



Cite this article: Patricelli D *et al.* 2015 Plant defences against ants provide a pathway to social parasitism in butterflies. *Proc. R. Soc. B* **282**: 20151111.

<http://dx.doi.org/10.1098/rspb.2015.1111>

Received: 15 May 2015

Accepted: 10 June 2015

Subject Areas:

ecology, behaviour, physiology

Keywords:

gene expression, plant volatiles, myrmecophily, host detection, ant, *Maculinea*

Author for correspondence:

Jeremy A. Thomas

e-mail: jeremy.thomas@zoo.ox.ac.uk

[†]These authors contributed equally to this work.

Electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2015.1111> or via <http://rsob.royalsocietypublishing.org>.

Plant defences against ants provide a pathway to social parasitism in butterflies

Dario Patricelli^{1,†}, Francesca Barbero^{1,†}, Andrea Occhipinti², Cinzia M. Berteà², Simona Bonelli¹, Luca P. Casacci¹, Simon A. Zebelo², Christoph Crocoll³, Jonathan Gershenson⁴, Massimo E. Maffei², Jeremy A. Thomas⁵ and Emilio Balletto¹

¹Zoology Unit, Department of Life Sciences and Systems Biology, University of Turin, Via Accademia Albertina 13, Turin 10123, Italy

²Plant Physiology Unit, Department Life Sciences and Systems Biology, University of Turin, Innovation Centre, Via Quarello 15/A, Turin 10135, Italy

³Department of Plant and Environmental Sciences, Faculty of Sciences, University of Copenhagen, Thorvaldsensvej 40, Frederiksberg C 1871, Denmark

⁴Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knoell-Strasse 8, Jena 07745, Germany

⁵Department of Zoology, University of Oxford, The Tinbergen Building, South Parks Road, Oxford OX1 3PS, UK

Understanding the chemical cues and gene expressions that mediate herbivore–host–plant and parasite–host interactions can elucidate the ecological costs and benefits accruing to different partners in tight-knit community modules, and may reveal unexpected complexities. We investigated the exploitation of sequential hosts by the phytophagous–predaceous butterfly *Maculinea arion*, whose larvae initially feed on *Origanum vulgare* flowerheads before switching to parasitize *Myrmica* ant colonies for their main period of growth. Gravid female butterflies were attracted to *Origanum* plants that emitted high levels of the monoterpenoid volatile carvacrol, a condition that occurred when ants disturbed their roots: we also found that *Origanum* expressed four genes involved in monoterpene formation when ants were present, accompanied by a significant induction of jasmonates. When exposed to carvacrol, *Myrmica* workers upregulated five genes whose products bind and detoxify this biocide, and their colonies were more tolerant of it than other common ant genera, consistent with an observed ability to occupy the competitor-free spaces surrounding *Origanum*. A cost is potential colony destruction by *Ma. arion*, which in turn may benefit infested *Origanum* plants by relieving their roots of further damage. Our results suggest a new pathway, whereby social parasites can detect successive resources by employing plant volatiles to simultaneously select their initial plant food and a suitable sequential host.

1. Introduction

A critical choice for many animals that eschew parental care is where and when to deposit their offspring. In most butterflies, this is seen in the females' use of visual, chemical [1] and microclimatic [2] cues to oviposit on small subsets of their host-plant(s) which possess attributes that optimize brood fitness [2,3]. For certain of the approximately 100 000 insect species [4,5], including perhaps a quarter of all butterflies, whose immature stages interact with ants (myrmecophiles), selection may also favour appropriate plants that harbour certain mutualistic ant species [6]. For the approximately 10% of myrmecophiles that infiltrate and exploit ant colonies as social parasites, egg-laying near colonies of their host-ant taxon is in general obligatory [5,7].

Many insect social parasites have evolved from phytophagous ancestors [6,8,9] and are constrained, during early larval development, by an obligate need to feed on specific plant tissues before infiltrating ant colonies for an extended final instar during which most of their ultimate biomass is acquired

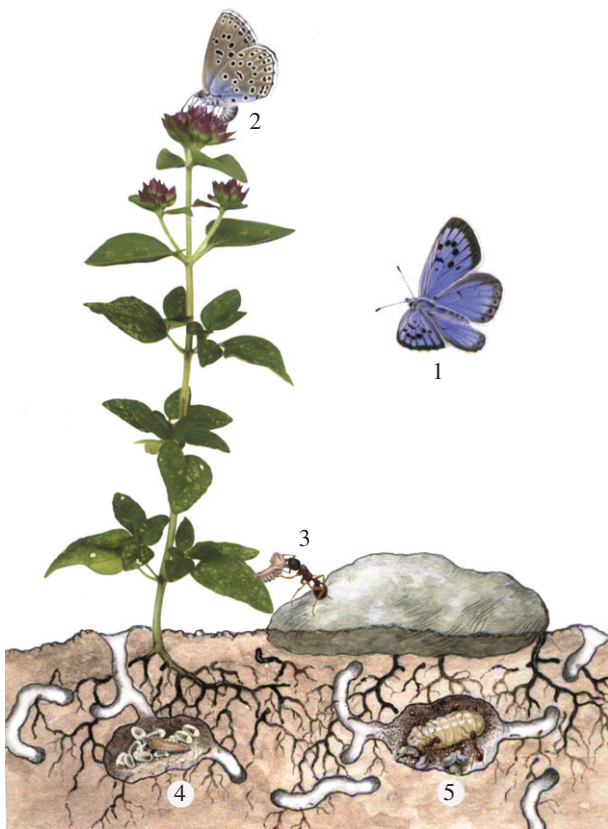


Figure 1. Life cycle of *Ma. arion* and its interaction with *Myrmica* ants and *O. vulgare*, indicated by this and previous [5,11–14] studies. (1) Female butterfly is attracted to flowers then, when close, by volatiles promoting oviposition (2) on flowerbuds of *O. vulgare* plants that co-occur with a nest of any *Myrmica* species; (3) final-instar larvae abandon *O. vulgare* and are adopted by *Myrmica* ants, in whose nests they live for 11 months, feeding on ant brood (4, 5) and acquiring more than 98% of their final biomass (5). Larval survival in the study race of *Ma. arion* is highest with *Myrmica scabrinodis* and *Myrmica sabuleti* and zero with ants of other genera [15].

[8,10–12]; figure 1). With average realized fecundities seldom exceeding 50 eggs laid per wild female [5,11], this lifestyle poses the logistical challenge of locating sufficient proximate hosts for a population to persist. That constraint is shared by many true parasites that sequentially exploit more than one unrelated host species, but most overcome it by producing thousands of propagules, by switching host between generations rather than within the same one, and often by having a primary host that interacts trophically with a secondary one (the vector) [16,17].

What guides oviposition in insect social parasites with sequential hosts is unknown, despite a longstanding controversy exemplified by whether female *Maculinea* (large blue butterfly) species can detect initial host-plants growing close to colonies of their specific host species of *Myrmica* ant, and if so, whether they respond directly to ant cues or indirectly to growth forms of their foodplants that inhabit the same niche as that (or those) species of ant [13,18–21]. An obstacle to using *Myrmica* odours as a cue is that the known hosts in this ant genus seldom forage at times when the butterfly oviposits (10–16 h), the workers lay ephemeral trails and rarely climb plants, and the female butterfly exhibits no behaviour to detect ant nests [8,13] (by contrast, certain lycaenids that coexist with other ant genera that possess other attributes have indeed evolved the ability to detect their obligate ant mutualist or host prior to oviposition [6,8,22,23]). Nevertheless, we recently established

that *Maculinea arion* females oviposit disproportionately often on *Origanum vulgare* plants situated near to a colony of *Myrmica* (of any species), and avoid other ant genera which their larvae cannot exploit [15]. It is well known that many plants synthesize deterrent volatile organic compounds (VOCs) in response to tissue damage by insects [24,25]. Here, we show that this social parasite can detect, and is attracted by, individual foodplants whose chemical profile has been modified by subterranean, non-trophic interactions with *Myrmica* ant nests.

2. Material and methods

(a) Establishing terraria

Myrmica sabuleti and *Myrmica scabrinodis* colonies (primary host of *Ma. arion*, respectively, in west Europe and Italy) and *O. vulgare* seeds were collected in the Parco Fluviale Gesso e Stura, North Italy, where a previous study of *Ma. arion* oviposition behaviour had been made [15]. Twelve terraria (electronic supplementary material, figure S1) were established, each containing four *O. vulgare* plants grown from seedlings maintained at 26°C, 60% humidity and 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light under a 16 L:8 D regime. Six also contained one *Myrmica* colony each, while six controls did not. *Myrmica* ants were fed on a standard diet of sugar and dead *Spodoptera littoralis* larvae [26].

(b) Collection and analysis of plant volatiles

Origanum vulgare VOC emissions were assayed by stir-bar sorptive extraction (SBSE) [27] using bars placed in each terrarium. SBSEs were conditioned for 1 h at 250°C in a helium flow, then placed above a jar covering phenologically similar *O. vulgare* plants for 48 h ($n = 41$). After absorption, each stir bar was rinsed with double distilled water (Millipore, USA), dried and stored into a sterile vial at -20°C before analysis within 2 days by extraction using the Gerstel TDU Desorption Unit System and gas chromatography coupled to mass spectrometry (GC-MS). Quantification was performed by external calibration curve. Initially, separate assays ($n = 20$) were made of the impact of *My. sabuleti* and *My. scabrinodis* but as no difference was detected in their induced emissions, their data were pooled in later analyses. Further details of the collection and analysis of *O. vulgare* emissions are given in the electronic supplementary material.

(c) Bioassays of responses of gravid female *Maculinea arion* to volatile organic compounds

Choice tests were performed on 10 gravid female butterflies per test in a 15 cm diameter Y-tube olfactometer housed in a blackened box with constant diffused fluorescent light shining directly above its centre. For 10 min, each female was offered a choice between two arms, each connected to a 4 l glass desiccator receiving a GC-grade constant air flow of 200 ml min^{-1} . Stimuli were presented simultaneously in each trial and were alternated in the two arms between tests. A positive choice was registered only when a female entered and remained 1 min in one arm, rather than stayed in the initial tube or flew between both arms. Between tests, all glassware was carefully washed and air-dried at 120°C for 4 h. Flasks contained control or treated *O. vulgare* plants, while in other experiments synthetic thymol and carvacrol were applied to assess whether females could discriminate between these isomers. To test whether *Ma. arion* might use host-ant nest volatiles as an oviposition cue [18,20], we also used *My. scabrinodis* nest soil collected at our experimental site. As a control, we used the same volume of the same soil that had been sterilized by autoclaving to remove volatiles. In both cases, ants were removed from the soil before testing.

(d) Electroantennogram measurements of gravid female *Maculinea arion*

Antennae of *Ma. arion* were excised at the base of the flagellum. The tip of the terminal segment was cut using micro-scissors, and the antennae ($n = 8$) were connected to an electrophysiology apparatus. The excised tip was placed on the antennal holder (two 1.7 mm diameter wells containing 0.1 M KCl); the recording electrode was connected to the base of each antenna with the reference electrode connected to its tip. Baseline responses of antennae were established from exposure to clean GC-grade air, and compared with responses when fluxing ant allomones, plant volatiles and synthetic pure compounds or mixtures. VOCs were fluxed towards the mounted antenna on the electroantennogram (EAG) apparatus with clean air as carrier gas. Synthetic carvacrol, thymol and Z-3-hexen-1-ol were obtained from Sigma-Aldrich (Milan, Italy) (10 μg per desiccator). VOCs from treated and untreated *O. vulgare* from 41 glass jars (see above), synthetic mixtures and extracts from solitary ant nests were flowed. Electrical signals were pre-amplified and their intensity measured using a 0.052 V step input; the amplified output was 6.49 V. Responses were expressed as mean differences to the standard (Z-3-hexen-1-ol), Dimethyl sulfoxide (Sigma-Aldrich, USA) and clean air after exposing the antenna alternately, every 30 s, to a test chemical or to clean air.

(e) Ant reactions to carvacrol

To assess whether significant differences of resistance rate occur upon acute exposure to carvacrol in the commonest ant species of the study site, two colonies each of five ant genera (*My. scabrinodis*, *Tetramorium caespitum*, *Lasius alienus*, *Tapinoma erraticum* and *Formica cinerea*) were kept for one week on standardized diets [26] before workers were exposed in sealed 6 cm Petri plates (representing a nest chamber) to an atmosphere containing 20 ppm carvacrol: survival was assessed as minutes before death. Parallel controls were tested using clean air but no ant died in the absence of carvacrol. At the end of the experiment, ants were frozen in liquid N₂ and kept at -80°C for gene expression analyses.

(f) Isolation of total RNA and gene expression analysis

(i) *Origanum vulgare* genes involved in terpene metabolism

To evaluate the effect of the presence of ants on terpenoid metabolism in *O. vulgare*, we analysed the expression level of terpene synthases and cytochrome P450s previously identified as being involved in the production of the main VOC components, carvacrol and thymol, in this plant species [28,29]. Full details about total RNA extraction, cDNA synthesis and gene expression analysis are given in the electronic supplementary material.

(ii) Ant genes involved in response to carvacrol

To understand the possible mechanism underlying the response to carvacrol exposure in different ant species, we analysed the expression level of known genes related to odour perception and xenobiotic detoxification in insects. Full details of the rationale used to choose candidate genes are given in the electronic supplementary material together with the methodologies used for total RNA extraction, cDNA synthesis and gene expression analysis.

(g) Competitive advantage of *Myrmica* ant species to inhabit ground near *Origanum vulgare* plants

Two hundred and seventy pitfall traps were placed on our main study site, 190 within 10 cm of an *O. vulgare* plant [15] and 80 beneath vegetation of similar composition, sward and soil structure but situated more than 2 m from any *O. vulgare* plant.

(h) Damage caused to *Origanum vulgare* by *Maculinea arion* larvae

The number of individual florets per flowerhead eaten by 13 *Ma. arion* larvae between hatching and the final moult before abandoning the foodplant was counted daily in the wild, together with the number of florets available per flowering plant spike, the number of flowering spikes per plant and the mean density of larvae surviving to final instar on a UK site that supported high densities of *Ma. arion*.

(i) Statistics

Kolmogorov–Smirnov tests were used to assess the data distribution type. Accordingly, parametric or non-parametric analysis of variance was used to assess differences in VOC amounts, although for consistency just one type of test is presented in each table. Choice data were analysed using χ^2 tests. Kruskal–Wallis analysis was used to test for differences in mean ant survival times; pairwise differences between species were also calculated using Bonferroni correction, and Benjamini–Hochberg procedure was used to control for false discovery rate (FDR) in multiple tests. For genomic (log-transformed data) and chemical analyses, the overall datasets were expressed as mean values of at least three biological replicates, each one repeated (technical replicates). Significance of differences observed in datasets was tested by ANOVA and pairwise comparisons were assessed with Bonferroni post hoc. All statistical analyses were performed using SPSS (v. 18.0, Chicago). Other tests are indicated in the text.

3. Results

(a) Response of *Origanum* plants to root disturbance by *Myrmica* ants

Origanum vulgare grown in the presence of *My. sabuleti* or *My. scabrinodis* had the same headspace concentrations of 20 major VOCs, including carvacrol (2-methyl-5-(1-methylethyl)-phenol) and thymol (electronic supplementary material, table S1; $p = 0.990$ and 0.583 , respectively): we therefore combined data from these two congeners in subsequent analyses. Compared with the controls, *O. vulgare* grown in the presence of a *Myrmica* species had a headspace concentration of carvacrol that was nearly twice that of plants grown without ants nesting around and disturbing their roots ($p = 0.00032$, table 1). This aromatic oxygenated monoterpene is well known for its antifungal and insecticidal properties [30,31]. By contrast, the headspace concentration of the 19 other major volatiles did not change apart from that of the carvacrol precursor, γ -terpinene (table 1). A similar pattern was obtained for leaf extracts from wild plants growing in the field, except that carvacrol's isomer thymol was also significantly elevated in *O. vulgare* growing near *Myrmica* colonies ($p < 0.0001$; electronic supplementary material, table S2).

We next measured the levels of certain plant hormones and genes associated with monoterpene biosynthesis. In leaves of *O. vulgare* plants interacting with *Myrmica* colonies (figure 2a), there was a significant increase in the defence hormone jasmonic acid (JA) (ANOVA, $F_{1,5} = 62.32$, $p = 0.001$), its conjugate (3*R*,7*S*)-jasmonoyl-L-isoleucine (JA-Ile) (ANOVA, $F_{1,5} = 141.625$, $p < 0.001$) and the JA precursor, 12-oxo phytodienoic acid (OPDA) (ANOVA, $F_{1,5} = 244.905$, $p < 0.001$) [32]. There was also a 10-fold induction in the presence of *Myrmica* (figure 2b) for a monoterpene synthase gene (*OvTPS2*) (ANOVA, $F_{1,5} = 273.055$, $p = 0.0008$) that encodes a

Table 1. The abundance of major compounds found in the headspace of *O. vulgare* grown in terraria with or without a *Myrmica* ant colony. (Mean values between treatment ($n = 20$) and control plants ($n = 21$) were compared by Kruskal–Wallis tests (K). Data are expressed as $\mu\text{g g}^{-1}$ of fresh weight \pm s.e.m. after 48 h of sorption with SBSE (see Material and methods). Asterisks (*) denote significant p -values controlled for FDR by using the Benjamini–Hochberg procedure.)

compound	Kovats index	plants with <i>Myrmica</i>	control plants without <i>Myrmica</i>	K	p -value
<i>p</i> -cymene	1024	0.912 \pm 0.209	0.916 \pm 0.198	0.083	0.773
γ -terpinene	1055	0.924 \pm 0.185	0.804 \pm 0.171	22.286	0.000002*
<i>trans</i> -sabinene hydrate	1098	1.016 \pm 0.242	0.923 \pm 0.240	2.871	0.090
methylthymyl ether	1173	1.064 \pm 0.284	1.067 \pm 0.255	0.062	0.804
thymol	1290	1.290 \pm 0.311	1.911 \pm 0.439	1.088	0.297
carvacrol	1299	1.310 \pm 0.291	0.892 \pm 0.202	12.957	0.000319*
α -cubebene	1351	0.867 \pm 0.193	0.833 \pm 0.180	1.046	0.307
α -copaene	1377	1.040 \pm 0.219	0.986 \pm 0.195	0.497	0.481
β -ylangene	1421	4.499 \pm 0.854	4.309 \pm 0.644	0.011	0.917
γ -elemene	1437	1.327 \pm 0.280	1.287 \pm 0.261	0.098	0.754
α -bergamotene	1413	0.831 \pm 0.198	0.824 \pm 0.182	0.393	0.531
γ -muurolene	1480	1.960 \pm 0.370	1.665 \pm 0.261	1.035	0.309
bicyclosesquiphellandrene	1484	1.282 \pm 0.251	1.175 \pm 0.213	0.787	0.375
germacrene-D	1485	5.722 \pm 1.128	5.514 \pm 0.784	0.006	0.938
α -amorphene	1485	1.052 \pm 0.251	0.960 \pm 0.219	2.532	0.112
(<i>E</i>)- β -farnesene	1457	4.638 \pm 0.854	4.389 \pm 0.909	0.197	0.657
bicydogermacrene	1500	1.056 \pm 0.216	0.960 \pm 0.186	1.503	0.220
α -bisabolene	1507	0.827 \pm 0.211	0.832 \pm 0.199	0.340	0.560
δ -cadinene	1523	0.941 \pm 0.211	0.969 \pm 0.206	0.360	0.548
α -humulene epoxide	1608	1.786 \pm 0.380	1.208 \pm 0.264	2.451	0.117

protein catalysing the formation of γ -terpinene, a direct precursor for thymol and carvacrol [28,33], as well as large increases in transcript levels of three cytochrome P450 genes involved in carvacrol and thymol biosynthesis: *CYP71D180* (ANOVA, $F_{1,5} = 48.913$, $p = 0.002$), *CYP71D179/182* (ANOVA, $F_{1,5} = 3388.328$, $p < 0.00001$) and *CYP71D178* (ANOVA, $F_{1,5} = 6919.464$, $p < 0.00001$). By contrast, the expression levels of genes involved in biosynthesis of other volatile terpenes (electronic supplementary material, table S3) showed no significant differences between ant-treated and control plants.

These results suggested that *Myrmica* ant colonies may stimulate *O. vulgare* plants, leading to elevated levels of the defence hormones JA and JA-Ile, and subsequently to increases in monoterpene biosynthesis via upregulation of selected gene transcripts [32].

(b) Effect of carvacrol emissions on *Maculinea arion* behaviour

Having demonstrated that *O. vulgare* plants in the optimum phenological state for *Ma. arion* oviposition [13] synthesize and emit elevated levels of aromatic oxygenated monoterpenes when *Myrmica* ants are present, we tested whether carvacrol, or its isomer thymol, affect *Ma. arion* behaviour. In the bioassays performed with a Y-tube olfactometer (figure 3a), gravid female butterflies selected tubes containing carvacrol ($\chi^2 = 9.8$, $p = 0.007$) or thymol ($\chi^2 = 10.4$, $p = 0.005$) significantly more often than pure air, and responded more frequently to a blend of carvacrol and thymol than to pure thymol ($\chi^2 = 10.4$, $p = 0.005$). Furthermore, females selected

O. vulgare grown without a *Myrmica* colony to which synthetic carvacrol had been applied in preference to untreated *O. vulgare* plants ($\chi^2 = 7.4$, $p = 0.024$) and showed a strong preference for the odour from plants grown in terraria containing ants compared with controls ($\chi^2 = 6.2$, $p = 0.045$). By contrast, females displayed no attraction to the odours of soil taken from a host *Myrmica* nest, or for sterilized soil, but remained in the main portion of the Y-tube or flew randomly between the two branches.

Finally, in EAG experiments, isolated antennae of *Ma. arion* females generated distinct electrophysiological pulses when exposed to air containing thymol and, especially, carvacrol compared with clean air (figure 3b) (ANOVA, $F_{7,1110} = 445.829$, $p < 0.001$, Tukey test_{Carvacrol versus Thymol}, mean difference = 14.45, $p < 0.001$). Antennae also responded much more strongly (figure 3c) to the induced VOCs of *O. vulgare* than to volatiles extracted directly from a *Myrmica* colony (Tukey test_{*O. vulgare*-treated-with-ants versus *Myrmica*}, mean difference = 32.38, $p < 0.001$), although they did show a weak hyperpolarization when exposed to the latter (Tukey test_{*Myrmica* versus control *O. vulgare*}, mean difference = 13.70, $p < 0.001$).

(c) Impact of carvacrol synthesis on *Myrmica* and other genera of ants

We had previously observed that *Ma. arion* eggs are laid disproportionately often on *O. vulgare* plants growing near the nests of *Myrmica* species in the wild [15], yet an attraction to carvacrol is unlikely to benefit *Ma. arion* if damage from

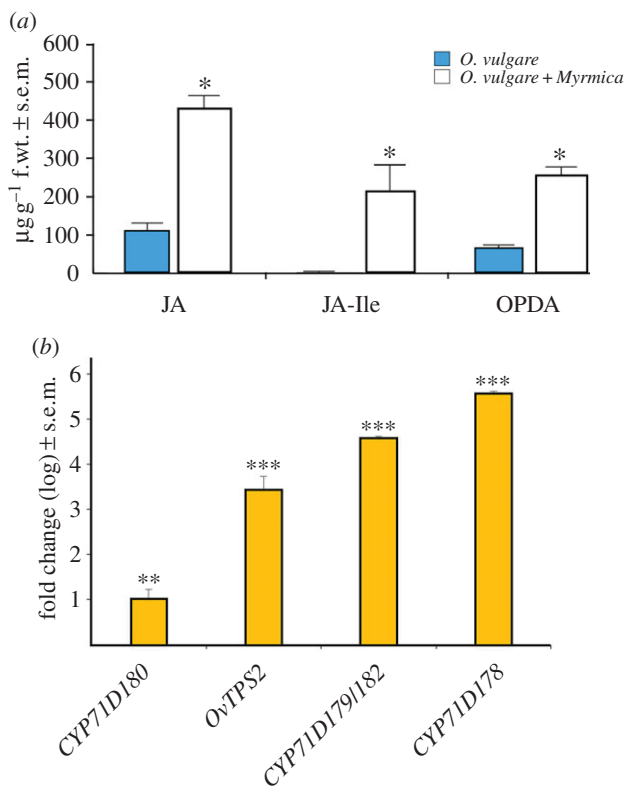


Figure 2. Effects of *Myrmica* ants on *O. vulgare* defence hormone production and gene induction. (a) *Myrmica* presence induces increases in the defence hormone JA, its conjugate JA-Ile and JA precursor OPDA; f.wt = fresh weight; * $p < 0.001$ between plants with and without *Myrmica*. (b) *Myrmica* presence causes a 10-fold induction for a monoterpene synthase gene (*OvTPS2*) that encodes a protein catalysing the formation of γ -terpinene, a direct precursor for thymol and carvacrol. Large increases also occurred in transcript levels of three cytochrome P450 genes involved in carvacrol and thymol biosynthesis: *CYP71D180*, *CYP71D179/182* and *CYP71D178*; ** $p < 0.01$, *** $p < 0.001$.

non-host-ant genera induces similar emissions. Knowing that carvacrol is a biocide for many insects [30,31], we tested its impact on the commonest ant genera on our *Ma. arion* sites by confining their workers in closed cells containing 20 ppm of this volatile. In this atmosphere, *My. scabrinodis* survived four to six times longer than workers from other genera (figure 4a). Moreover, gene expression of the *My. scabrinodis* odourant-binding protein *OBP6* (ANOVA, $F_{1,5} = 619.095$, $p < 0.0001$) and odourant receptor 1 (*OR1*) (ANOVA, $F_{1,5} = 12.659$, $p = 0.024$) showed a significant upregulation in workers exposed to carvacrol (figure 4b), suggesting *Myrmica*'s ability to react to the compound at the molecular level. Furthermore, three detoxification-related genes were significantly more upregulated in *My. scabrinodis* compared with other ant genera (figure 4c) upon exposure to carvacrol: acetylcholinesterase (*AChE*; ANOVA, $F_{4,14} = 43.04$, $p < 0.00001$), which is responsible for the hydrolysis of acetylcholine at synaptic regions of cholinergic nerve endings in the ant central nervous system [34]; glutathione *S* transferase (*GST*; ANOVA, $F_{4,14} = 51.55$, $p < 0.00001$), a primary defence against xenobiotics [35]; and a cytochrome P450 (*CYP4509E2*; ANOVA, $F_{4,14} = 29.42$, $p < 0.00001$), known to metabolize synthetic chemicals (insecticides/pesticides) and host-plant allelochemicals [36].

Although workers inhabiting natural nests move freely between nest chambers and above-ground where concentrations

may be lower, we tested ant responses to 20 ppm of carvacrol (=3.4 times higher than that emitted into *Origanum* headspace but one-third of the induced concentration within leaf tissues) to compare differences in species' tolerance to acute exposure to this biocide (figure 4a). Taken together, our data suggest that, while *O. vulgare* may produce carvacrol as an insect repellent, *My. scabrinodis* possesses a higher capability to detoxify this volatile than ant species from other genera, and an inducible ability to recognize and bind the molecule.

(d) Benefits and costs

For *Myrmica* species, the apparent benefit of their relative tolerance to carvacrol is an ability to inhabit the competitor-free space surrounding *O. vulgare* plants (table 2; *Myrmica* versus non-*Myrmica*, $\chi^2_1 = 52.0$, $p < 0.001$). A cost is parasitism in the few sites occupied by *Ma. arion* (an International Union for Conservation of Nature-listed rarity [11]), as infection by the butterfly typically destroys the host species' colony or causes it to desert, owing to the often total loss of brood to parasitic caterpillars that acquire more than 98% of their ultimate biomass from devouring ant larvae [14]. From the plant's perspective, the attraction of gravid *Ma. arion* females has a much smaller negative impact as an individual caterpillar eats approximately 17 florets before abandoning its foodplant, representing 2% of seed production of the individual plants supporting caterpillars on high-density *Ma. arion* sites (table 3).

4. Discussion

We have yet to determine how a colony of omnivorous *Myrmica* ants causes sufficient damage to trigger increased defence hormones and monoterpene biosynthesis in *O. vulgare*, but a likely cause is their cutting of roots and rootlets to excavate cells. Although the specialized domestic root-aphids nurtured by *Myrmica* workers [37] might amplify plant defences in the field, these aphids were excluded from our laboratory experiments. Elsewhere in Europe, *Ma. arion* oviposits on flowers of the genus *Thymus*, a close relative of *O. vulgare*. *Thymus* species also emit thymol and carvacrol when damaged [38], and *Ma. arion* presumably employs the same cues in its interaction with *O. vulgare* or *Thymus* plants and *Myrmica* ant species throughout its range.

These results help to resolve a controversy as to whether female *Maculinea* butterflies, prior to oviposition, could select initial foodplants that coexist with their specific host species of *Myrmica* ant by using ant semio-chemicals as a cue [18–21], or whether they simply choose distinctive growth forms of plant that inhabit a similar niche to that of their host ant [13]. We show that neither hypothesis is correct, at least in the case of *Ma. arion*, and that although the butterfly antenna can respond weakly (in comparison to *Origanum* VOCs) to *Myrmica* odours, these exert no attraction for gravid females (figure 3). We therefore suggest that, after initially fluttering around pink flowers, the butterfly responds to plant VOCs induced by the presence of nests of various species in the genus *Myrmica* living in close proximity to *Origanum* plants, before further biasing egg distribution to a second subset of *Origanum* plants whose growth-form occupies a similar niche to that of the host species of *Myrmica* [13].

We consider that the described interactions are adaptive for all members of the *Ma. arion* community module (figure 1). An ability, when ovipositing, indirectly to detect the presence of

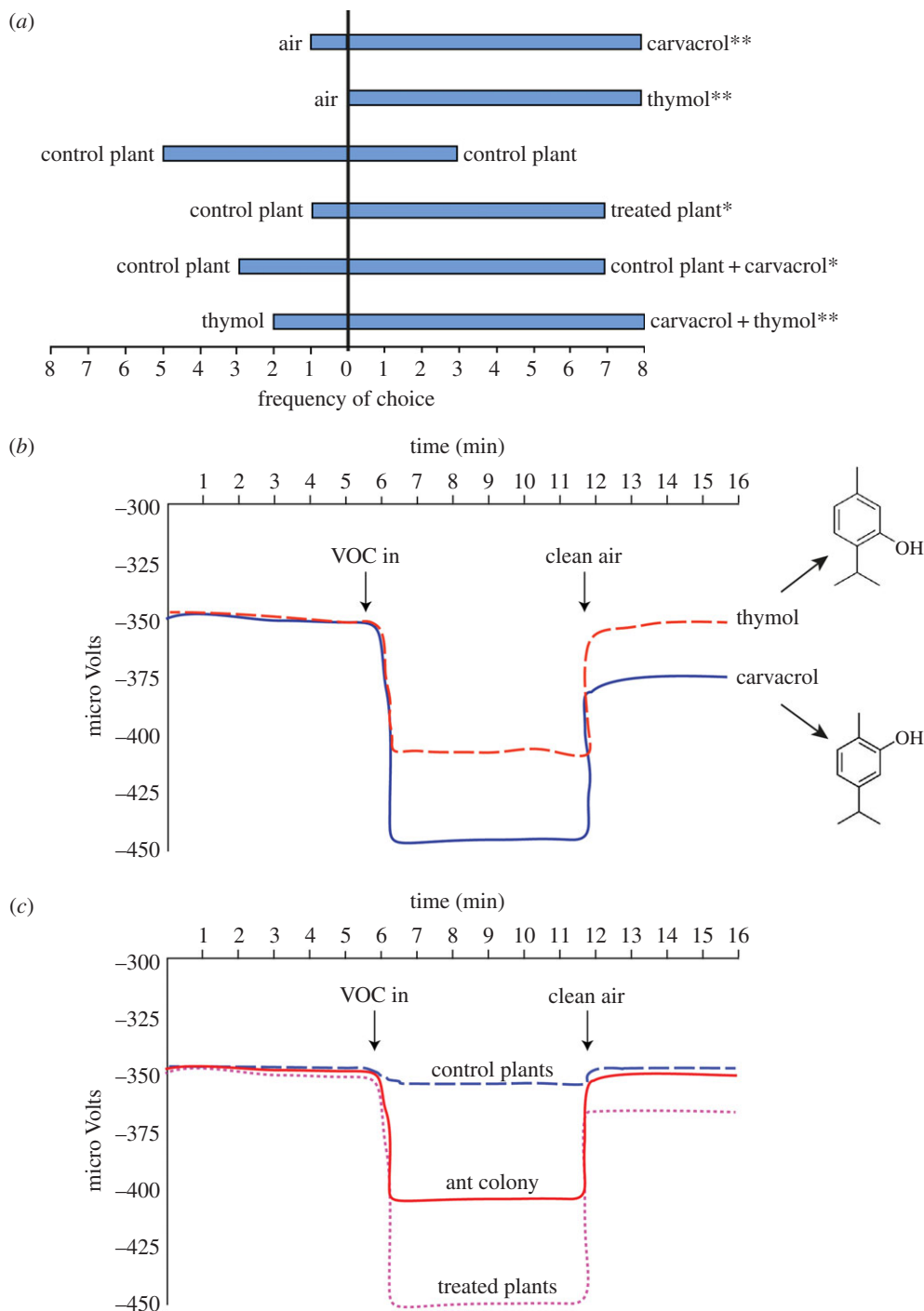


Figure 3. Responses of *Ma. arion* to ant-induced plant volatiles. (a) Olfactometry choice bioassays of gravid female *Ma. arion* show a strong preference for carvacrol and its isomer thymol over clean air, as well as for the odour of *O. vulgare* exposed to ants (=treated plant) or of controls dosed with carvacrol rather than that of isolated control plants. Females also chose a blend of carvacrol and thymol rather than thymol alone; * $p < 0.05$, ** $p < 0.01$. (b) EAG of *Ma. arion* antennal responses to the two main aromatic monoterpenes emitted by *O. vulgare*. (c) Antennal responses in EAGs to odours from plants grown with *Myrmica* among their roots (=treated) and to the odour of *My. scabrinodis* ant colonies.

the genus *Myrmica* benefits the butterfly because, on sites capable of supporting its populations, the large majority of *Myrmica* nests that coexist with the foodplant will belong to the primary host species [5,11]. Moreover, for the minority of eggs laid near a less appropriate congener species, there is a small (approx. 80% lower) chance of survival compared with none for individuals placed near other genera of ants [11,14]. For *Myrmica* ants, the highly localized cost of colony destruction by *Ma. arion* is presumably outweighed by an ability to dominate competitor-free spaces surrounding *Origanum*. While for the plant, the minor loss of seed to *Ma. arion* is

presumably outweighed by the subsequent removal of any *Myrmica* colony damaging its roots and the cost of continuing to synthesize carvacrol. However, other aspects of these interactions remain unquantified, for example the precision with which carvacrol synthesis signals the proximity of a *Myrmica* colony to *Origanum*, or whether carvacrol is emitted more generally in response to insect herbivores. On current evidence, the latter seems unlikely, as herbivory is not cited as a cause in other studies of carvacrol synthesis, nor did we detect it when studying *Origanum* responses to the leaf-eating herbivore *S. littoralis* (A. Occhipinti 2014, personal communication).

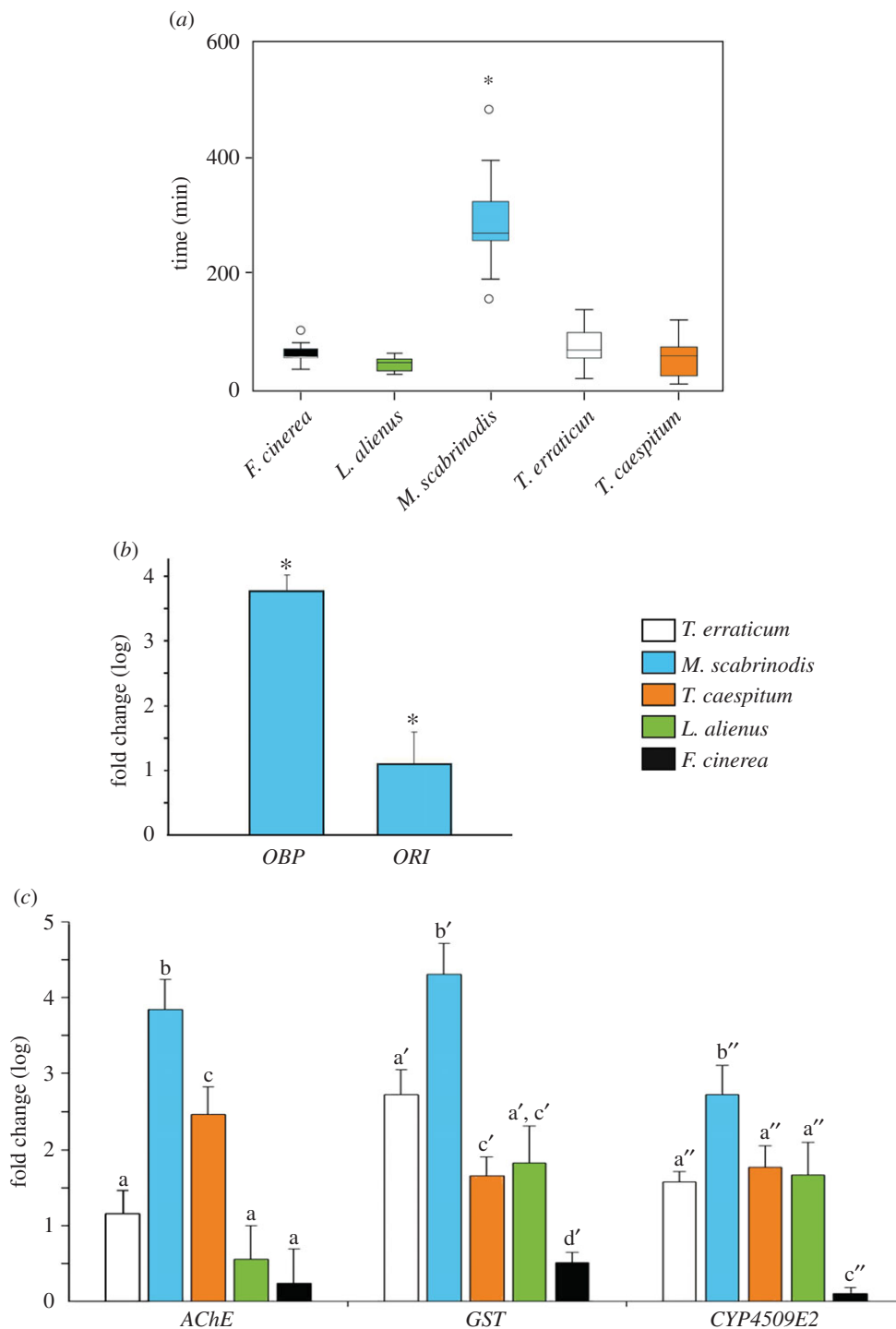


Figure 4. Tolerance of different ant genera to carvacrol. (a) Worker survival times when entrapped in cells containing 20 ppm pure carvacrol. Boxplots show median, quartile, maximum and minimum survival; outliers are open circles; Kruskal–Wallis test_{all ants} $H_4 = 37.71$, $n = 63$, d.f. = 4, $p < 0.001$; pairwise: *My. scabrinodis* \neq *L. alienus* ($U = 41.577$, $p < 0.001$), \neq *T. caespitum* ($U = 33.154$, $p < 0.001$), \neq *F. cinerea* ($U = 27.077$, $p = 0.002$), \neq *T. erraticum* ($U = 22.864$, $p = 0.023$). (b) Gene expression of odourant receptor 1 and odourant-binding protein (*OBP6*) in *My. scabrinodis* ants after exposure to carvacrol. (c) Expression of detoxification-related genes in the five ant genera tested after exposure to carvacrol: acetylcholinesterase (*AChE*), *GST* and a cytochrome P450 (*CYP4509E2*). Error bars indicate s.e.m. for each gene, different letters indicate significant ($p < 0.05$) differences.

In addition, we have no data on whether *Ma. arion*'s early larval instars are exposed to carvacrol within *Origanum* flowerheads, and if so whether—like *Myrmica* (figure 4)—they can detect, bind and detoxify it.

More broadly, these results suggest that *Ma. arion* resembles many true endo-parasites in specializing on sequential hosts that interact closely with each other. It differs, however, in exploiting a non-trophic interaction that apparently emits so precise a signal that a typical butterfly, with an average

realized fecundity of just 50 eggs per female, can nevertheless place sufficient eggs within the approximately 6 m² territory of a suitable *Myrmica* colony for her offspring to experience a positive rate of intrinsic population growth [11]. We suspect that it may have been the ability to detect this, or comparable plant–ant interactions, that enabled the *Maculinea*, and others among multiple independent lines of phytophagous insect myrmecophiles [5,6,8], to evolve from mutualistic ancestors to enjoy the rewards of social parasitism (abundant food in

Table 2. The distribution of *Myrmica* species and other (non-*Myrmica*) genera of ants beneath *O. vulgare* plants and in equivalent habitat more than 2 m away from *O. vulgare*.

habitat patches	N	<i>Myrmica</i> species present	non- <i>Myrmica</i> ant genera present	no ants
beneath <i>Origanum</i>	190	139 (73%)	18 (9%)	36 (19%)
more than 2 m from <i>Origanum</i>	80	36 (45%)	44 (55%)	0

Table 3. The impact of *Ma. arion* larvae on seed production by *O. vulgare* plants on which females laid one or more eggs. (Sampling was from the site with the highest density of *Ma. arion* known in Europe; mean values are given \pm s.e.m.)

density of <i>Ma. arion</i> larvae per occupied plant	no. florets eaten per <i>Ma. arion</i> larva	no. florets per flowering spike of occupied plants	no. flowering spikes per plant	% florets eaten by <i>Ma. arion</i> on occupied plants
1.45 \pm 0.1	17.08 \pm 0.6	310 \pm 36	5.23 \pm 0.53	2.02 \pm 0.31

an enemy-free space for the main period of growth) while retaining their ancestral traits of low fecundity [11] and possession of vulnerable phytophagous early young stages that are ill-adapted [9,12] to penetrate the formidable inner defences of ant host societies [37].

Although it is well known that plant volatile compounds provide reliable and easily detectable cues both in host-plant selection by insect herbivores [39] and in the attraction of herbivore enemies to herbivore-damaged plants [24,25], the *Maculinea–Origanum–Myrmica* interaction is the first example, to our knowledge, in which the same volatile signal directs both trophic and parasitic behaviours. The plant monoterpene carvacrol acts as an allomone with respect to *Myrmica* ants, while simultaneously attracting butterflies as a kairomone [25]. In this way, the butterfly chooses its initial host-plant not only for its intrinsic qualities as food for its larvae, but also for propinquity to its subsequent animal resource. These results suggest a new pathway and mechanism, whereby

social and other parasites that possess sequential hosts can detect and exploit their successive resources.

Data accessibility. All raw data concerning bioassays, other experiments, chemical analysis and gene expression are lodged with the Dryad database, file: DATASET_Patricelli_Barbero *et al.* <http://dx.doi.org/10.5061/dryad.p14jc>.

Authors' contributions. E.B., M.E.M., S.B. and L.P.C. conceived and designed the experiments; D.P., F.B., M.E.M., L.P.C., A.O., C.M.B., C.C., J.G., S.B., J.A.T. and S.A.Z. performed and analysed experiments; F.B., J.A.T., D.P., L.P.C., S.B., A.O., C.M.B., S.A.Z., C.C., J.G., M.E.M. and E.B. wrote the paper.

Competing interests. We declare we have no competing interests.

Funding. Research was funded by the FP6 BiodivERsA Eranet project CLIMIT, by project RBAP10A2T4_003 of the Italian Ministry of Education, University and Research (MAMB), for D.P. by The Italian Ministry for the Environment, by the Doctorate School of Pharmaceutical and Biomolecular Sciences (S.A.Z.) and by the Max Planck Society (C.C., J.G.).

Acknowledgements. We thank MAMB and Parco Gesso Stura for permits and Richard Lewington for artwork (figure 1).

References

- Thompson JN, Pellmyr O. 1991 Evolution of oviposition behaviour and host preference in Lepidoptera. *Annu. Rev. Entomol.* **36**, 65–89. (doi:10.1146/annurev.en.36.010191.000433)
- Thomas JA, Simcox DJ, Hovestadt T. 2011 Evidence based conservation of butterflies. *J. Insect Conserv.* **15**, 241–258. (doi:10.1007/s10841-010-9341-z)
- Renwick JAA, Chew FS. 1994 Oviposition behavior in Lepidoptera. *Annu. Rev. Entomol.* **39**, 377–400. (doi:10.1146/annurev.en.39.010194.002113)
- Elmes GW. 1996 Biological diversity of ants and their role in ecosystem function. In *Biodiversity research and its perspectives in East Asia* (eds BH Lee, TH Kim, BY Sun), pp. 33–48. Jeonju, Korea: Chonbuk National University.
- Thomas JA, Schönrogge K, Elmes GW. 2005 Specializations and host associations of social parasites of ants. In *Insect evolutionary ecology* (eds ME Fellowes, GJ Holloway, J Rolff), pp. 479–518. Wallingford, UK: CABI.
- Pierce NE, Braby MF, Heath A, Lohman DJ, Mathew J, Rand DB, Travassos MA. 2002 The ecology and evolution of ant association in the Lycaenidae (Lepidoptera). *Annu. Rev. Entomol.* **47**, 733–771. (doi:10.1146/annurev.ento.47.091201.145257)
- Schönrogge K, Napper EKV, Birkett MA, Woodcock CM, Pickett JA, Wadhams L, Thomas JA. 2008 Host recognition by the specialist hoverfly *Microdon mutabilis*, a social parasite of the ant *Formica lemmani*. *J. Chem. Ecol.* **34**, 168–178. (doi:10.1007/s10886-007-9417-8)
- Fiedler K. 1998 Lycaenid-ant interactions of the *Maculinea* type: tracing their historical roots in a comparative framework. *J. Insect Conserv.* **2**, 3–14. (doi:10.1023/A:1009618721239)
- Cottrell CB. 1984 Aphytophagy in butterflies: its relationship to myrmecophily. *Zool. J. Linn. Soc.* **79**, 1–57. (doi:10.1111/j.1096-3642.1984.tb02318.x)
- Als TD, Vila R, Kandul NP, Nash DR, Yen S, Hsu Y, Mignault AA, Boomsma JJ, Pierce NE. 2004 The evolution of alternative parasitic life histories in large blue butterflies. *Nature* **432**, 386–390. (doi:10.1038/nature03020)
- Thomas JA, Simcox DJ, Clarke RT. 2009 Successful conservation of a threatened *Maculinea* butterfly. *Science* **325**, 80–83. (doi:10.1126/science.1175726)
- Elmes GW, Thomas JA, Munguira ML, Fiedler K. 2001 Larvae of lycaenid butterflies that parasitise ant colonies provide exceptions to normal insect growth rules. *Biol. J. Linn. Soc.* **73**, 259–278. (doi:10.1111/j.1095-8312.2001.tb01362.x)
- Thomas JA, Elmes GW. 2001 Food-plant niche selection rather than the presence of ant nests explains oviposition patterns in the myrmecophilous butterfly genus *Maculinea*. *Proc. R. Soc. Lond. B* **268**, 471–477. (doi:10.1098/rspb.2000.1398)
- Thomas JA, Wardlaw JC. 1992 The capacity of a *Myrmica* ant nest to support a predacious species of *Maculinea* butterfly. *Oecologia* **91**, 101–109. (doi:10.1007/BF00317247)

15. Patricelli D, Barbero F, Morgia VL, Casacci LP, Witek M, Balletto E, Bonelli S. 2011 To lay or not to lay: oviposition of *Maculinea arion* in relation to *Myrmica* ant presence and host plant phenology. *Anim. Behav.* **82**, 791–799. (doi:10.1016/j.anbehav.2011.07.011)
16. Godfray HCJ. 2013 Mosquito ecology and control of malaria. *J. Anim. Ecol.* **82**, 15–25. (doi:10.1111/1365-2656.12003)
17. Guzman MG. 2010 Dengue: a continuing global threat. *Nat. Rev. Microbiol.* **8**, S7–S16. (doi:10.1038/nrmicro2460)
18. Van Dyck H, Oostermeijer JGB, Talloen W, Feenstra V, van der Hidde A, Wynhoff I. 2000 Does the presence of ant nests matter for oviposition to a specialized myrmecophilous *Maculinea* butterfly? *Proc. R. Soc. Lond. B* **267**, 861–866. (doi:10.1098/rspb.2000.1082)
19. Musche M, Anton C, Worgan A, Settele J. 2006 No experimental evidence for host ant related oviposition in a parasitic butterfly. *J. Insect Behav.* **19**, 631–643. (doi:10.1007/s10905-006-9053-0)
20. Wynhoff I, Grutters M, Van Langevelde F. 2008 Looking for the ants: selection of oviposition sites by two myrmecophilous butterfly species. *Anim. Biol.* **58**, 371–388. (doi:10.1163/157075608X383683)
21. Fürst MA, Nash DR. 2009 Host ant independent oviposition in the parasitic butterfly *Maculinea alcon*. *Biol. Lett.* **6**, 174–176. (doi:10.1098/rsbl.2009.0730)
22. Pierce NE, Elgar MA. 1985 The influence of ants on host plant selection by *Jalmenus evagoras*, a myrmecophilous lycaenid butterfly. *Behav. Ecol. Sociobiol.* **16**, 209–222. (doi:10.1007/BF00310983)
23. Fraser AM, Tregenza T, Wedell N, Elgar MA, Pierce NE. 2002 Oviposition tests of ant preference in a myrmecophilous butterfly. *J. Evol. Biol.* **15**, 861–870. (doi:10.1046/j.1420-9101.2002.00434.x)
24. Dicke M, Baldwin IT. 2010 The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends Plant Sci.* **15**, 167–175. (doi:10.1016/j.tplants.2009.12.002)
25. Arimura GI, Matsui K, Takabayashi J. 2009 Chemical and molecular ecology of herbivore-induced plant volatiles: proximate factors and their ultimate functions. *Plant Cell Physiol.* **50**, 911–923. (doi:10.1093/pcp/pcp030)
26. Wardlaw JC, Elmes GW, Thomas JA. 1998 Techniques for studying *Maculinea* butterflies. I. Rearing *Maculinea* caterpillars with *Myrmica* ants in the laboratory. *J. Insect Conserv.* **2**, 79–84. (doi:10.1023/A:1009648908035)
27. Splivallo R, Bossi S, Maffei M, Bonfante P. 2007 Discrimination of truffle fruiting body versus mycelian aromas by stir bar sorptive extraction. *Phytochemistry* **68**, 2584–2598. (doi:10.1016/j.phytochem.2007.03.030)
28. Crocoll C, Asbach J, Novak J, Gershenzon J, Degenhardt J. 2010 Terpene syntheses of oregano (*Origanum vulgare* L.) and their roles in the pathway and regulation of terpene biosynthesis. *Plant. Mol. Biol.* **73**, 587–603. (doi:10.1007/s11103-010-9636-1)
29. Crocoll C. 2010 Biosynthesis of the phenolic monoterpenes, thymol and carvacrol, by terpene syntheses and cytochrome P450s in oregano and thyme. PhD dissertation, Friedrich-Schiller-Universität, Max-Planck-Institut für chemische Ökologie, Jena, Germany.
30. Rao A, Zhang Y, Muend S, Rao R. 2010 Mechanism of antifungal activity of terpenoid phenols resembles calcium stress and inhibition of the TOR pathway. *Antimicrob. Agents Chemother.* **54**, 5062–5068. (doi:10.1128/AAC.01050-10)
31. Xiaorong T, Shaoling C, Ling W. 2011 Purification and identification of carvacrol from the root of *Stellera chamaejasme* and research on its insecticidal activity. *Nat. Prod. Res.* **25**, 320–325. (doi:10.1080/14786419.2010.532796)
32. Maffei ME, Gertsch J, Appendino G. 2011 Plant volatiles: production, function and pharmacology. *Nat. Prod. Rep.* **28**, 1359–1380. (doi:10.1039/C1NP00021G)
33. Poulou AJ, Croteau R. 1978 γ -Terpinene synthetase key enzyme in biosynthesis of aromatic monoterpenes. *Arch. Biochem. Biophys.* **191**, 400–411. (doi:10.1016/0003-9861(78)90104-2)
34. Casida JE, Durkin KA. 2013 Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. *Annu. Rev. Entomol.* **58**, 99–117. (doi:10.1146/annurev-ento-120811-15364534)
35. Zhang Y, Yan H, Lu W, Li Y, Guo X, Xu B. 2013 A novel omega-class glutathione S-transferase gene in *Apis cerana cerana*: molecular characterisation of GSTO2 and its protective effects in oxidative stress. *Cell Stress Chap.* **18**, 503–516. (doi:10.1007/s12192-013-0406-2)
36. Scott JG, Liu N, Wen Z. 1998 Insect cytochromes P450: diversity, insecticide resistance and tolerance to plant toxins. *Comp. Biochem. Physiol. C Pharm. Toxicol.* **121**, 147–155. (doi:10.1016/S0742-8413(98)10035-X)
37. Hölldobler B, Wilson EO. 1990 *The ants*. Berlin, Germany: Springer.
38. Bischof-Deichnik C, Holtuijzen J, Stahl-Biskup E. 2000 Multivariate statistical analysis of the essential oil composition of *Thymus praecox* Opiz ssp. *polytrichus* (Kern. ex Borb.) Ronn. collected in the Tyrolean alps. *Flavour Frag. J.* **15**, 1–6. (doi:10.1002/(SICI)1099-1026(200001/02)15:1<1::AID-FFJ845>3.0.CO;2-8)
39. Renwick JAA. 1989 Chemical ecology of oviposition in phytophagous insects. *Experimentia* **45**, 223–228. (doi:10.1007/BF01951807)

Electronic Supplementary Material

Plant defenses against ants provide a pathway to social parasitism in butterflies

Dario Patricelli, Francesca Barbero, Andrea Occhipinti, Cinzia M. Berteà, Simona Bonelli,
Luca P. Casacci, Simon A. Zebelo, Christoph Crocoll, Jonathan Gershenzon, Massimo E.
Maffei, Jeremy A. Thomas* and Emilio Balletto

* E-mail: jeremy.thomas@zoo.ox.ac.uk

Supplementary Material and Methods.

1. Analysis of volatiles from *Origanum vulgare*
2. Analysis of phytohormones by liquid chromatography-tandem mass spectrometry (LC-MS) of control and *Myrmica*-infested *Origanum vulgare*.
3. Total RNA extraction from control and *Myrmica*-infested *Origanum vulgare* leaves, cDNA synthesis and qPCR conditions and analysis.
4. Total RNA extraction from different ant species, cDNA synthesis and qPCR conditions and analysis.
5. The rationale for choosing candidate genes involved in the putative perception and detoxification of carvacrol by ants.

Supplementary references

Tables S1-S4

Figure S1

1. Analysis of volatiles from *Origanum vulgare*

O. vulgare leaves were collected in the experimental site Parco Fluviale Gesso e Stura, Cuneo, North Italy. Samples were immersed in liquid nitrogen and stored at -80°C until used. The extraction was performed with a 2:1 mixture of pentane/diethyl ether (Carlo-Erba, Italy) in a ratio 1:10 p/v, three times. Pulegone (TCI-Europe) was used as internal standard. Qualitative and quantitative analyses were performed by GC-MS (6890N-5973A, Agilent Technologies, USA) and GC-FID (6890 Agilent Technologies, USA), respectively. VOCs were separated on a Zebron ZB5-MS column (30 m length, 250 µm diameter, 0.25 µm thickness of stationary phase) (Phenomenex, USA). The injector was set up in splitless mode at 250°C. Helium was used as carrier gas at constant flow (1 mL min⁻¹). The thermal gradient was: 60°C for 5 min, from 60°C to 250°C at 3°C min⁻¹, and 250°C for 5 min. For GC-MS analyses, the transfer line to MSD was at 280°C, ionization energy (EI) was 70 eV and the acquisition scan was from 50 to 250 m/z. The separated compounds were identified by comparisons with pure standards, by comparisons of their mass spectra and Kováts retention indexes with those of reference substances, and by comparison with the NIST mass spectral search software v2.0 using the NIST 98 and Adams' libraries [S1].

2. Analysis of phytohormones by liquid chromatography-tandem mass spectrometry (LC-MS) of control and *Myrmica*-infested *Origanum vulgare*

Leaves were collected from control and *Myrmica scabrinodis* infested *O. vulgare* plants, placed immediately in liquid nitrogen and kept at -80 °C until extraction. Leaf samples (200-300 mg) were finely ground and 1 ml of methanol (Carlo-Erba, Italy) was added. Dihydrojasmonic acid (200 ng) (TCI-Europe, Belgium) was added as internal standard.

Samples were kept on ice and extracted for 30 min and mixed by vortex every 5 minutes. Plant material was removed by centrifugation for 5 min at 9000 g at room temperature. Sample clean-up was performed by C18 SPE (SampliQ C18 Endcapped, 500mg, Agilent Technologies, U.S.A.). Phytohormones were eluted with 500 µl of methanol (Carlo-Erba, Italy).

Phytohormones were analysed by liquid chromatography (1200 HPLC, Agilent Technologies, U.S.A.) equipped with a reversed-phase column Luna C18 (3.00 µm, 150 x 3.0 mm, Phenomenex, U.S.A.). The binary solvent system was: (A) acidified double distilled water (Millipore, U.S.A.) with 0.1% v/v formic acid (Sigma-Aldrich, USA), and (B) acetonitrile (Carlo-Erba, Italy) with 0.1% v/v of formic acid. Separation was performed at 0.2 ml min⁻¹ flow rate, 25°C as column temperature and the following solvent gradient: 40% of B for 1 min, 55% B at 4.5 min, 62% B at 5 min, 65% B at 14 min, 98% B at 16 min and 40% of B at 18 min. The initial mobile phase was re-established for 8 min before the next injection. Mass spectrometry analyses were performed with a 6330 Series Ion Trap LC-MS/MS System (Agilent Technologies, USA) equipped with an electrospray ionization source (ESI).

Quantitative analyses were made in negative mode with 1.5 kV ion spray voltage, nebulizer gas (N₂) at 10 L min⁻¹, 325 °C and 1.00 V fragmentation amplitude. The monitored mass transitions in MRM mode were 209→59, 211→59, 322→130 and 291→273 m/z for Jasmonic acid (JA), Dihydrojasmonic acid (IS), (3R,7S)-Jasmonoyl-L-isoleucine (JA-Ile) and cis-(+)-12-oxophytodienoic acid (OPDA), respectively. Spectral data were processed and analyzed by DataAnalysis for 6330 Series Ion Trap LC/MS 4.0 software (Bruker Daltonik, Germany).

External calibration curves were made with standard solutions of Jasmonic acid (Sigma-Aldrich, USA), JA-Ile and OPDA (Cayman, USA).

3. Total RNA extraction from control and *Myrmica*-infested *Origanum vulgare* leaves, cDNA synthesis and qPCR conditions and analysis

Total RNA was extracted from 50 mg of ant-induced and control *O. vulgare* leaves ($n = 6$) using the Agilent Plant RNA Isolation Mini Kit (Agilent Technologies, USA). To remove residual genomic DNA, RNA was treated with RNase-free DNase I set (Qiagen, Germany). RNA quality was checked using the Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip. Quantitative analysis was performed using the Nano Drop ND-1000 micro-scale spectrophotometer (Thermo Fisher Scientific, USA).

For cDNA synthesis, High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used according to manufacturer's instructions. Reactions were prepared by adding 1.5 µg total RNA, 2 µl of 10X RT Buffer, 0.8 µl of 25X dNTPs mix (100 mM), 2 µl 10X RT random primer, 1 µl of Multiscribe™ Reverse Transcriptase and nuclease-free sterile water up to 20 µl. Reaction mixtures were incubated at 25°C for 10 min, 37°C for 2 h, and 85°C for 5 sec. Samples were stored at -20°C for further analyses. Primer pairs for cytochrome P450s CYP71D178-182 were designed using BeaconDesigner (version 5.0; PremierBiosoft, Palo Alto). Primers were designed for regions with the largest possible difference between the P450 sequences whilst primer binding sites were chosen for identical regions of all known alleles for the respective genes from *O. vulgare* and *Thymus vulgaris*. Each primer pair was tested for potential cross-hybridisation with the other P450s as templates; none was observed at a 60 °C annealing temperature except that the CYP71D179 primers cross-reacted to some extent with the CYP79D178 and D182 sequences due to their close sequence similarities. Primers are listed in table S4. Primer efficiencies for all pairs were calculated using the standard curve method [S2, S3].

qPCR analysis was on a Stratagene Mx3000P Real-Time System (La Jolla, CA, USA) using SYBR green I with ROX as internal loading standard, using 25 μ l of mixture consisting of 12.5 μ l 2X Maxima™ SYBR Green qPCR Master Mix (Fermentas), 0.5 μ l cDNA and 100 nM primers (Integrated DNA Technologies). Controls included non-RT controls (using total RNA without reverse transcription to monitor genomic DNA contamination) and non-template controls (water template). PCR conditions were: 10 min at 95°C, 40 cycles of 15 s at 95°C, 20 sec at 60°C, 30 sec at 72°C. Fluorescence was read after each annealing and extension phase. All runs were followed by a melting curve analysis from 55°C to 95°C. The linear range of template concentration to threshold cycle value (Ct value) was determined by performing a dilution series using cDNA from three independent RNA extractions analyzed in three technical replicates. qPCR reactions were run using specific primers [S4]. Three distinct reference genes: elongation factor 1 alpha [S2], actin and 18S rRNA [S5] were used to normalize the results of the real-time PCR. The most stable gene, selected using Normfinder software [S6], was the elongation factor 1 alpha. All amplification plots were analysed with MX3000P™ software to obtain Ct values. Relative RNA levels were calibrated and normalized with the level of the elongation factor 1 alpha mRNA.

4. Total RNA extraction from different ant species, cDNA synthesis and qPCR conditions and analysis

Frozen ants' total RNA was isolated using Qiagen RNeasy Micro kit and RNase-Free DNase set to remove residual genomic DNA. Sample quality and quantity was checked by using the RNA 6000 Nano kit and Agilent 2100 Bioanalyzer, and confirmed spectrophotometrically using a NanoDrop ND-1000. High-capacity cDNA Reverse Transcription Kit was used for cDNA synthesis. Reactions were prepared by adding 500 ng total RNA and following the same protocol as in §3 (above). All qPCR experiments were performed on a Stratagene Mx3000P

Real-Time System using SYBR green I with ROX as an internal loading standard. The reactions mixtures were prepared as described in §3 (above). PCR conditions were: tubulin (*TUB*), Acetylcholinesterase (*AChE*), actin (*ACT*): 10 min at 95°C, 40 cycles of 15 s at 95°C, 20 sec at 55°C, and 45 sec at 72°C; odorant receptor 1 (*OR1*), cytochrome P450 9E2 (*CYP450 9E2*), Glutathione S transferase (*GST*), odorant binding protein 6 (*OBP6*): 10 min at 95°C, 40 cycles of 15 s at 95°C, 30 sec at 57°C, and 30 sec at 72°C. Procedures for fluorescence reading, melting curve analysis and determination of the linear range of template concentration to Ct value were as in §3 (above).

qPCR reactions were run using specific primers designed on Genbank-available ant sequences using the Primer3 software [S7] (table S1), with efficiencies for all pairs calculated as above [30]. Two reference genes, actin and tubulin, were used to normalize results of the real time PCR, from which actin was selected as in §3 (above) [S6]. Ct values of amplification plots and the calibration and normalization of relative RNA levels (using actin) were as in §3 (above).

5. The rationale for choosing candidate genes involved in the putative perception and detoxification of carvacrol by ants.

The selection of genes analysed in this study was based on their known or suspected function in odour perception and on sequences available from the different ant genomes [S8-S12]. We analysed the expression levels of AChE, GST and a CYP9 for their suspected involvement in the detoxification of carvacrol in different ant species, along with OBP6 and OR1 as potential candidates in the perception of this compound.

The detoxification-related genes we used were previously known from other insects and shown to be involved in the detoxification of different xenobiotics. In particular, the functions

of acetylcholinesterases (of insect and mammalian origin) are discussed by Casida and Durkin [S8]. Inhibitor specificity between the AChE of insects and between susceptible and tolerant species of insects contributes to selective toxicity and resistance. Since insect acetylcholinesterase is often targeted by insecticides and by compounds showing insecticidal properties (such as terpenoids), we deemed it advisable to analyse the expression level of this gene in the different ant species following treatment with carvacrol. But whereas the effect of this compound has been studied on AChE enzyme activities [S9], to the best of our knowledge no study on AchE expression had been carried out following exposure to this compound.

Glutathione S-transferases (GSTs) in insects are a family of enzymes that play a critical role in insecticide detoxification. Since carvacrol and other terpenoids have insecticidal properties, we suspected that they may be able to modulate GST gene expression.

Cytochrome P450s are a class of heme proteins that may also be involved in the oxidation of exogenous substances, such as environmental contaminants. In insects, cytochrome P450s are important detoxification enzymes of organophosphate, carbamate insecticides and other types, including terpenoids with insecticidal properties. Several cytochrome P450s have been reported in insect antennae and may play a role in odorant processing and/or xenobiotic metabolism [S10]. It has recently been reported that CYP4, CYP6 and CYP9 in *Bombyx mori* participate in the metabolism of plant toxins and insecticides [S11].

Proteins coded by several different gene families contribute to the reception of chemical messages. Growing evidence suggests that odorant binding proteins (OBP) are involved in solubilizing and transporting odorants and pheromones through aqueous haemolymph in insects. The chemical messages carried by the OBPs are decoded when odorant receptors (OR) selectively bind different chemicals [S12].

Supplementary References

- S1. Adams RP. 2007 *Identification of essential oil components by gas chromatography/mass spectrometry*. Illinois, Allured Publishing.
- S2. Crocoll C, Asbach J, Novak J, Gershenzon J, Degenhardt J. 2010 Terpene synthases of oregano (*Origanum vulgare* L.) and their roles in the pathway and regulation of terpene biosynthesis. *Plant. Mol. Biol.* **73**, 587-603. (doi: 10.1007/s11103-010-9636-1)
- S3. Pfaffl MW. 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, 2002-2007. (doi: 10.1093/nar/29.9.e45)
- S4. Crocoll C (2010) *Biosynthesis of the phenolic monoterpenes, thymol and carvacrol, by terpene synthases and cytochrome P450s in oregano and thyme*. Ph.D dissertation, Friedrich-Schiller-Universität. Max-Planck-Institut für chemische Ökologie.
- S5. Zebelo SA, Berteza CM, Bossi S, Occhipinti A, Gnani G, Maffei ME. 2011 *Chrysolina herbacea* modulates terpenoid biosynthesis of *Mentha aquatica*. *PLoS One* **6**, e17195. (doi: 10.1371/journal.pone.0017195)
- S6. Andersen CL, Jensen JL, Ørntoft TF. 2004 Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64** 5245-5250. (doi: 10.1158/0008-5472.CAN-04-0496)
- S7. Rozen S, Skaletsky H. 2000 Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols* (eds S Krawetz, SA Misener), pp 365–386. Totowa: Humana Press.
- S8. Casida JE, Durkin KA. 2013 Neuroactive insecticides: Targets, selectivity, resistance, and secondary effects. *Annu. Rev. Entomol.* **58**, 99-117. (doi: 10.1146/annurev-ento-120811-153645)

- S9. Jukic M, Politeo O, Maksimovic M, Milos M, Milos M. 2007 In Vitro Acetylcholinesterase Inhibitory Properties of Thymol, Carvacrol and their Derivatives Thymoquinone and Thymohydroquinone. *Phytotherapy Res.* **21**, 259-261. (doi: 10.1002/ptr.2063)
- S10. Pottier MA, Bozzolan F, Chertemps T, Jacquin-Joly E, Lalouette L, Siaussat D, Maibèche-Coisne, M. 2012 Cytochrome P450s and cytochrome P450 reductase in the olfactory organ of the cotton leafworm *Spodoptera littoralis*. *Insect Mol. Biol.* **21**, 568-580. (doi: 10.1111/j.1365-2583.2012.01160.x)
- S11. Gu ZY, Li FC, Wang BB, Xu KZ, Ni M, Zhang H, Shen WD, Li, B. 2015 Differentially expressed genes in the fat body of *Bombyx mori* in response to phoxim insecticide. *Pesticide Biochemistry and Physiology* **117**, 47-53. (doi:10.1016/j.pestbp.2014.10.007)
- S12. McKenzie SK, Oxley PR, Kronauer DJC. 2014 Comparative genomics and transcriptomics in ants provide new insights into the evolution and function of odorant binding and chemosensory proteins. *BMC Genomics* **15**, 718. (doi:10.1186/1471-2164-15-718)

Table S1. Comparison of volatiles produced by *Origanum vulgare* plants after experiencing root disturbance by *Myrmica sabuleti* and *My. scabrinodis* in experimental terraria. Mean values between treatments ($n = 20$, 1df) were compared by ANOVA; data are expressed as $\mu\text{g g}^{-1}$ fresh weight \pm s.e.m..

Compound	Kovats index	Plants with <i>M. sabuleti</i>	Plants with <i>M. scabrinodis</i>	Statistical test	p-value
<i>p</i> -Cymene	1024	0.877 \pm 0.272	0.947 \pm 0.311	F=0.862	0.366
<i>γ</i> -Terpinene	1055	0.930 \pm 0.261	0.919 \pm 0.263	F=0.167	0.687
<i>trans</i> -Sabinene hydrate	1098	1.086 \pm 0.366	0.946 \pm 0.310	F=0.972	0.337
Methylthymyl ether	1173	0.919 \pm 0.288	1.210 \pm 0.458	F=1.725	0.206
Thymol	1290	1.369 \pm 0.452	1.210 \pm 0.434	F=0.312	0.583
Carvacrol	1299	1.312 \pm 0.429	1.309 \pm 0.401	F=0.001	0.990
α -Cubebene	1351	0.854 \pm 0.262	0.880 \pm 0.283	F=0.301	0.590
α -Copaene	1377	1.058 \pm 0.310	1.022 \pm 0.312	F=0.131	0.722
β -Ylangene	1421	4.777 \pm 1.219	4.221 \pm 1.240	F=0.158	0.695
γ -Elemene	1437	1.246 \pm 0.366	1.408 \pm 0.425	F=0.536	0.473
α -Bergamotene	1413	0.818 \pm 0.279	0.844 \pm 0.282	F=0.222	0.643
γ -Muurolene	1480	2.214 \pm 0.575	1.706 \pm 0.447	F=1.688	0.210
Bicyclosesquiphellandrene	1484	1.248 \pm 0.349	1.317 \pm 0.365	F=0.181	0.676
Germacrene-D	1485	6.503 \pm 1.721	4.941 \pm 1.484	F=0.653	0.429
α -Amorphene	1485	1.114 \pm 0.395	0.989 \pm 0.293	F=0.597	0.450
(<i>E</i>)- β -Farnesene	1457	3.977 \pm 1.139	5.299 \pm 1.276	F=0.931	0.347
Bicyclogermacrene	1500	1.068 \pm 0.322	1.045 \pm 0.287	F=0.063	0.805
α -Bisabolene	1507	0.864 \pm 0.322	0.791 \pm 0.254	F=0.814	0.379
δ -Cadinene	1523	0.886 \pm 0.281	0.995 \pm 0.310	F=1.919	0.183
α -Humulene epoxide	1608	1.730 \pm 0.564	1.841 \pm 0.524	F=0.067	0.799

Table S2. The abundance of major compounds found in leaf extracts of *Origanum vulgare* growing naturally in the field with or without a *Myrmica* ant colony in the surrounding soil. Mean values between treatment and control plants ($n = 10$) were compared by ANOVA; significant increases were reported. tr = traces detected, nc = not computable. Data are expressed as $\mu\text{g g}^{-1}$ fresh weight \pm s.e.m.. * Significant p -values controlled for False Discovery Rate (FDR) by using the Benjamini-Hochberg procedure

Compounds	Kovats index	Plants with <i>Myrmica</i>	Control plants without <i>Myrmica</i>	Statistical test	p -value
α -Thujene	912	1.91 \pm 0.07	0.94 \pm 0.48	F=4.034	0.079
α -Pinene	924	0.79 \pm 0.04	Tr	Nc	Nc
1-Octen-3-ol	976	11.55 \pm 1.97	8.09 \pm 0.33	F=3.008	0.121
Myrcene	989	7.31 \pm 0.76	6.74 \pm 0.32	F=0.484	0.506
α -Terpinene	1016	7.66 \pm 0.62	Tr	Nc	Nc
β - <i>Trans</i> -ocimene	1035	30.53 \pm 5.75	41.24 \pm 0.52	F=3.439	0.101
γ -Terpinene	1055	84.60 \pm 4.00	13.61 \pm 0.80	F=302.332	<0.0001*
Allo-ocimene	1120	28.22 \pm 0.59	50.43 \pm 12.81	F=2.997	0.122
Thymol	1312	17.16 \pm 0.40	1.83 \pm 0.61	F=437.157	<0.0001*
Carvacrol	1319	72.89 \pm 1.09	37.09 \pm 1.87	F=272.937	<0.0001
Bicycloelemene	1348	20.61 \pm 3.40	25.56 \pm 2.95	F=1.212	0.303
β -Caryophyllene	1417	46.93 \pm 13.34	132.45 \pm 24.29	F=9.522	0.015
β -Copaene	1430	16.53 \pm 1.19	21.58 \pm 1.96	F=4.856	0.059
Germacrene D	1484	35.41 \pm 1.04	38.17 \pm 1.94	F=1.578	0.244
Bicyclgermacrene	1500	31.21 \pm 1.12	34.70 \pm 1.57	F=3.283	0.108
α -Farnesene	1505	10.75 \pm 3.34	57.92 \pm 20.28	F=5.266	0.051
Germacrene D-4-ol	1574	10.64 \pm 3.32	23.16 \pm 4.30	F=5.317	0.050

Table S3. Expression of *Origanum vulgare* terpene biosynthesis related genes from plants treated with *Myrmica scabrinodis* ants compared with untreated control plants. Log-transformed data are expressed as mean values of at least three biological replicates (\pm s.e.m.). Three technical replicates were run for each biological replicate. ANOVA was used to determine significant differences between treatments and controls (n=6). Gene descriptions not given in figure 2: *GPS*, geranyl diphosphate synthase; *OvTPS6*, a sesquiterpene synthase producing (*E*)- β -caryophyllene and α -humulene.

Gene	Plants with <i>Myrmica</i>	Control plants without <i>Myrmica</i>	Statistical test	<i>p</i>-value
<i>OvTPS6</i>	0.185 \pm 0.030	0.200 \pm 0.027	F=0.127	0.740
<i>GPS</i>	0.739 \pm 0.258	0.893 \pm 0.102	F=0.307	0.609

Table S4. Primers used in this work

<i>Origanum vulgare</i> PRIMERS		
Gene	Forward primer (5'→3')	Reverse primer (5'→3')
<i>OvEF1alpha</i>	CTCCAGTTCTTGATTGCCACAC	GTCCTTTCCAGACCTCCTATC
<i>18S rRNA</i>	ATGATAACTCGACGGATCGC	CTTGATGTGGTAGCCGTTT
<i>Act</i>	GCTCCAAGGGCTGTGTTCC	TCTTTCTGTCCCATGCCAAC
<i>OvTPS2</i>	GTGGCTGAGTTTGGTGAAGG	TTGGCGTTCTCTAGGTATTCTGC
<i>OvTPS6</i>	AGGGCGTATCGGAGGAAGAAG	GGTCGTAAGGATTGGAACAGAGG
<i>GPS</i>	ATGATAAGCGGGCTGCATAG	CCGAAATTCCTCAGCTTCTG
<i>CYP71D178</i>	CAAGGAATGACTGCTGCTGAC	TTGGATTGTGGATTGTTGGAACC
<i>CYP71D178/179/182</i>	CGTGGCTTCTCAACCTTCTC	CGCTCTTCTCACCTATGC
<i>CYP71D180</i>	GCAAAGAAGAATGCGAGGTC	GATTGAACGTGTCGGGATCT
ANT PRIMERS		
Gene	Forward primer (5'→3')	Reverse primer (5'→3')
<i>ACT</i>	CTACGGCTGAGCGAGAAATC	AAGGAAGGCTGGAAAAGAGC
<i>TUB</i>	CGGCTATTCAGGAGCTGTTC	CTGCTGATACTCGGACACGA
<i>OR1</i>	AAGCCAGCTGGAAAAAGGAT	CTGCTTGAAGCCAGTTAGG
<i>OBP</i>	GAGTCGCCACAGCTGACATA	TTTCTCAACCTCGGCTCTGT
<i>AChE</i>	ACACCGCCGTCAATAAAGAG	GCTGCGGCAGATACTTTTTC
<i>GST</i>	GTGGTTACGCAGTCGGTTCT	ATGAGGTCGTCGTGATAGCC
<i>CYP4509E2</i>	GGAGCCTACCTTCTTTTTGG	ATCGGCATCGATGTCTTTTC

Figure S1. Establishing the terraria used for experiments

