two equipment controls and the one treatment taken with the side-biased setup (*Hesperis matronalis)*.

Times spent in the treatment side of the olfactometer were calculated, converted to proportions and square root arcsine transformed for use in the model. As temperature readings were not taken before all olfactometer trials, where reasonable the temperature information was extrapolated to allow the inclusion of more data points into this analysis. For example, for a trial where temperature readings were taken 15 minutes before and 15 minutes after the trial, the mean of the two recorded values was used as a reading for the trial. This analysis was carried out using SPSS Version 19.

2.1.2. Floral Volatile Analysis of *Petasites fragrans*

Study species and location

Petasites fragrans (Winter Heliotrope) was selected for this study because previous work using the elicitation of aggressive behaviour in *Formica aquilonia* ants as a proxy for floral repellence found strong evidence that *P. fragrans* male flowers emit repellent floral volatiles (Willmer *et al.* 2009) (N.B. in that study, *P. fragrans* was incorrectly named as *P. hybridus*). *P. fragrans* is an introduced but naturalised plant, originating from North Africa and is dioecious with only the male plants found in Britain (Toman 1972, 1983). It is winter flowering with racemes 10-25cm tall containing several flower heads ~15 mm diameter, with many pale pink/white florets (Rose *et al.* 2006). The flowers have a distinctive vanilla/marzipan scent. A single male plant was sampled, in situ, located on the banks of the Kinnessburn in St Andrews, OS Grid reference: NO 507 163.

Sampling method

Floral volatiles were sampled using solid-phase micro-extraction (SPME) fibers with 85 µm carboxen and polydimethylsiloxane (CAR/PDMS) coating, Supelco UK, (Sigma-Aldrich 57295-U). SPME fibers are highly sensitive to trace volatiles (Faldt *et al.* 2000) and PDMS/CAR fiber coatings are particularly suited for floral volatile analysis (Bicchi *et al.* 2000). Fibers were conditioned at 300° C for one hour prior to use.

For sampling, a headspace was created around the flower using a wire frame supporting an inert PET plastic film, modelled on similar enclosures used by (Shepherd *et al.* 2009) for sampling raspberry cane volatiles. Chemical residues were removed from the frame before use by immersing in an ultrasonic bath with GLC grade methanol for 20 minutes and rinsing with methanol, and for a subsequent 20 minutes with GLC grade isohexane and rinsing with isohexane. After washing, the frame was dried thoroughly for an hour and a half in an oven (t < 80°C). Throughout sampling, the frame and film were handled wearing nitrile gloves. The frame was designed to enclose the flower stem and several flowers - the inflorescence enclosed for sampling had 12 flower heads, 11 opened and 1 unopened. The enclosure was

supported above using a plastic bag tie to attach the top of the film to a retort stand support and below by being held securely to the stem of the flower (Figure 2.3).

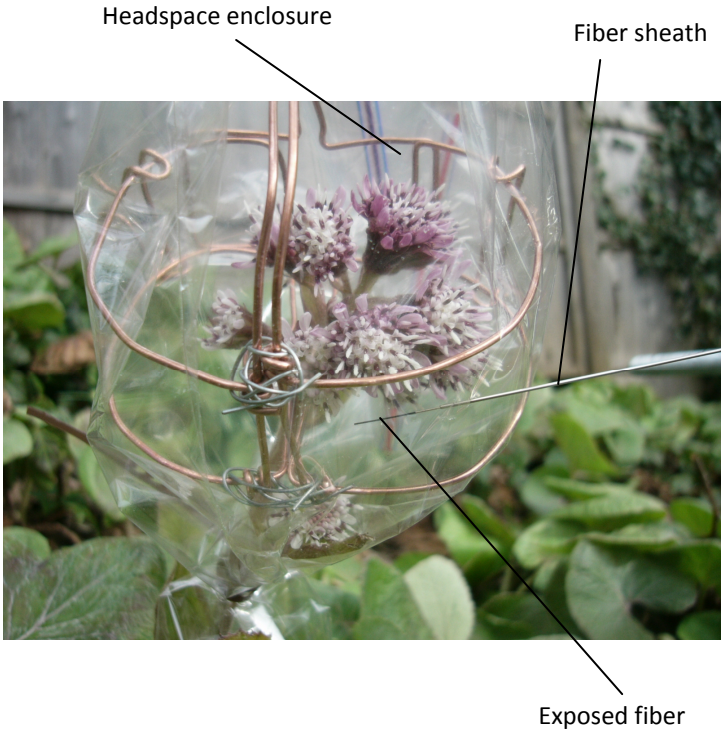


Figure 2.3. *P. fragrans* and headspace enclosure.

For sampling, SPME fibers, fitted into fiber holders (Supelco UK, Sigma-Aldrich 57347-U), were positioned using a retort stand support so the end of a sheathed SPME fiber was protruding into the enclosure through a small hole in the film, within 10 mm of a recently opened flower head with newly dehisced anthers (Figure 2.3). The fiber was exposed for 25 minutes and then re-sheathed. Concurrently with the exposure of the first floral sample fibre, as a control for background volatiles a second fiber was exposed in a similar manner for 25 minutes within an identical enclosure of an empty airspace in the same location as the floral sample fiber. After the floral sample and control sample fibers were re-sheathed and removed from the enclosure, a second sample was taken from each (floral and control) enclosure using a further two fibers exposed for 25 minutes. The first floral and control sample fibers were exposed from 11:55 AM and the second from 12:31 PM. Before, and after exposure, and until desorption into the GC-MS, the fibers were stored in glass vials pre-flushed with nitrogen.

GC-MS analysis

The fibers were desorbed via a GC-MS autosampler and PTV injector and analysed using GC-MS consisting of a ThermoFisher (UK) DSQII quadrupole mass spectrometer with a Trace gas chromatograph, and a CombiPal autosampler (CTC Analytics, Switzerland). The samples were desorbed for 2 minutes into a PTV injector assembly operating in splitless mode at temperatures of 280°C with subsequent separation of volatiles on a DB 1707 GC column (30 m x 0.25 mm I.D. x 0.25 µm) using a helium carrier gas with flow rate 1.5 ml min^{-1}. The gas chromatograph was run on a temperature program with an initial hold at 40°C for two minutes followed by a temperature increase of 10°C min^{-1} for 20 minutes up till 240°C. The mass spectrometer was run in electron impact mode at 70 eV with a data acquisition rate of 4 spectra s^{-1}. Data were acquired and analysed using the Xcalibur™ software package, version 2.07.

One prominent compound, present in the floral samples only, and potentially the main component of the floral bouquet of *P. fragrans*, was tentatively identified using a combination of direct interpretation of the respective mass spectrogram and library database searching. To conclusively identify this compound and reject the possibility of two other possible contenders (structural isomers of the first compound), standards of the compound in question, 4-methoxybenzaldehyde, as well as its two isomers 3-methoxybenzaldehyde (97%, Aldrich 129658) and 2-methoxybenzaldehyde (98%, Aldrich 109622), were analysed using the GC-MS. Three standards were prepared by introducing a droplet of the respective chemicals in three 20 ml headspace vials using a microsyringe. The standards were prepared as follows: **1:** 1 µl 2-methoxybenzaldehyde; **2:** 1 µl 3-methoxybenzaldehyde; **3:** A combination of 1 µl 3-methoxybenzaldehyde and 2 µl 4-methoxybenzaldehyde. Volatiles were adsorbed by exposing newly conditioned CAR/PDMS SPME fibers for 10 seconds in the headspace vials and desorbed and analysed using exactly the same protocols as with the floral and background control fibers above except that the PTV injector was operated using a CT split with ratio 10-1.

From the chromatograms and associated retention times of the three compounds run as standards, the floral volatile was conclusively identified as 4-methoxybenzaldehyde (see Results) and thus this was used for testing with the olfactometer.

4-methoxybenzaldehyde olfactometer repellence testing

4-methoxybenzaldehyde, identified from the floral bouquet of *Petasites fragrans*, was tested, using the olfactometer, for repellence against *F. aquilonia*. The olfactometer was set up as in the previous floral and chemical assays. As calibration of the amounts of 4-methoxybenzaldehyde being released by *P. fragrans* was not attempted, the quantity selected for testing was based on similar floral origin chemical trials carried out by Junker and Bluthgen (2008). With reference to this, the 4-methoxybenzaldehyde was prepared by emulsifying 8 µl of 4-methoxybenzaldehyde (Sigma A88107) in 3856 µl of low volatile liquid paraffin oil (Sigma 18512) in an ultrasonicating bath. Using a micropipette, 25 µl of this solution was placed on a glass slide in one of the small incubation chambers so that approximately 50 nl of 4-methoxybenzaldehyde was present in the chamber. For the control, 25 µl of paraffin oil only was placed on the slide.

2.2. Results

2.2.1. Olfactometer testing of floral volatiles

Initial equipment set up

With the initial olfactometer setup, a significant side bias was present (Figure 2.4). The mean percentage time spent by *Formica aquilonia* on each side of the olfactometer was split 31.5 - 68.5% in one side compared to the other, equating to a side bias of almost 20% ($p = 0.010$). As noted in the methods, *Hesperis matronalis* was analysed using this setup; however, all subsequent analyses were taken using the revised setup.

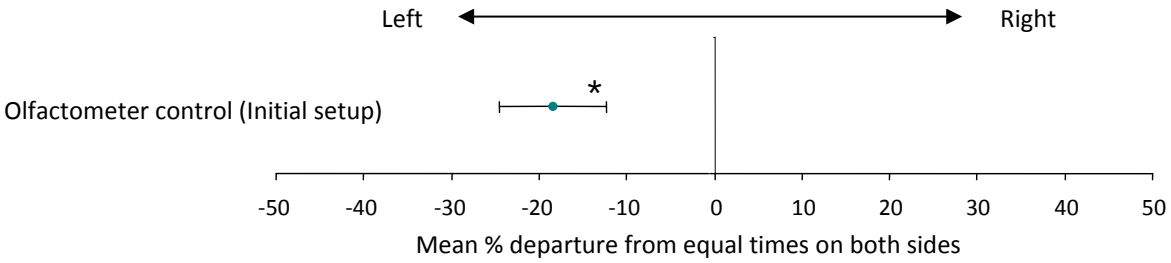


Figure 2.4. Mean (±SE) percentage side bias of *F. aquilonia* to left (negative values) or right (positive values) from equal time spent in both sides of the olfactometer in Initial Trial Setup Configuration with blank air introduced both sides of the olfactometer (n=16). * = significant ($p<0.05$) departure from 50% (Randomisation Test).

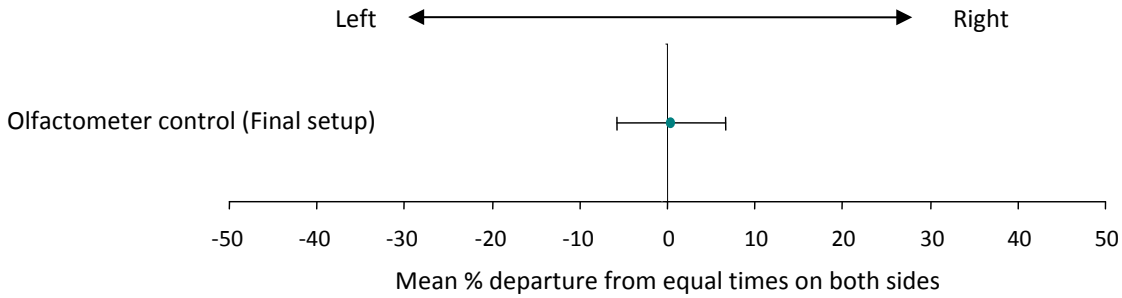


Figure 2.5. Mean (±SE) percentage side bias of *F. aquilonia* to left (negative values) or right (positive values) from equal time spent in both sides of the olfactometer in Final Setup Configuration, with blank air was introduced both sides of the olfactometer (n=17). There was no significant departure from 50% (Randomisation Test).

Final equipment set up

No significant side bias was identified with the revised olfactometer setup (Figure 2.5). For the mean percent time spent in each side, there was only a 0.4% departure from 50% which was not significant (p = 0.948).

Table 2.2. Summary of all olfactometer trials. Figures in bold represent significant (p < 0.05) departures from 50% (equal time in both sides for treatment vs control airspace (or left vs right sides for equipment controls)) (Randomisation Test).

Treatment	N	Date(s) [# reps]	Mean temperature (°C)	Mean % time in treatment airspace; (for equipment controls, this figure indicates mean bias to left (–ve) or right (+ve) from 50%)	
				Mean ±SE	p (testing departure from 50%)
Control (trial setup)	16	26/5 [13] 31/5 [3]	--	**-18 ± 6.0**	**0.010**
Control (final setup)	17	29/6 [17]	26.5	0.4 ± 6.2	0.948
Formic acid (5 µl)	16	9/7 [4] 14/7 [3] 15/7 [9]	22.5	**29 ± 6.2**	**0.008**
Formic acid (2 µl)	16	1/7 [10] 2/7 [6]	26.5	**9.7 ± 3.9**	**<0.001**
Formic acid (2 µl, 8%)	16	16/7 [7] 19/7 [9]	24.0	53 ± 11.1	0.759
Armeria maritima	16	13/7 [16]	22.5	**33 ± 6.5**	**0.029**
Hesperis matronalis	13	17/6 [6] 24/6 [7]	25.0	41 ± 8.1	0.301
Digitalis purpurea	15	6/7 [8] 9/7 [7]	24.0	48 ± 8.2	0.834
Linaria purpurea	16	20/7 [15] 26/7 [1]	25.0	**29 ± 7.3**	**0.017**
Buddleja davidii	9	19/7 [9]	24.0	33 ± 11.3	0.167
Sambucus nigra	16	2/7 [7] 5/7 [9]	27.0	**36 ± 4.3**	**0.008**
Centranthus ruber	16	12/7 [16]	22.5	47 ± 8.8	0.779
4-methoxybenzaldehyde	16	11/8 [4] 12/8 [4] 13/8 [4] 19/8 [4]	23.0	**20 ± 7.7**	**0.004**

Formic acid standardised testing

For both the higher quantities of formic acid tested (2 µl and 5 µl, 63%), the mean percent time spent by *F. aquilonia* in the treatment airspace was significantly less than 50%; however, for the lowest quantity of formic acid (2 µl, 8%) no significant difference from 50% was present (p = 0.755) (Figure 2.6).

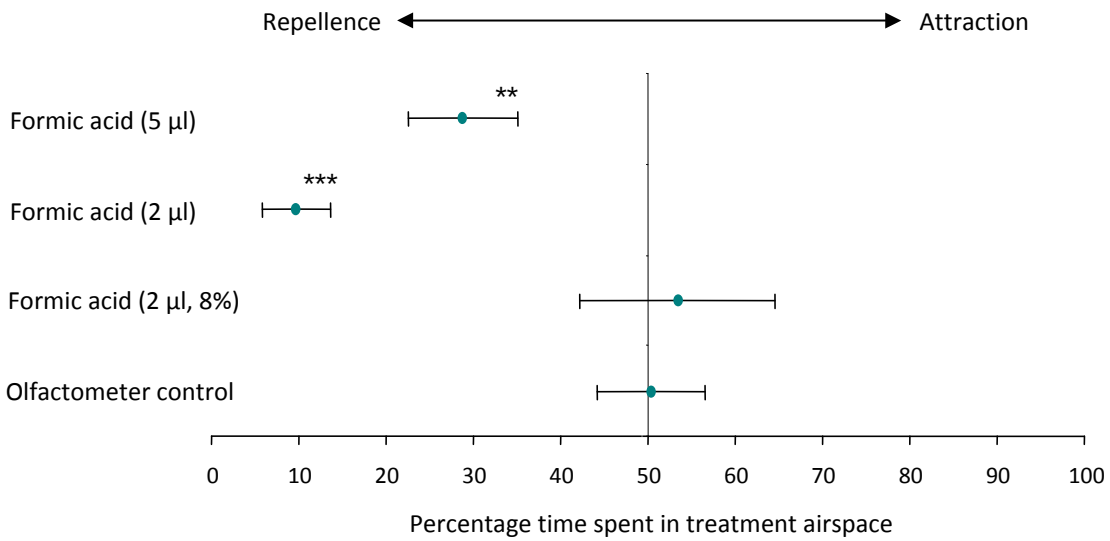


Figure 2.6. Mean (±SE) percentage time spent by *F. aquilonia* in treatment airspace of olfactometer for 5 µl formic acid (n=16), 2 µl formic acid (n=16) and 2 µl 8% formic acid (n=16). Significant departure from 50% (equal time in both sides) indicated by * = p<0.05, ** = p<0.01, *** = p<0.001.

Interestingly, the mean percent time in treatment airspace was lower for 2 µl, 63% formic acid (10%, p<0.0001, n = 16) than for 5 µl, 63% formic acid (29%, p = 0.008, n = 16). However, the mean temperature during the assays was 4 °C higher for 2 µl formic acid than for 5 µl (Table 2.2).

Impact of temperature on repellence

On modelling all (appropriate) treatments together, no significant effect of temperature on repellence was apparent (GLM, p = 0.895); however there was a marginally significant

difference between treatments in the time spent in the treatment airspace (GLM, p = 0.031), as expected following the results above (Table 2.2).

Floral testing

F. aquilonia significantly avoided floral volatiles in three out of the seven species tested, *Sambucus nigra*, *Armeria maritima*, and *Linaria purpurea* (Figure 2.7). The mean percentage time in the treatment airspace was lowest for *L. purpurea* at 29% (p = 0.017, n = 16), 33% for *A. maritima* (p = 0.029, n = 16), and 36% for *S. nigra* (p = 0.008, n = 16). For *Buddleja davidii*, the mean percentage time was also notably low at 33%; however, this was not a significant departure from 50% (Table 2.2) and should be interpreted with care given the low number of replicates.

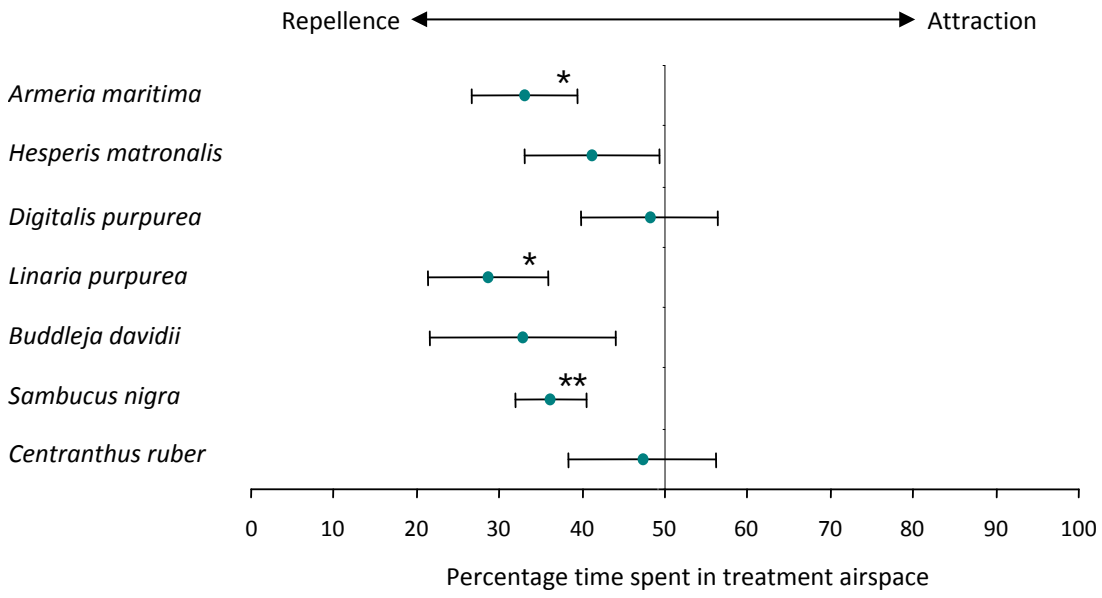


Figure 2.7. Mean (±SE) percentage time spent by *F. aquilonia* in treatment airspace of olfactometer for *A. maritima* (n=16), *H. matronalis* (n=13), *D. purpurea* (n=15), *L. purpurea* (n=16), *B. davidii* (n=9), *S. nigra* (n=16) & *C. ruber* (n=16). Significant departure from 50% (equal time in both sides) indicated by * = p<0.05, ** = p<0.01.

Ants spent slightly less than 50% of their time in the treatment airspace for *Hesperis matronalis* at a mean of 41%, but this was not significant (Table 2.2). There was negligible avoidance of floral volatiles for *Centranthus ruber* and *Digitalis purpurea* with only a very slight non-significant departure from 50% time in treatment airspace (Table 2.2).

2.2.2. Floral Volatile Analysis of Petasites fragrans

The majority of compounds desorbed from the SPME fibers and eluted from the chromatograph column were a mixture of background volatiles and fiber breakdown products and were identical for the floral and background control fibers from both samples. However, there was one compound, with a retention time of 14.20 minutes, notable in its presence in both floral sample fibers, which was absent from both background control sample fibers (Figure 2.9). This suggests that the floral bouquet of *P. fragrans* is largely composed of one prominent volatile. The relative abundance of this volatile was higher in the second floral sample than the first, possibly due to longer headspace equilibration time. Through a combination of direct examination of the mass spectrum of the compound and database library searching, this was tentatively identified as 4-methoxybenzaldehyde.

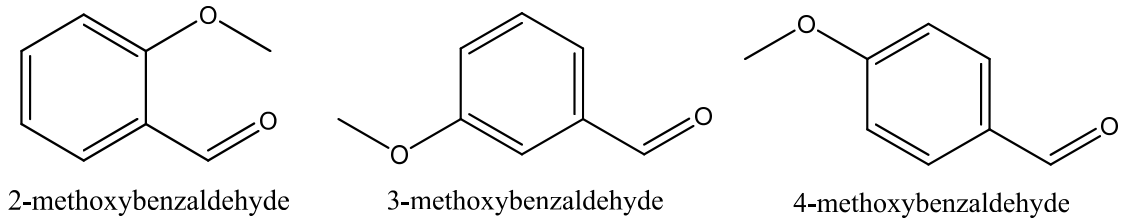
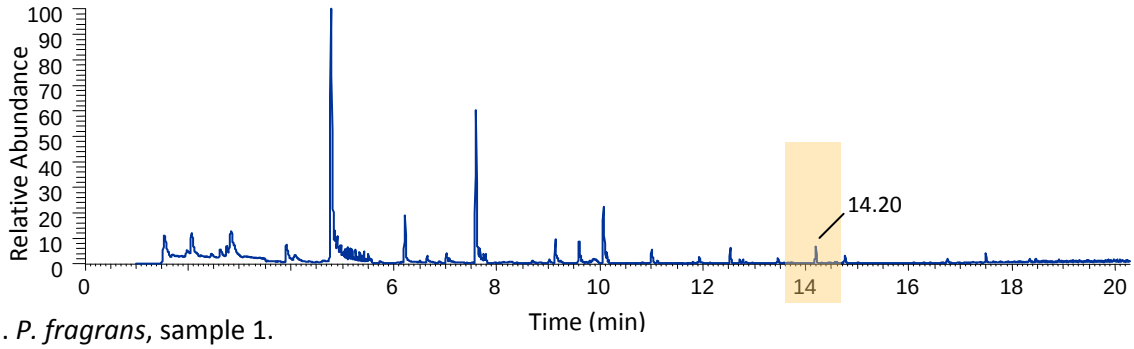
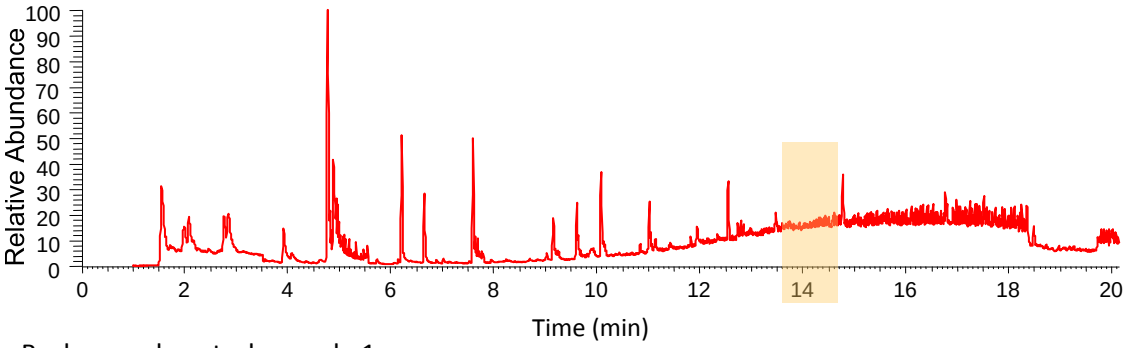


Figure 2.8. 4-methoxybenzaldehyde and the two structural isomers, 2- and 3-methoxybenzaldehyde.

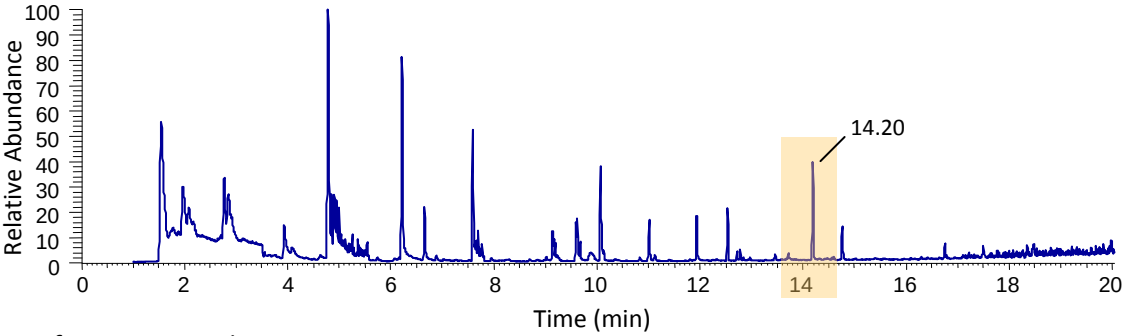
Of the three isomers that were run as standards and analysed using the GC-MS, 2-methoxybenzaldehyde had a retention time of 13.92 minutes, 3-methoxybenzaldehyde had a retention time of 13.16 minutes, and 4-methoxybenzaldehyde had a retention time of 14.20 minutes confirming its identity as the dominant floral volatile (Figure 2.10).



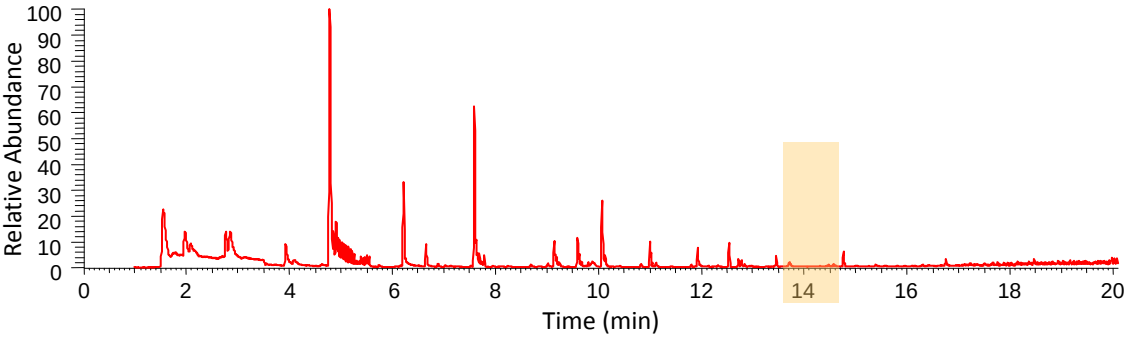
a. *P. fragrans*, sample 1.



b. Background control, sample 1

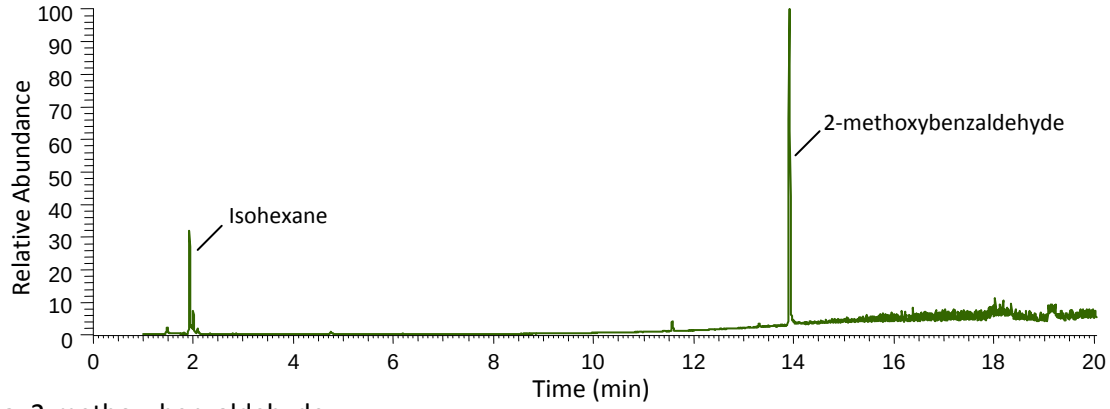


c. *P. fragrans*, sample 2.

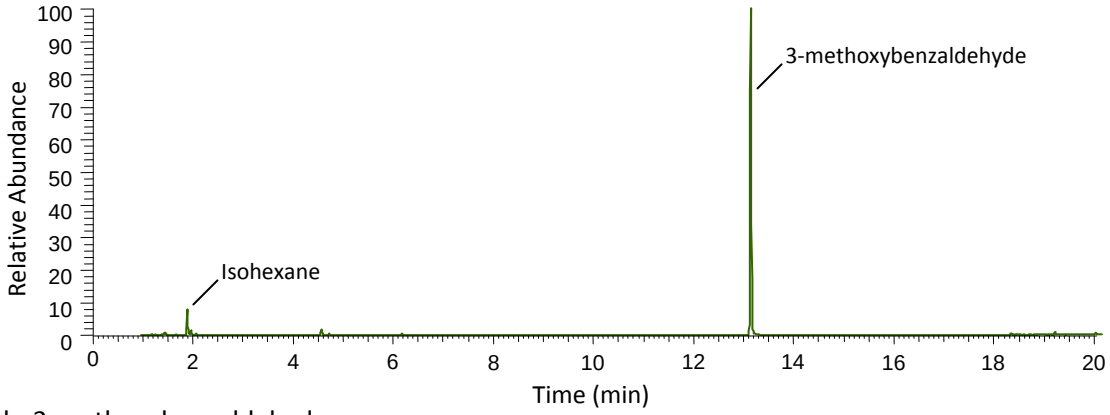


d. Background control, sample 2.

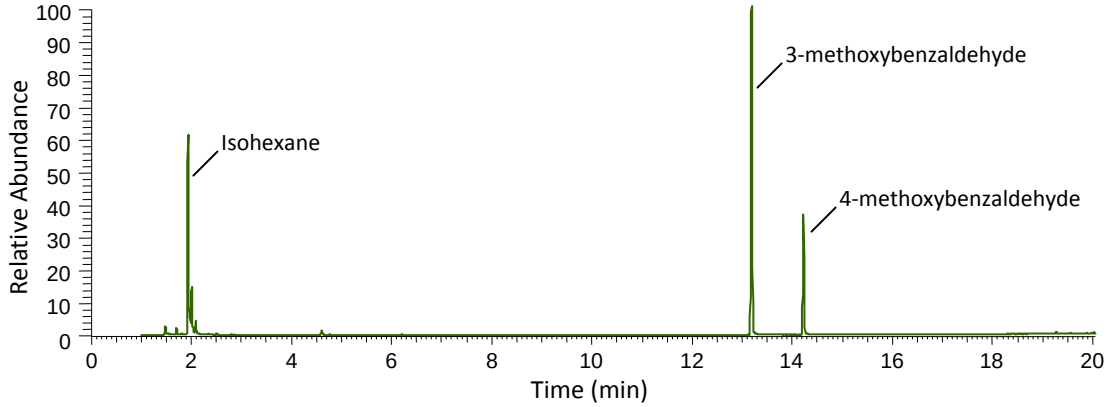
Figure 2.9. Chromatogram traces from four desorbed SPME fiber samples of either *P. fragrans* floral headspace or background control headspace. The region with the chemical of potential interest is highlighted on each chromatogram with the respective peak labelled: Sample 1: **a.** *P.* fragrans, **b.** Background control Sample 2: **c.** *P.* fragrans, **d.** Background control floral.



a. 2-methoxybenzaldehyde



b. 3-methoxybenzaldehyde



c. 3- and 4-methoxybenzaldehyde

Figure 2.10. Chromatogram traces for standards: **a.** 2-methoxybenzaldehyde **b.** 3-methoxybenzaldehyde **c.** 3- and 4-methoxybenzaldehyde. Peaks are labelled with the corresponding chemical. N.B. Isohexane is present as it was used to clean equipment pre-testing.

4-methoxybenzaldehyde olfactometer testing

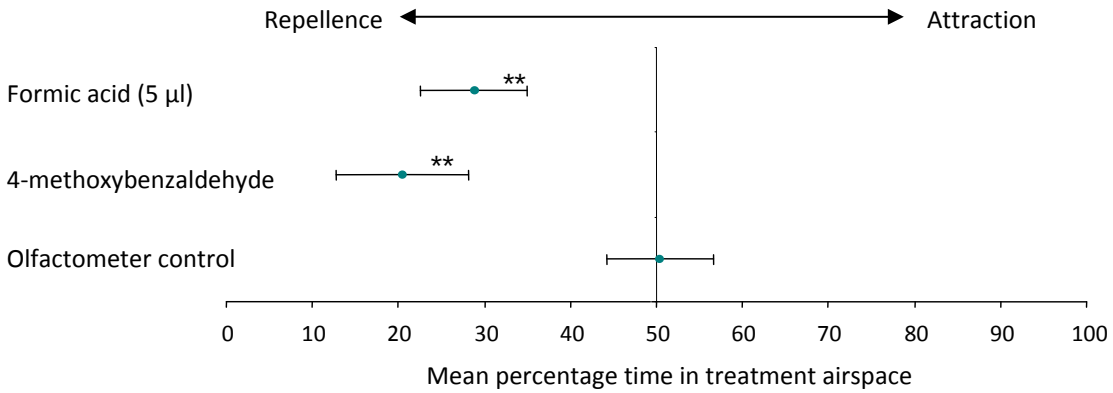


Figure 2.11. Mean (±SE) percentage time spent by *F. aquilonia* in treatment airspace of olfactometer for 4-methoxybenzaldehyde (n=16) with 5 µl formic acid (n=16) and equipment control (untreated air introduced both sides of the olfactometer) (n=17) for comparison. Significant departure from 50% (equal time in both sides) indicated by * = $p<0.05$, ** = $p<0.01$.

F. aquilonia significantly avoided olfactometer airspace containing 4-methoxybenzaldehyde, with a mean percent time in treatment airspace of 20% ($p = 0.004$, $n = 16$) (Figure 2.11).

Chapter 3: Flower visitors and their effects on pollen viability: comparisons between ants and bees.

3.1. Methods and materials

Pollen from a *Lilium sp.* was subjected to four different conditions. Three of these were treatments exposing the pollen to the integument of one of three Hymenopteran species: two bees – *Apis mellifera* and *Bombus pascuorum*, and an ant - *Formica aquilonia.* The 4th condition was a control, where pollen was just left exposed for the same amount of time as the insect treatments.

Collection and preparation of insects

Apis mellifera workers were obtained between 11:00-12:00 on the day of the procedure from a privately owned hive located in the St Andrews Botanic Garden. They were collected individually in plastic vials as they left the hive, and subsequently transferred to glass vials sealed with gauze and brought into the laboratory in preparation for use.

Foraging *Bombus pascuorum* workers were collected directly off plants in the St Andrews Botanic Garden also between 11:00-12:00. They were then kept individually in containers of the same type as the *A. mellifera* workers. Although it was not possible to formally identify the bees collected as *B. pascuorum*, as this would have involved sedating or killing them, the only British bumblebee species they are visibly similar to is *Bombus muscorum* which are rare and has not been reported in this location (Prŷs-Jones & Corbet 2011). Therefore, a general visual inspection was deemed sufficient.

Formica aquilonia workers were collected from the surface of the laboratory formicarium shortly before use in the experiment and contained individually in glass vials until use. For original collection location and care information for *F. aquilonia* see Chapter 2.

Collection and preparation of pollen

A cutting of an ornamental *Lilium* sp. (Oriental variety) was obtained from a commercial supplier on the morning of the experiment and kept in a vase of water throughout the pollen application procedure. The plant was selected to ensure a healthy flower with newly dehisced anthers.

Pollen application procedure

The insects were held stationary throughout pollen application using a retaining device constructed from modified 50 ml plastic syringes. These had the end cut off and replaced with a fine synthetic gauze seal held in place using a cable tie. With the plunger removed, an insect was introduced into the plunger end of the syringe and then sealed in by replacing the plunger. By depressing the plunger the insect could be held in place against the gauze, keeping it stationary throughout the pollen application.

Lily pollen was collected onto a glass slide by gently brushing an anther using a metal seeker. A small quantity of pollen was picked up on the tip of the seeker, gently applied to the integument of the insect through the gauze and left for 20-22 minutes. For the two bee species, pollen was applied to the dorsal side of the thorax; for *F. aquilonia*, the pollen was applied to the dorsal side of the gaster (abdomen), and for the control, pollen was placed directly onto gauze with no insect present. After exposure to the insects, a portion of the pollen (~100-200 grains) was transferred to the germination solution using the seeker. In all replicates, the pollen was in the germination solution within 25 minutes of first being exposed to the insect or gauze. Between each movement of pollen (both application and subsequent transfer to solution) the seeker was rinsed firstly in ethanol >80%, then distilled water, and dried using paper towel.

Six repeats were carried out for each of the three treatments and the control. By staggering the pollen applications at two minute intervals, it was possible to carry out four replicates at the same time; thus, six sets of four repeats were carried out, with each set containing each treatment in an order pre-selected randomly. Within each set, the pollen came from the same

anther, and a new anther was used for each replicate set. A full set of four repeats took approximately 30 minutes with the first set started at 13:20 and the sixth finished at 17:30.

Although efforts were taken to minimise stress to the insects, unfortunately four *A. mellifera* workers died during the course of the experiment, most likely due to starvation. As no replacements were available, they were still used for the procedure. The workers which died were those used in replicates 2, 4, 5, and 6.

Pollen germination solution and incubation procedure

The germination solution was 10% w/w sucrose with 2 x 10^{-3} M H_3BO_3 and 4.4 x 10^{-3} M $Ca(NO_3)_2$ and this and the incubation procedure were based on a methodology in Dafni *et al.* (2005). 10% sucrose solution was selected as a suitable sugar concentation for germinating the chosen *Lilium sp.* pollen following several preliminary germination trials.

The solution was prepared by dissolving 0.1235 grams of H_3BO_3 and 1.044 g of $Ca(NO_3)_2.4H_2O$ in 1 litre of distilled water. 10 g of sucrose was dissolved in 100 ml of this solution, which was refrigerated until a couple of hours before the experimental procedure. Prior to the pollen application procedure, the sucrose concentration was checked with a hand-held refractometer (Bellingham and Stanley) giving a reading of 8.5%.

The pollen was incubated in petri dishes (base diameter 32 mm) with one repeat per dish. The dishes were prepared by adding 2 ml of germination solution to the base and, using a micropipette, a 10 µl droplet of germination solution to the lid. The portion of ~200 grains of treated pollen was transferred, using the seeker, to the droplet. To create a seal between the lid and base of the dish, the upper edge of the base was smeared with Vaseline. Hence once the lid was replaced, the droplet (with pollen) was suspended above the solution in the base with this solution acting as a well to maintain humidity inside the dish and minimise changes in the solution concentration of the droplet.

Pollen was incubated at room temperature (20-23°C) for 24 hours. After incubation the lid was removed and the pollen in the droplets was prepared for counting by staining with 1 µl of

1% methylene blue, leaving for 10 minutes and then mounting by placing glass cover slips directly over the droplet in the lid.

Pollen counting/germination scoring

Shortly after staining, a digital photo was taken of each mounted lid through the eyepiece of a dissecting microscope set up so that all pollen grains were visible in the field of view. The stained and mounted pollen was then refrigerated within 4 hours of staining until analysis.

Germination was assessed by observing the stained and mounted lids through a binocular dissecting microscope and using the printed photos of each lid as a guide. Working from the left-hand side of each photo, the first 50 grains closest to the left were scored, into two categories: Germinated -- germinated pollen with a pollen tube length > 4 x length of an individual grain; Not Germinated -- no pollen tube growth or germinated pollen with a pollen tube length < 4 x length of an individual grain. The dividing point of 4 x length of an individual grain was chosen as germinated pollen with healthy pollen tubes were observed to have a pollen tube length well in excess of this value, whereas grains with poorly formed pollen tubes almost always had a pollen tube length <4 x length of an individual grain.

In some cases, it was difficult to determine the germination status of pollen grains, owing to grains overlapping one another or poor uptake of stain. If it was not possible to score a grain reliably then this grain was missed and analysis moved onto the next grain. The number of grains omitted in this way was recorded.

Statistical methods and data analysis

A proportion of the pollen germinated in all replicates except for one replicate of pollen exposed to *F. aquilonia*. This replicate was left out of the analysis as it was inconsistent with all the others and the pollen grains looked abnormal under visual inspection with the dissecting microscope.

The data were modelled using a GLMM with a binomial error structure and logit link function using the lmer function in the lme4 package (Bates & Maechler 2010) in R Version 2.10.0 (R Development Core Team 2010), with differences between conditions tested using Wald tests.

There was the potential for substantial variance between the six replicate sets, firstly because each set of the four conditions used pollen from a different anther and also because the 24-hour incubation periods of the different replicate sets were out of synchrony with each other, with each having a different light/dark cycle. To take account of this, 'replicate set' was entered in the model as a random factor.

3.2. Results

A proportion of pollen grains germinated in all but one of the 24 replicates, replicate no. 2 for *F. aquilonia*. This outlier was not included in the analysis as the pollen concerned appeared abnormal on visual inspection (see Methods and Materials, Chapter 3.1). The mean percentage germination (pollen viability) for the control *Lilium* sp. pollen was 74%. This was around 10-15% higher than the mean for the three insect exposure treatments, at 63% for both *Apis mellifera* and *Bombus pascuorum* and 58% for *Formica aquilonia* (Table 3.1).

Table 3.1. A summary of the number of pollen grains germinated for all trials, including timeframe within which the pollen was exposed to the treatment or control, with the mean (± standard error) percentage germination for each condition.

Set #	Time of replicate	Number / 50 of pollen grains germinated			
		Control	*Apis mellifera*	*Bombus pascuorum*	*Formica aquilonia*
1	1319 -1350	37	41	40	40
2	1416 -1447	39	37	37	0†
3	1431 -1500	42	23	35	32
4	1620 -1632	31	19	14	9
5	1718 -1749	34	33	32	24
6	1730 -1758	39	35	31	39
Mean % Germination ± SE		74 ± 3.2	63 ± 7.0	63 ± 7.5	58 ± 11.4‡

† This replicate was not included in the statistical analysis or in the calculation of the mean in this table (see Methods).
‡ Replicate no. 2 was not included in the calculation of this value.

There was a substantial variation to the individual number of grains germinated within conditions, making investigation of any overall effect difficult. A general pattern does emerge when examining each treatment on a replicate set by replicate set basis, as for all but one of the sets, the control condition has the greatest (or joint highest in one case) proportion of germinated pollen (Figure 3.1).

By including this between replicate-set variability in the GLMM model, a difference was detected between conditions; however, this was only marginally significant, (Figure 3.2) Wald Test, Chi-squared = 7.864, p = 0.0489.

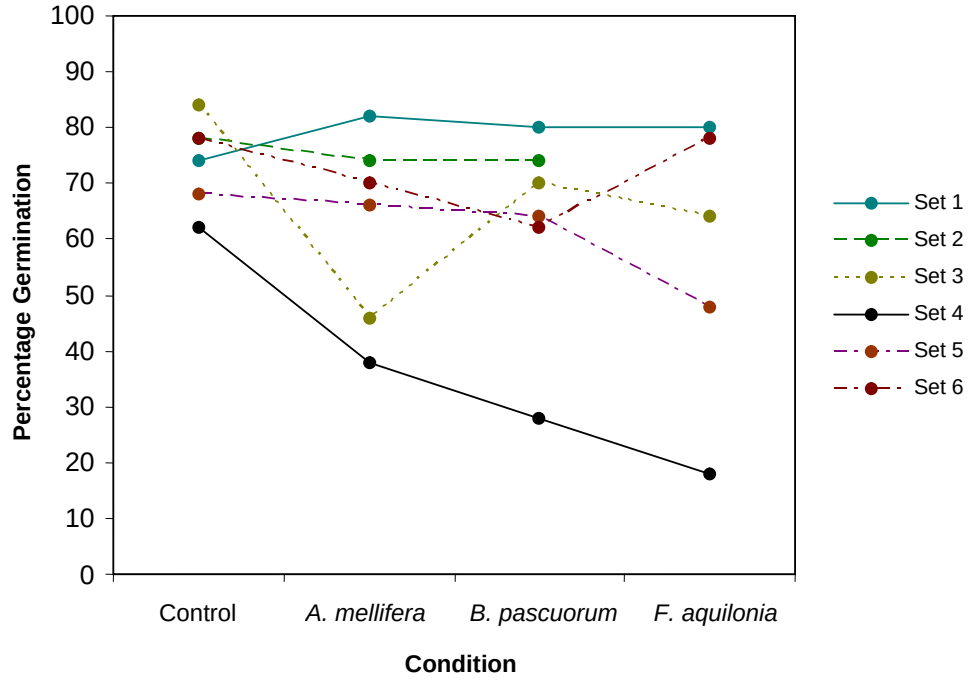


Figure 3.1. Percentage germination for all trials for the four conditions, with individual values from the same replicate set linked together. N.B. Rep no. 2 with *F. aquilonia* is left out (see methods).

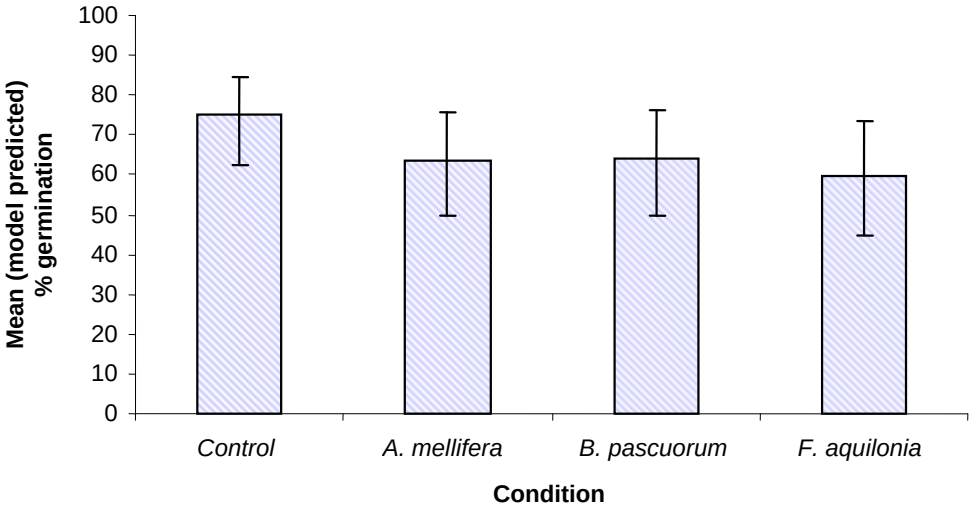


Figure 3.2. Mean % germination and 95% confidence intervals (values back-transformed from GLMM model) for the three treatments and control. The effect of condition on % germination was marginally significant (Wald, Chi-squared = 7.864, p = 0.0489).

On more detailed analysis of comparisons between treatments, the lower pollen viability exhibited by the three insect treatments compared to the control was found to be marginally significant, Wald Tests, Control - *A. mellifera*, Z = -2.087, p = 0.037, Control - *B. pascuorum*, Z = -2.052, p = 0.040, Control - *F. aquilonia*, Z = -2.568, p = 0.010. No significant difference was detected between the three insect treatments, Wald Test, Chi-square = 0.506, p = 0.7763.

Although pollen viability was lower for the three treatments than the control, the significance was only marginal. Additionally as this drop in pollen viability was relatively small at <15% and there was a large amount of variance in the data it is debatable whether this represents any overall effect.

Chapter 4: Discussion

4.1. Overview of olfactometer assays

Many distinct patterns were evident from the responses of *Formica aquilonia* to the different treatments. Whilst *F. aquilonia* showed significant avoidance of several of the treatment volatiles, there was no evidence of attraction (Table 2.2). It can be assumed that the significant avoidance responses of *Formica aquilonia* were not simply due to a general neophobic avoidance of all volatiles in the context of olfactometer assays as the volatiles of several flower species elicited no significant repellence or attraction (Figure 2.7).

4.2. Responses of *F. aquilonia* to formic acid

The formic acid assays allowed further testing of the equipment with a chemical known to influence *F. aquilonia* behaviour. Significant repellent effects were elicited by the two higher amounts (2 µl and 5 µl, 63%) of volatilised formic acid (Figure 2.6), confirming the ability of the olfactometer to detect repellent volatiles.

It is interesting that formic acid elicits such a repellent response, as previous investigations have shown that wood ants of the *Formica rufa* group (which includes *F. aquilonia*) typically respond to formic acid by aggressively approaching the source of the chemical (Löfqvist 1976). General responses to pheromones are known to be context-dependent (e.g. Hölldobler 1999) and it may be that when tested individually away from their nest ants are more likely to exhibit an avoidance or panic reaction than aggression. Additionally, although the volume and concentration of the two higher absolute amounts tested are of similar values to formic acid stored in individual ants (Löfqvist 1976, 1977), the amounts tested may be below the threshold for typical alarm behaviour. Löfqvist (1976) tested alarm responses with different volumes of formic acid-saturated air, making direct comparisons with the quantities used here tricky, but his estimate of 1 cm^3 of acid-saturated air as being just above the response threshold is likely to be in excess of the maximum quantity I tested. Also, the tested formic acid used here did not contain any of the supplementary compounds reported to be present in *F. rufa* group defence secretions (Wilson & Regnier 1971; Hölldobler & Wilson 1990).

This may explain the curious result that the highest absolute amount of formic acid (5 µl, 63%) appeared to be less repellent than 2 µl of the same concentration (Figure 2.6). This makes sense if the assay at 5 µl formic acid was approaching the threshold at which the response changes to approach/aggression, and indeed the behaviour of the ants at 5 µl appeared more indicative of an aggressive response. Alternatively, as the mean recorded temperature whilst assaying 2 µl formic acid was 4°C higher than when testing 5 µl (Table 2.2), this could potentially explain the greater repellence.

Higher temperatures are likely to increase ant activity as well as potentially increasing the volatilisation of chemicals tested in the olfactometer. Although no significant effects of temperature on magnitude of repellence were detected (See 2.2.1. Olfactometer testing of floral volatiles), there were only relatively small differences between the repellent effects of the different treatments where repellence was present (Figure 2.6, Figure 2.7), and additionally it was not possible to include all the treatments in the temperature analysis. It would therefore be useful to specifically test the effect of temperature on repellence in a controlled way to account for this more thoroughly as a possible effect.

Although olfactometer assays may not be suitable for quantifying behavioural responses other than general repellence or attraction, this apparent avoidance of formic acid may be something missed from previous work as to my knowledge a similar study has not been carried out.

4.3. Floral repellence testing

Formica aquilonia exhibited significant repellent responses to floral volatiles of 3/7 plant species tested (Figure 2.7), with all three being native to Britain. This supports previous work identifying floral ant-repellence as a common phenomenon in temperate angiosperms (Willmer *et al.* 2009). For two further species ants spent noticeably less time on average in the floral volatile treatment airspace; however, the sample size was lower for both of these and they were not significant.

This brings up an important point about the olfactometer assays and associated statistical testing. When determining the magnitude of repellent effects, the mean % time in treatment airspace can be somewhat misleading, particularly as often around one to three ants per floral assay spent the entire duration of recording at one or other side of the olfactometer. As mentioned in the Methods and Materials (Chapter 2), the randomisation test accounts for this because when calculating significance, multiple replicates producing a small but consistent repellent effect will have much more weighting than just a few replicates with times at the extremes of the range.

Two of the three species eliciting repellent responses - *Sambucus nigra* (elder) and *Digitalis purpurea* (foxglove) - were chosen for olfactometer testing after observing that they had interactions with *Lasius* sp. (probably *L. niger*) ants in the field. *Lasius* sp. tend aphids on both *S. nigra* (pers. obs.) and *D. purpurea* (Ballantyne 2011). On *D. purpurea* they additionally forage for floral nectar. *S. nigra* has extrafloral nectaries (EFNs) (Fahn 1987); however, the flowers contain no nectar (Atkinson & Atkinson 2002) and I did not observe any visits by ants to flowers. The incidence of floral repellence as assayed here with *F. aquilonia* correlates with the pattern of ant visitation in the field. *F. aquilonia* spent significantly less time in the treatment airspace for floral volatiles of *S. nigra* but had negligible responses to *D. purpurea* (Figure 2.7). Information from field observations were however from *Lasius* sp. rather than *F. aquilonia*, but as *Lasius niger* has previously been found to exhibit repellence related behaviours to floral volatiles from some plants also eliciting these behaviours in *F. aquilonia* (Willmer *et al.* 2009), it is possible the floral repellence detected with *F. aquilonia* could help explain the distribution of *Lasius* sp. on these two plants.

Although ants are not as widely diverse in Britain as in other ecosystems, British plant species still have frequent interactions with ants, in particular through ant-attraction via EFNs and/or to ants tending aphids (e.g. Mahdi & Whittaker 1993; Oliver *et al.* 2007). Plants occupied by aphid-tending or EFNs feeding ants may benefit from this interaction through protection against herbivores (Styrsky & Eubanks 2007), but any encounters with flowers are potentially detrimental to the plant (Galen 1999; Galen & Butchart 2003). Therefore, as in other ecosystems, plants stand to benefit from possessing ant-repellent floral volatiles.

In this example, as well as more generally, repellent floral volatiles are a particularly useful mode of preventing the potentially damaging effects of ants on flowers. Unlike morphological deterrents, repellent floral volatiles are able to act specifically where and when they are needed (Willmer *et al.* 2009), are not subject to simple circumvention (e.g. Junker *et al.* 2011), and are less costly than morphological deterrents. This efficiency and specificity of repellent floral volatiles allows plants to moderate their interactions with ants, maintaining beneficial aspects of the interaction and also tipping the balance more towards favouring the establishment of new beneficial interactions that would otherwise not be possible.

It is interesting that plants with frequent interactions with ants (such as *D.* purpurea) do not necessarily have floral ant-repellents (Figure 2.7). Ballantyne (2011) found no evidence that bumblebee pollinators were deterred by *D. purpurea* flowers previously visited by *L. niger*, and it may be that costs of ant visits to *D. purpurea* flowers are minimal, thus correlating with the observed lack of repellence.

Whereas volatiles from *D. purpurea* flowers elicited no repellent response from *F. aquilonia*, the ants significantly avoided floral volatiles of *Linaria purpurea* in the same family, indicating (as in previous investigations) that there appears to be no clear phylogenetic pattern to floral ant-repellence.

The susceptibility of different floral forms to ant visits may be a predictor for floral ant-repellence, as there is evidence that various ant-deterring traits may trade off against each other (Willmer *et al.* 2009; Junker *et al.* 2011). *Centranthus ruber* has narrow corollas that would restrict ant access and correspondingly had no detectable repellent effect. On the other hand, *Armeria maritima* plants grow very close to the ground and have open inflorescences leaving them more susceptible to ant exploitation. The significant ant-repellent effect identified in *A. maritima* may be a consequence of this vulnerability. *Linaria purpurea* was unusual in this respect in that it elicited ant-repellent effects and also has a closed floral morphology.

Ants spent less time in the treatment airspace for both *Hesperis matronalis* and *Buddleja davidii*; however these data were not significant. For *H. matronalis* although the mean time in treatment airspace was lower than 50%, a combination of small sample size and equipment

bias when testing meant that reliable conclusions cannot be drawn from this finding. The assay with *B. davidii* had a particularly low sample number so although there were still indications of a repellent effect, this was not significant (Figure 2.7). More replicates would be useful here as *B. davidii* is very invasive in the UK and it would be interesting to investigate if possession of ant-repellent floral volatiles have a role to play in this.

Although floral volatiles from several of the plant species tested in the olfactometer were found to be repellent to *F. aquilonia*, this may not necessarily translate to repellence in the wild. *F. aquilonia* don't show a large polymorphism within the worker caste; however, there are differences in sizes between the workers (Sorvari & Hakkarainen 2009) and these may reflect polyethism (different behavioural roles) in workers (Wright *et al.* 2000). Apparent repellent floral volatiles may cause a different reaction in a worker specialised for foraging to one with the role of soldier, but no attempt was made to record these differences in the workers tested. Thus, the potential inclusion, in olfactometer trials, of workers unlikely to come into direct contact with flowers in the wild may obscure the reaction present in the wild.

The reaction of ants to particular volatiles in the olfactometer is also likely to depend on their behavioural state. Previous work has suggested that elicitation of aggressive behaviours on exposure to floral volatiles can correlate with repellence (Willmer *et al.* 2009) and so it is possible that repellence from volatiles in the olfactometer is eliciting behaviours from the aggression-panic spectrum of alarm behaviours (Hölldobler & Wilson 1990) as suggested by the formic acid assays above. If this is the case with the floral volatiles, then a panicked, repellent response in the olfactometer may not translate to a similar response if the ant is in its own territory, where it may react with a more aggressive behaviour. Testing in the olfactometer was also carried out without any food source and the presence of this is likely to change the response of an ant to repellent volatiles. If repellent floral volatiles were present in a flower also possessing high nectar volumes, it is possible that ants may be able to overcome the repellence in order to reach the nectar.

Finally, while testing ants individually in the olfactometer helps avoid magnification of any potential effect through one following pheromone trails of another (Willmer *et al.* 2009), in the field ants often forage in association with many others (Dornhaus & Powell 2010). The reaction of an ant to a potentially repellent volatile encountered along an already established

pheromone-based trail is likely to be different to that exhibited by a solitary ant foraging in a new location. Further investigation into how repellence varies between these conditions would also be useful for further understanding this effect.

4.4. Analysis and behavioural testing of *Petasites fragrans* floral bouquet

Only one prominent chemical was identified from the floral bouquet of *Petasites fragrans*, 4-methoxybenzaldehyde (4-MoB), and this is almost certainly the main floral volatile in this species. Whilst the volatile components of many flowers are typically made up of a mixture of volatiles (e.g. Dotterl *et al.* 2005), it is not uncommon for one chemical to dominate the floral signature. For example 2-phenylethanol accounts for 50% of volatile emissions from flowers of *Polemonium viscosum* (Galen *et al.* 2011).

It is possible that other minor components could be present in the floral bouquet of *P. fragrans*. Additional sampling of flowers with a longer sampling and/or headspace equilibration time and using SPME fiber coatings with a different chemical sensitivity could help to identify if these were present.

Olfactometer testing with 4-MoB showed significant and substantial repellent effects in *F. aquilonia* (Figure 2.11). This strongly implicates it as the cause of the ant-repellent effect in *P. fragrans* that was originally observed by Willmer *et al.* (2009). The only uncertainty with this conclusion is how well the amount of chemical tested in isolation represents the actual emissions of 4-MoB by *P. fragrans*. While the amount tested was of the same order of magnitude as typical floral volatiles, volatile emissions can vary greatly and thus the experimental response may over- or under-estimate the contribution of 4-MoB to the floral repellence in this species. Nevertheless, the prevalence of 4-MoB in the floral signature of *P. fragrans* is strong evidence that it is the cause of the repellent effect.

A combination of two interesting observations, one by Toman (1983) and one by myself, sheds further light on several aspects of the source of the repellent volatiles in *P. fragrans*. So far evidence points towards the anthers or pollen as emitting these volatiles, since detached anthers tested in isolation can produce repellent effects (Willmer *et al.* 2009). The vanilla-marzipan-like odour of 4-MoB strongly resembled the distinctive smell of (the male) *P.*

fragrans flowers, yet Toman (1983) observed that the female flowers were unlike the male flowers in that they did not possess the same "pleasant" aroma. Taken together, this again suggests that the pollen or anthers are producing the repellent effect and that it is indeed 4-MoB that is responsible.

Despite being one of the strongest elicitors of floral repellent-type behaviour in a study of over 60 species tested (Willmer *et al.* 2009) nothing is known about the natural interactions of *P. fragrans* with ants. Although now naturalised in the UK, it is an introduced species (Rose *et al.* 2006) originating from North Africa (Toman 1972, 1983). Obviously, further investigation into *P. fragrans* in its original habitat could provide insights into the adaptiveness of the floral ant-repellence in this species.

4-MoB is of a different class of volatiles to previously identified ant-repellent floral volatiles (Shepherd pers comm.) supporting the view that these compounds are as wide ranging as the occurrence of floral ant-repellence itself. In a study by Theis (2006), 4-MoB was found to be significantly attractive to the common pollinators *Apis mellifera* and *Lasioglossum* spp. but not to several florivorous species, suggesting it may function as both a repellent and attractant in *P. fragrans*.

To my knowledge there are only a few other species reported as possessing 4-MoB as a floral volatile (Andersson *et al.* 2002; Knudsen *et al.* 2006; Theis 2006; Willmer *et al.* 2009). Intriguingly, one of these, the mutualistic ant-plant acacia species *Acacia seyal fistula* also has ant-repellent flowers (Willmer *et al.* 2009). Although in this species it looks as if E,E-α-farnesene is responsible for the repellent effect, it would be interesting to test 4-MoB for repellence against its resident ants. 4-MoB has also been identified from flowers of the thistle *Cirsium arvense* (Andersson *et al.* 2002; Theis 2006) a plant commonly visited by aphid tending ants (Fischer *et al.* 2005), but here 4-MoB only accounts for 0.5% of the bouquet (Theis 2006).

4.5. Pollen viability and effects of insect exposure

The control mean pollen viability of the *Lilium* sp. was of a similar magnitude to previous studies assessing pollen germination in vitro. For example Hull and Beattie (1988) found a

slightly higher "average" viability of 82.7% in *Lilium candidum* but a slightly lower viability in *Narcissus pseudonarcissus* of 67.6%.

The reduction in lily pollen viability after exposure to *F. aquilonia* is also similar to the findings of previous studies (e.g. Beattie *et al.* 1984; Galen & Butchart 2003; de Vega *et al.* 2009) which showed that pollen viability can be reduced following contact with ants. Many ant species produce antimicrobial chemicals to aid nest hygiene (Ortius-Lechner *et al.* 2000; Fernandez-Marin *et al.* 2006) and it is these that are assumed to be the cause of negative effects on pollen and the infrequency of ant pollination (Gómez *et al.* 1996).

Interestingly a similar reduction in viability was also found after contact with both bee species. There is very little information on the how the viability of pollen is influenced by contact with bees, though storing pollen in scopa can reduce viability (Beattie *et al.* 1984) and there is also evidence that the bee species *Trigona carbonaria* has negative effects on pollen (Harriss & Beattie 1991). The bee-induced reductions in pollen viability were not significantly different from those induced by *F. aquilonia*, and if this finding were replicated on comparing bees with other plant and ant species then this has obvious negative implications for the pollen inviability hypothesis of why ants are poor pollinators.

However, the reductions in pollen viability here are small and only marginally significant so it is hard to draw any specific conclusions without further study. Moreover, it is not known how these reductions would affect seed set and as this is a more accurate measure of any potential fitness consequences of a reduction in pollen viability, it would be prudent to take this into account in follow on work. Even if a strong reduction in pollen viability by bees were found, it is not possible to extend this to plants in general without first analysing a substantial range of species. There is already known to be considerable variance in how different ant species can impact pollen viability and a similar variability in the susceptibility of different plant species (Peakall & Beattie 1989; de Vega *et al.* 2009).

There are also a few experimental factors that could impact the observed effects on pollen viability. It is possible that viability was affected by the additional manipulation required to apply the pollen to insects compared with the relatively easy application procedure in the control assays. Additionally, it is not known how the effect varies with different quantities of

pollen attached to the insect. With large numbers of pollen grains, this may dilute the negative effects of any chemicals present, leading to an underestimation of any pollen viability reductions. An investigation into these effects would help establish their significance. Stress on the insects as a result of the pollen application procedure could potentially cause a change in integument chemicals present, and thus potentially affect pollen viability, increasing the magnitude of any reduction in viability and perhaps masking any between insect differences. Other methods of exposing pollen to insects such as containing the insect in a vial with pollen used by Beattie *et al.* (1984) are likely to be less stressful and it would be interesting to compare these approaches.

4.6. Summary: General Implications and Future Directions

Here, I identified a further three temperate angiosperm species (out of seven tested) as possessing ant-repellent floral volatiles. Where data were available, this correlated with patterns of ant visits to flowers in the field, and is in agreement with previous work suggesting floral ant-repellence is a common phenomenon (Junker & Bluthgen 2008; Willmer *et al.* 2009; Junker *et al.* 2011). Although one species tested here - *Linaria purpurea* - possesses repellent floral volatiles and a closed floral morphology, both features with the potential to exclude ants from flowers, no substantial evidence was found to contradict previous suggestions that there appears to be a trade-off between morphological and chemical mechanisms for deterring ants from flowers in temperate angiosperms.

Very few studies have investigated the chemicals responsible for floral ant-repellence (Junker & Bluthgen 2008; Willmer *et al.* 2009; Galen *et al.* 2011) and here I added to this small body of knowledge by identifying 4-methoxybenzaldehyde as the likely source of the ant-repellent effect in *Petasites fragrans*. Intriguingly, 4-methoxybenzaldehyde is present in the floral bouquet of two other plant species that are known to have frequent interactions with ants and so testing this compound for repellent effects against other ant species would be particularly interesting, as previous work has not found any evidence of a pattern to the identity of chemicals producing floral ant-repellents.

There is increasing evidence that repellent floral volatiles are adaptive with the function of preventing potential negative effects of ant visits to flowers (Junker *et al.* 2011; Ballantyne & Willmer 2011 (in press)); however, in many cases the significance of repellent volatiles and other mechanisms for excluding ants is still unknown. One frequently cited way in which ants can damage flowers is the negative effect many species have on pollen viability, yet no studies have investigated if this also occurs in other pollinators. To my knowledge, this investigation is the first to directly compare the effects of ants on pollen viability with other hymenopterans.

I found that although pollen viability does decrease after contact with *F. aquilonia* ants, a similar decrease was also observed after contact with two common bee pollinators, suggesting that adverse effects on pollen may be common to many hymenopterans. However, the observed decrease in viability was only marginally significant and thus any effects of this in the field are likely to be minor.

If pollen viability reductions are not unique to ants, with effects of similar magnitude produced by other common pollinators, then this casts doubt on the pollen inviability theory of why ant pollination is rare.

Negative effects of ants on pollen can be of significant impact in ant-plant interactions (Galen & Butchart 2003) but without comparisons with other pollinators, the general significance of this phenomenon as a potential force for structuring ant-plant interactions remains unknown. A comprehensive survey of a range of pollinator and ant species with pollen from several plant species all from the same ecosystem, ideally using ants with several different lifestyles, is still badly needed and would help to resolve the importance of ant reductions in pollen viability.

Even if ant effects on pollen viability are generally not as important as assumed, ant visits to flowers can be detrimental for many other reasons and there is still great potential for work investigating how the potential factors producing these detrimental effects and the mechanisms to avoid them interact. More generalised comparative studies taking these factors into account would allow further exploration of this phenomenon.

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