


A dual function for 4-methoxybenzaldehyde in *Petasites fragrans*? Pollinator-attractant and ant-repellent

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Abstract Ant-repellent floral volatiles offer one method through which plants can mediate the detrimental effects of ants on flowers. Although the repellence itself is well documented, the volatiles involved are less well explored. Here, we investigated the floral bouquet of ant-repellent male flowers of *Petasites fragrans*, identifying 4-methoxybenzaldehyde as the main component. 4-methoxybenzaldehyde significantly repelled ants when presented in isolation in an olfactometer and thus is the likely source of the repellent effect. As 4-methoxybenzaldehyde has previously been shown to attract pollinators, it may therefore have a dual function in *P. fragrans*, pollinator-attractant and ant-repellent. Additionally, 4-methoxybenzaldehyde is particularly interesting as an ant-repellent as it has been observed in the bouquets of other plant species with specific ant interactions.

Keywords *Petasites fragrans* · Ant-repellent floral volatiles · 4-methoxybenzaldehyde · p-anisaldehyde

Introduction

Ant-repellent floral volatiles are a well-documented phenomenon, with examples from many different ecosystems (Junker and Blüthgen 2008; Willmer et al. 2009; Junker et al. 2011a). Their presence has typically been linked to the detrimental effects of ants on flowers. Ants are generally poor pollinators, but frequently visit flowers for nectar where, in addition to nectar theft, they can deter legitimate pollinators with their aggressive behaviour, damage pollen with antimicrobial chemicals present on their integument, and cause physical damage to floral parts (see Willmer et al. 2009 for a review).

The prevalence of ant-repellent volatiles has been used as an interesting context for exploring the general functions and evolution of floral scent (e.g. Junker and Blüthgen 2010). Although individual volatiles can be targeted towards specific flower visitors, an alternative, broader explanation of their function is that they act as generalised filters, with some components restricting access to potentially detrimental visitors whilst others act as attractants (Raguso 2008; Junker and Blüthgen 2010). To simultaneously attract beneficial visitors and deter antagonists, plants may deploy a complex blend of chemicals, containing repellent and attractant volatiles, both of which can act to increase plant fitness (Kessler et al. 2008; Kessler et al. 2013). Deterrents may also act against pollinators; however, this is not necessarily detrimental to the plant as it can improve pollinator movement between flowers, increasing visitation rate (Kessler and Baldwin 2007).

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Investigations into the structures of repellent volatiles have failed to reveal any clear patterns in their function (Junker and Blüthgen 2010; Junker et al. 2011a). Junker and Blüthgen (2010) suggested that terpenoids may be generally repellent and benzenoids generally attractive; however, they note that exceptions to this are known (and see Junker et al. 2011b). The situation is made more complex as individual chemicals can act as both repellent and attractant to different species, and chemicals that have no effect in isolation may be repellent when presented in a blend (Junker and Blüthgen 2008). Furthermore, the response of an insect to volatiles can vary depending on the chemical background to the volatile concerned (Reinhard et al. 2010).

Here, we analysed the bouquet of male flowers of *Petasites fragrans* (Winter heliotrope). *P. fragrans* was previously found to elicit particularly strong repellence-related behaviours in *Formica aquilonia* ants (Willmer et al. 2009) (although there misnamed as *P. hybridus*). The single identified volatile was subsequently tested for ant-repellence using a two-way olfactometer.

Methods and materials

Study species

Petasites fragrans (Vill.) C.Presl, (Asteraceae), is a dioecious, herbaceous perennial, introduced in Britain, although naturalised and distributed over much of the country (Toman 1983). Flowering occurs in late winter/early spring and the male flowers have a distinctive vanilla–marzipan-like scent.

Volatile collection and analysis

Floral volatiles were collected in the field from four male *P. fragrans* plants from static headspace enclosures of mature inflorescences of 9–14 open flower heads/capitula. Enclosures were made from a copper frame supporting a plastic PET oven bag (Stewart-Jones and Poppy 2006). The frames were cleaned with a polar (methanol or acetone) and non-polar (isohexane) solvent and dried thoroughly before use. Volatiles were sampled with SPME fibers with 85 μm carboxen and polydimethylsiloxane (CAR/PDMS) coatings (Supelco UK), positioned within 20 mm of a dehiscing capitulum. Samples were taken on 18/03/2010 (1 inflorescence) and between 11–15/02/2012 (3 inflorescences) from two nearby sites on the banks of the Kiness Burn (a stream) in St Andrews, Fife, UK, OS Grid reference: NO 507 163. Each headspace had c. 30-min equilibration time. For the first inflorescence, sampling time was c. 25 min. Only one volatile was detected, so to increase

the likelihood of detecting additional volatiles, the three subsequent inflorescences were each sampled for 50 min. An empty control headspace, located within 2 m of the floral headspace, was sampled concurrently with each floral sample. Air temperature at sampling was 6–13 °C.

Fibers were desorbed and volatiles analysed using a ThermoFisher (UK) DSQII quadrupole mass spectrometer with a Trace gas chromatograph, and a CombiPal autosampler (CTC Analytics, Switzerland), as described in McMenemy et al. (2012). Data were acquired and analysed using the Xcalibur™ software package, version 2.0.7. Identification of plant volatiles was confirmed by running standards. Relative quantities of volatiles in floral samples were estimated by integrating chromatogram peaks and calculating the sample:background ratio.

Informal field observations were made of the floral visitors, size of floral display, and presence of nectar. Nectar volume was measured using 1 μl microcapillaries.

Olfactometer testing

The single prominent identified volatile, 4-methoxybenzaldehyde (syn. p-anisaldehyde), was tested for ant-repellence with a two-way olfactometer. This consisted of a 230-mm-length, 19-mm-diameter, hollow acrylic cylinder with PTFE-piping air inlets connected via channelled silicone stoppers to either end of the cylinder, and a similarly channelled stopper for the air outlet at the centre. Each inlet was connected to custom-made acrylic incubation chambers of volume c. 80 cm^3 . Airflow to each chamber was adjusted to 100 $\text{cm}^3 \text{min}^{-1}$ pre-testing using two flowmeters (Influx 5–100 $\text{cm}^3 \text{min}^{-1}$, Caché Instrumentation Ltd). By introducing a volatile into one incubation chamber, a control and treatment airspace could be created in either side of the olfactometer.

As volatile emission rate data for *P. fragrans* were not collected, we used a similar dilution of floral volatiles to the olfactometer trials of Junker and Blüthgen (2008). Hence, 8 μl of 4-methoxybenzaldehyde (Sigma A88107) was emulsified in 3856 μl of low-volatile liquid paraffin oil (Sigma 18512), 25 μl of which was pipetted onto a glass slide in one incubation chamber. For the control chamber, the slide contained 25 μl of paraffin oil only.

Formica aquilonia ants came from Abernethy Forest, Inverness-shire. Around 200 workers were collected from the surface of a nest. They were maintained in a glass formicarium (L \times W \times H) (200 \times 450 \times 200 mm) and fed with honey and dead insects. Although workers came from only one colony, *F. aquilonia* are polygynous and so genetic relatedness between workers within a nest is very low (Pamilo et al. 2005).

For each replicate ($n = 16$), an ant was introduced into the centre of the olfactometer. With the airflow on, the location

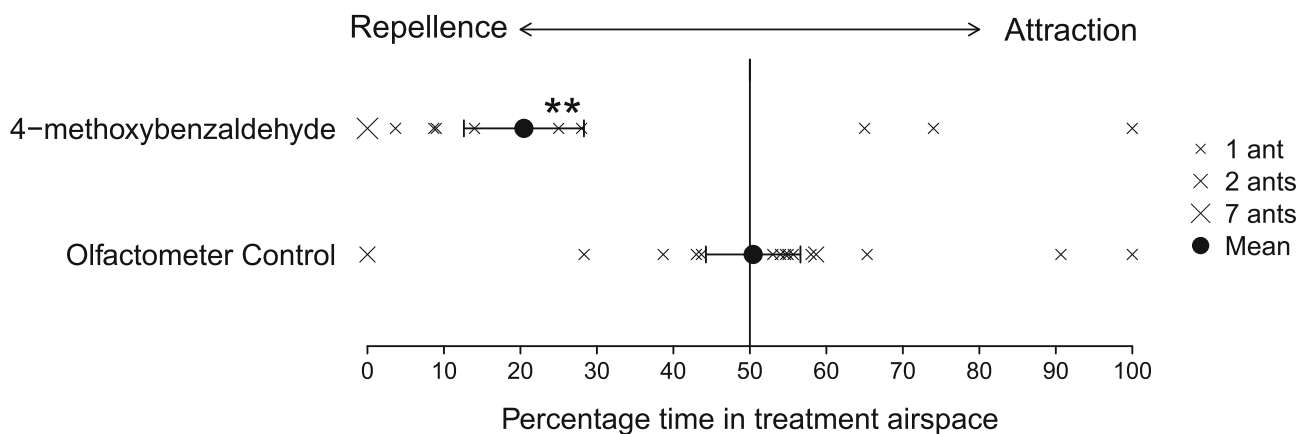


Fig. 1 Mean (\pm SE) percentage time spent by *F. aquilonia* in treatment airspace of the olfactometer for 4-methoxybenzaldehyde ($n = 16$) and the equipment control ($n = 17$). Percentage times for the individual ants are represented by crosses. Significant departure from 50% (equal

time in both sides) indicated by $**p < 0.01$. Ants significantly avoided 4-methoxybenzaldehyde, (randomisation test, $p = 0.004$, percentage time in treatment airspace (\pm SE) = $20.5\% \pm 7.7$)

of the ant (treatment/control airspace) was recorded for 5 min using a webcam. The olfactometer side receiving the test volatiles was changed randomly between replicates, with the constraint that each side received the treatment airflow an equal number of replicates. Pre-testing, the olfactometer design was tested for side bias by running an equipment control trial of 17 ants with no test volatiles in the incubation chambers. The incubation chambers and all equipment downflow of the incubation chambers were washed using water and dried between replicates.

Statistical analyses

Departure from equal proportions of time spent in the treatment and control airspaces was tested with a two-sided randomisation test (Manly 2006) using R version 3.1.1 (R Core Team 2014). As the data had relatively high ceiling and floor tendencies, and were constrained to sum to 300 s, standard parametric tests were not appropriate and a randomisation test gave more power than comparative non-parametric tests (Manly 2006). A T^2 test statistic was calculated from the mean proportion of time in treatment airspace. A null distribution of 100,000 T^2 values was calculated using the observed data but re-randomising the side assignments and a p value obtained by calculating the proportion of T^2 values from this distribution greater than the observed test statistic.

Results

One prominent volatile, 4-methoxybenzaldehyde, was detected in all four floral samples, from four separate plants, with samples from two separate years. Chromatogram peak areas for 4-methoxybenzaldehyde were c.

200–1800 \times greater in floral samples than in corresponding background samples.

We observed honey bees, *Apis mellifera*, at least one species of Bumblebee, *Bombus* sp., and *Sepsis* sp. flies visiting inflorescences. Nectar (> 50 nL) was present in the majority of disc florets sampled. The mean number of capitula per inflorescence was 16.8 (SD = 5.7) and number of disc florets per capitulum 39.4 (SD = 7.3), giving a mean of 660 disc florets per inflorescence.

In olfactometer trials, ants avoided 4-methoxybenzaldehyde (Fig. 1). The mean (\pm SE) percentage time ants spent in the treatment airspace was $20.5\% \pm 7.7$, significantly less than 50% (Randomisation Test, $p = 0.004$, $n = 16$). For the equipment control, there was no evidence of any side bias, with the mean (\pm SE) percentage time spent in the ‘treatment’ airspace $50.4\% \pm 6.2$ (Randomisation Test, $p = 0.941$, $n = 17$).

Discussion

The floral volatiles of male *Petasites fragrans* plants have previously been shown to elicit repellence-related behaviour in *Formica aquilonia* ants (Willmer et al. 2009). Here, we detected one prominent volatile, 4-methoxybenzaldehyde (hereafter 4-MoB), in the floral bouquet of *P. fragrans*. In olfactometer trials, *F. aquilonia* significantly avoided 4-MoB suggesting it is responsible for the repellent effect. *P. fragrans* is winter-flowering (here sampled in February and March), and may represent a significant nectar source at this time of year; thus, repelling nectar thieves could be of substantial benefit to the plant. 4-MoB has previously been found to be strongly attractive to pollinators (Theis 2006). As we observed pollinators visiting *P. fragrans*, and 4-MoB was the only floral volatile

detected, we suggest that 4-MoB may have a dual function in *P. fragrans*, as pollinator-attractant and ant-repellent.

Some caution is needed here, as although *P. fragrans* is common in the UK, its interactions with ants in its natural range of N. Africa (Toman 1983) are unknown. In particular, it is not clear whether *F. aquilonia* interacts with *P. fragrans* in the wild. Lab colonies may show behavioural differences from those in the wild, so a repellent effect in the lab may not be indicative of one in the field. To further investigate the ecological significance of the ant-repellence, it would be useful to test the effect of the volatiles on additional ant species, either from the natural range of *P. fragrans* in N. Africa, or a British ant species with a wider distribution than *F. aquilonia*. Although 4-MoB is attractive to pollinators (Theis 2006), to prove that 4-MoB functions as a pollinator-attractant in *P. fragrans* would require pollinator assays with *P. fragrans* inflorescences, decoupling the visual cues from the floral scent.

It is possible that the ants left chemical trails in the olfactometer which were not removed by the cleaning process, influencing the subsequent ants tested. However, given that the ants were repelled even though the side of the olfactometer receiving the treatment airflow was randomised between replicates, this is good evidence that the repellent effect is an actual one.

P. fragrans is dioecious, and here we used only male plants as females are not present in the UK (Toman 1983). The flowers of female plants are described as lacking the distinctive odour of the males (Toman 1983). Given that one possible function of ant-repellent floral volatiles is to prevent ants contacting and damaging pollen (Willmer et al. 2009), this raises interesting questions about the specific role and possible source of 4-MoB in *P. fragrans*.

In this study, *P. fragrans* inflorescences were sampled in the field. This allowed volatiles to be collected from flowers in their natural condition. However, environmental factors can affect the efficiency of volatile collection, and here we only detected a single volatile, perhaps indicating a low volatile yield. To investigate if there are other components to the floral bouquet of *P. fragrans*, a complementary technique such as dynamic headspace analysis carried out under more controlled lab conditions could provide greater resolution. This technique is also more suited to quantification of volatile emission (Tholl et al. 2006) and would allow determination of volatile emission rates to be used for subsequent olfactometer trials. Additional volatiles may also have been detected using SMPE fibers with different coating compositions (Bicchi et al. 2000); however, it is highly likely that 4-MoB is the major component to the bouquet of *P. fragrans*. As a benzenoid that is both attractive and repellent, 4-MoB appears to be another exception to the general functional dichotomy of

terpenoids as attractants and benzenoids as repellents, as suggested by Junker and Blüthgen (2010).

Intriguingly, 4-MoB has also been identified from floral volatiles of other angiosperms with specific ant interactions, notably *Cirsium arvense* (Theis 2006) and *Acacia seyal fistula* (Willmer et al. 2009). Ant-repellent flowers of the ant-acacia *A. seyal fistula* emit 4-MoB, and although E,E- α -farnesene has been proposed as the main repellent for this species (Willmer et al. 2009), it is possible that 4-MoB contributes to the effect.

More widely, 4-MoB is known from floral bouquets of several angiosperm families (Knudsen et al. 2006), and an ant-repellent function might be present in these other species and apply more generally to other detrimental flower visitors. Floral volatiles may have evolved primarily for a defensive function which potentially beneficial visitors have since overcome and used as a cue for reward (Pellmyr and Thien 1986; Knudsen et al. 2006). The identification of single volatiles that are attractive to a few pollinators but repellent to a wide range of potentially detrimental facultative floral visitors would support this hypothesis.

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