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Supplementary Materials for

Conserved Class of Queen Pheromones Stops Social Insect Workers from Reproducing

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Correction (24 November 2015): Table S2 was missing several rows in part (d), now at the top of page 14; they have been restored. The originally posted version and revision 1 can be seen here.

Supplementary Materials

Materials and Methods:

Analysis of cuticular extracts and identification of putative queen pheromones

We identified candidate queen pheromones by comparing the chemical profiles of workers, queens and gynes (young unmated queens) of *Vespula vulgaris* wasps (*20*), *Bombus terrestris* bumblebees (N_{queens}=12; worker data from (*21*)) and *Cataglyphis iberica* ants (N_{queens}=7, N_{gynes}=9, $N_{workers}$=18). Cuticular chemicals were extracted by immersing freeze-killed individuals in 1 mL HPLC grade pentane (Sigma-Aldrich, Belgium) for 10 minutes. This solvent was chosen as it extracts a wide array of both polar and non-polar compounds, including hydrocarbons, esters, alcohols, triglycerides, aldehydes, and lower molecular weight compounds such as oxygenated terpenoids and the aromatic components of mandibular gland pheromones, particularly in the microgram to nanogram ranges in which they are typically present (*22-24*). The solvent was evaporated and the residue was taken up in 150 µL pentane for ants and 500 µL pentane for bumblebees. Subsequently, 2 µL of this solution was injected into a gas chromatograph (Shimadzu 2010 Plus), equipped with a 30 m DB-5ms column (internal diameter 0.25 mm, film thickness 0.25 µm), and interfaced to a Shimadzu QP 2010 Ultra mass spectrometer. After an initial hold of 1 minute at 70°C, the oven temperature was increased to 150°C at a rate of 20° C min^{-1}, and then to 320°C at a rate of 3°C min^{-1}. The final temperature of 320°C was held for 15 minutes. We used helium carrier gas at a flow rate of 1mL min^{-1}, with splitless injection, an inlet temperature of 280°C, and a final pressure of 75 kPa. The electron ionization voltage was auto-tuned to enhance the acquisition performance according to the molecular weight of the compounds, and the ion source temperature was 230°C. Peaks in the chromatogram were integrated using GCMS Solutions software, and tentatively identified by matches with the mass spectral database (NIST 11). Subsequently, identifications were confirmed by matching retention times and mass spectra with those of known standards, or by the use of retention indices and diagnostic mass spectral fragments. Identifications that could not be confirmed by these methods were listed as "tentative". The concentrations of extracted chemicals were quantified using an external alkane ladder standard (49452-U, Supelco, C_7-C_{40} saturated alkane standard) in serial dilutions of 1:10, 1:100 and 1:1,000. Linear regressions on a log-log scale were used to determine the relationship between peak area and concentration as a function of chain length. Peak areas were normalized to relative concentrations with a *Z*-transformation (*25*).

Potential pheromones were used in the bioassays if they were highly queen caste-specific. This was defined in terms of the Cohen's *d* effect size, i.e. the difference in mean abundance of the focal chemical between castes divided by the pooled standard deviation, and which we required to be greater than 2 (ant and wasp data: Table S1; bumblebee data: see ref. (*21*)). In addition, we only retained compounds that comprised at least 1.5% of the queen cuticular chemical profile and excluded mixtures of co-eluting chemicals, as these could not be accurately quantified. Finally, to test our hypothesis that structurally similar compounds would be biologically active, we tested the top compounds that belonged to a similar biosynthetic series, namely the linear alkanes *n*-C_{27} and *n*-C_{29} in *V. vulgaris* and *C. iberica*, *n*-C_{28} in *V. vulgaris* and *n*-C_{25} in *B. terrestris*, and the 3-methyl alkanes 3-MeC_{29} and 3-MeC_{27} in *V. vulgaris* and *C. iberica*, respectively (Table S1). In addition, in bumblebees, we also tested 4 queen-specific compounds that belonged to a non-conserved group of queen-produced compounds, namely tetracosyl oleate, hexacosyl oleate, eicosyl oleate and docosyl oleate (*21*). The absolute amount present on the queen's cuticle, i.e. one queen equivalent (QE), was calculated as follows: for bumblebees: 232.5 µg *n*-C_{25}, 64 µg tetracosyl oleate, 57.5 µg hexacosyl oleate, 5.3 µg eicosyl oleate, 4.6 µg docosyl oleate; wasps: 118 µg *n*-C_{27}, 14 µg 3-MeC_{29}, 19 µg *n*-C_{29}, 6.1 µg *n*-C_{28}; ants: 2.2 µg *n*-C_{29}, 1.4 µg 3-MeC_{29}, 0.7 µg *n*-C_{27}, 1.3 µg 3-MeC_{27}.

Experimental bioassays of synthetic putative queen pheromones

General procedures

Linear alkanes were purchased (Sigma Aldrich, Belgium), whereas esters and methyl-alkanes were synthesized as described below. Two queen equivalents of each synthetic candidate pheromone dissolved in HPLC grade pentane (Sigma Aldrich, Belgium) were applied every 24 h (see below for details) to colonies from which the queen was artificially removed. This dose was chosen based on the fact that in the honey bee, the queen produces up to ca. 2 queen equivalents of queen pheromone (9-ODA) per day (*26*). To allow for maximum worker ovary development, treatment continued for 14 days for the wasps and bumblebees (*27, 28*) and for 21 days for the ants (*29*). Queenless control colonies were handled identically to the treatment colonies but received only the solvent (pentane). Workers were then freeze-killed (-20°C) and dissected to assess ovary development, which was scored on the scale of ref. (*28*) as either undeveloped (stages 0-II), developed (stages III-IV) or regressed (stage V). Researchers were blind to treatment, and colonies were randomly assigned to each treatment.

Vespula vulgaris bioassays

We collected five large *Vespula vulgaris* colonies in the vicinity of Leuven, Belgium, in August and September 2012. After collection, colonies were lightly anaesthetized with carbon dioxide and transferred to experimental wooden nest boxes (35 × 14.5 × 30 cm). Nest boxes consisted of two compartments, one containing *ad libitum* water, food (sugar syrup and mealworms) and wood for comb construction, and the other containing wire to support a piece of wasp comb. Four colonies were large enough to be split across all five treatments (Table S1) and a queenless control, while a fifth colony was split in two and used to replace two sub-colonies that did not survive the experiment. Each treatment was thus replicated four times. When splitting colonies, we selected pieces of comb of size 102 ± 11 cm^2 (mean ± SD), and placed one comb on a metal wire in the nest box along with 200-300 workers per treatment. The experimental nests were kept under natural light (the top panels were made of transparent Perspex) and were warmed with an infrared heat lamp (temperature inside the nest box: 30 ± 2°C). Each day, 100 μL of treatment solution was pipetted onto the back of the comb through five holes in the lid of the nest box.

Bombus terrestris bioassays

Queen pheromone bioassays were carried out in April and May 2012 on 28 bumblebee colonies obtained from Biobest Belgium N.V. At the start of the experiment, colonies were ca. 30 days old and contained about 20.2 ± 1.3 (mean ± SE) workers. We chose young colonies because these had not yet reached the point at which queens or workers would have started to lay male-destined haploid eggs (*30-32*). Bumblebee nests were kept at a constant temperature of 27°C and a constant humidity of 60 % in ventilation hives, which consisted of plastic nest boxes with a cardboard exterior and ventilation holes. The colonies were fed *ad libitum* with pollen and BIOGLUC® via a BIOGLUC® feeding system (Biobest N.V., Belgium). The experiment comprised four replicates each of five candidate queen pheromone treatments (n-C_{25}, eicosyl oleate, docosyl oleate, tetracosyl oleate, and hexacosyl oleate) as well as queenright and queenless pentane-treated controls. An unpaired experimental design was used, such that individual colonies were used only once. Each day for 14 days, we applied a total of 200 μL of treatment solution (or pentane for the controls) onto four glass slides that were distributed evenly across the nest, as well as onto a cotton ball in the centre of the nest.

Cataglyphis iberica bioassays

Ant colonies were collected in June 2012 in the vicinity of Madrid and Zaragoza (Spain), and were kept at a constant temperature of 27°C and a humidity of 60% in plastic nest boxes (20 × 40 × 60 cm) with a plaster floor. The ants were fed *ad libitum* with sugar syrup and mealworms. We divided five colonies across the four treatments (Table S1) and a queenless control, such that there were 32.3 ± 3.3 (mean ± SE) workers in each subcolony. Each day for 21 days, we administered 40µL of treatment solution to a glass slide inside the nest box.

Statistics

Effects of treatments on the proportions of workers with developed and regressed ovaries were analyzed using generalized linear mixed models (GLMM) with binomial errors in R package lme4 (*33*). In these analyses, colony was included as a random factor and the number of workers (colony size) and males present in the colony were included as covariates when they were found to significantly affect worker ovary development. Wald tests were used to test the significance levels of the fixed effects.

Comparative analysis and ancestral state reconstruction (ASR)

We conducted a systematic review of published data documenting chemical differences between queens and workers. Chemicals were scored as queen or fertility signals if they were significantly over-produced by queens or dominant egg-layers relative to workers, although in a few cases we also included chemicals that were more abundant on the surface of laying versus non-laying queens, or queen-laid versus worker-laid eggs. We excluded data from species in which workers lack ovaries (e.g. *Solenopsis invicta*). Using a variety of relevant search terms on ISI Web of Science and Google Scholar and exhaustively checking reference lists, we collected data on 69 species (Table S2).

The phylogenetic tree was constructed based on previously published phylogenies of ants (*34-38*), honeybees (*39*), stingless bees (*40*), and wasps (*41-45*), using higher-level relationships as given in (*13, 46-51*). Subsequently, marginal ancestral state reconstructions at different nodes in the phylogeny were calculated with function rayDISC in R package corHMM using maximum likelihood under a symmetrical transition rate model. For the root we either used a flat prior (Table S5a) or a prior that reflected the frequencies of the states at the tips (Table S5b). Branch lengths were calculated according to Grafen (*52*), but results were robust with respect to various other suggested branch length transformations (e.g. setting all branch lengths equal, corresponding to a punctuational mode of evolutionary change).

Most empirical studies compared non-volatile compounds produced by queens and workers. Nevertheless, this does not introduce any bias in our analyses, given that in bumblebees, polistine and vespine wasps, and ants, the queen pheromones mediating worker sterility have all been shown to be of low volatility (Table S4). Only in honeybees have bioassays shown the queen pheromone to be partly volatile (Table S4). This result fits with bioassays demonstrating that a number of volatile honeybee queen chemicals inhibit worker reproduction (Table S3).

Synthesis of esters

To synthesize docosanyl oleate, oleic acid (0.564 g, 2 mmol), docosanol (0.59 g, 1.8 mmol; Lancaster Synthesis, Pelham, NH, USA), and *p*-toluenesulphonic acid (50 mg) were refluxed in 20 ml benzene with a Dean-Stark trap for 3 hr. After cooling, the solution was diluted with 50 ml hexane, washed twice with dilute aqueous $NaHCO_3$, once with brine, dried over anhydrous Na_2SO_4 , and concentrated. The residue was purified by vacuum flash chromatography on silica gel in a 60 ml sintered glass funnel, eluting with hexane (50 ml), then 3% ethyl acetate in hexane (7 x 40 ml). The product eluted in fractions 6 and 7, which were combined and treated with decolorizing charcoal to remove the faint yellow color. After filtration and concentration, the residue was taken up in 30 ml hexane and chilled to -20ºC overnight, yielding 0.69 g (65%) of fluffy white crystals (melting point 37ºC). ^{1}H NMR ($CDCl_3$): δ 5.35 (M, 2H), 4.06 (t, 2H, J = 6.8 Hz), 2.30 (t, 2H, J = 7.6 Hz), 2.01 (m, 4H), 1.64 (m, 4H), 1.4-1.23 (M, 54 H), 0.89 (t, 2 × 3H, J = 6.8 Hz).

Eicosanyl oleate (62% yield, melting point 31ºC), tetracosanyl oleate (81% yield, melting point 43ºC), and hexacosanyl oleate (72% yield, melting point 48ºC) were prepared in analogous fashion by substituting eicosanol (Lancaster Synthesis), tetracosanol (TCI America, Portland, OR, USA), or hexacosanol (TCI America) for docosanol.

Synthesis of methylalkanes

General conditions

Tetrahydrofuran was distilled from sodium-benzophenone ketyl under argon. Unless specified otherwise, solutions of crude reaction products were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure. Flash or vacuum flash chromatography purifications were carried out with 230-400 mesh silica gel. ^{1}H- and ^{13}C-NMR spectra were taken on a Varian INOVA-400 spectrometer (Palo Alto, CA) (400 and 100.5 MHz, respectively), as $CDCl_3$ solutions. Electron impact ionization mass spectra were obtained with a Hewlett-Packard (HP, Avondale, PA) 5890 GC equipped with a DB5-MS column (25 m x 0.20 mm ID x 0.25µ film) interfaced to an HP 5971 mass selective detector, in EI mode (70 eV) with helium carrier gas.

Synthesis of 3-methyl-1-iodopentane

A dry flask was charged with 3-methyl-1-pentanol (5.8 g, 56 mmol), triethylamine (15.7 ml, 113 mmol), and 250 ml anhydrous diethyl ether, and the mixture was cooled under argon to -5°C in an ice-salt bath. Methanesulfonyl chloride (5.2 ml, 68 mmol) was added dropwise over 10 min, and the resulting mixture was slowly warmed to room temperature and stirred for one hour. The mixture was then quenched with aqueous $NaHCO_3$ and the ether layer was separated. The aqueous residue was extracted again with ether, and the combined ether extracts were washed sequentially with 1M HCl, water, and brine. The ether solution was dried and concentrated. The residue was taken up in dry acetone (200 ml) and powdered NaI (29.5 g, 200 mmol) was added in one portion. The mixture was stirred for 3 days at room temperature, and subsequently most of the acetone was removed under reduced pressure. The residue was taken up in 250 ml water and extracted with pentane. The pentane extract was washed with dilute $Na_2S_2O_3$ and brine, dried, concentrated, and Kugelrohr distilled (44°C at 10 mm Hg), yielding the iodide as a colorless oil (24.24 g, 81%). ^{1}H NMR ($CDCl_3$): δ 3.23.3.3 (m, 1H, H1), 3.21-3.14 (m, 1H, H1'), 1.94-1.84 (m, 1H, H2), 1.7-1.6 (m, 1H, H2'), 1.53-1.44 (m, 1H, H3), 1.42-1.32 (m, 1H, H4), 1.24-1.13 (m, 1H, H4'), 0.89 (t, 3H, J = 7.4 Hz, H5), 0.88 (d, 3H, J = 7.4 Hz, methyl). EI-MS, *m/z* (abundance): 212 (M^+, 4), 155 (4), 127 (4), 85 (61), 57 (42), 55 (22), 43(100), 41 (73).

Synthesis of 14-methylhexadec-10-yn-1-ol

A solution of 2-(10-undecyn-1-yloxy)tetrahydro-2H-pyran (7.1 g, 28.3 mmol) in dry THF (120 mmol) under argon was cooled to -78°C and treated with butyllithium (2.3 M in hexanes, 13.5 ml, 31 mmol) over 15 min. The resulting mixture was allowed to warm to room temperature, and 3-methyl-1-iodopentane (9.0 g, 42 mmol) was added dropwise. The mixture was then refluxed for 36 hours, and then cooled and quenched with saturated aqueous NH_4Cl. The layers were separated and the aqueous layer was extracted 3 times with ether. The combined ether layers were backwashed with brine, dried, and concentrated. The residue was heated under vacuum in a Kugelrohr distillation unit (oven 110°C at 0.9 mm Hg) to remove the unreacted starting materials. The residue was then taken up in MeOH with a catalytic amount of p-toluenesulphonic acid (0.25 g) and stirred at room temperature for 2 hours. Most of the MeOH was then removed on a rotary evaporator and the mixture was partitioned between dilute aqueous $NaHCO_3$ and ether. The ether layer was washed with brine, dried, concentrated, and Kugelrohr distilled (oven temp 110°C, 0.44 mm Hg), yielding 6.28 g of the desired alcohol (88%). ^{1}H NMR ($CDCl_3$): δ 3.65 (t, 3H, J = 8.4 Hz), 2.10-2.21 (m, 4H, H9, H12), 1.44-1.61 (m, 6H, H2, H8, H13), 1.23-1.42 (m, 12H, H3-7, H15), 1.12-1.2 (m, 1H, H14), 0.88 (t, 3H, J = 7.6 Hz, H16), 0.87 (d, 3H, J = 6.0 Hz, methyl). EI-MS, *m/z* (abundance): 223 (M^+-29), 163 (1), 149 (2), 135 (6), 124 (11), 121 (9), 109 (29), 95 (75), 81 (73), 67 (63), 55 (72), 43 (55), 41 (100).

Synthesis of 3-methylheptacosa-6,17-diyne

A dry flask was loaded with 14-methylhexadec-10-yn-1-ol (1.0 g, 4 mmol), pyridine (0.32 ml, 4 mmol), and CH_2Cl_2 (16 ml), and the solution was cooled to -20°C under argon. Trifluoracetic anhydride (0.8 ml, 4.8 mmol) was added dropwise, and the resulting mixture was warmed to 0°C and stirred 1 hour, then diluted with 30 ml hexane and filtered through a short plug of Celite, rinsing well with hexane. The solution was concentrated, and the crude product was used immediately. A solution of 1-undecyne (0.39 g, 2.56 mmol) in 11 ml dry THF under argon was cooled to 0°C and butyllithium (2.3 M in hexane, 1 ml, 2.3 mmol) was added over 5 minutes. The resulting solution was cooled to -78°C and the crude triflate (0.9 g, 2.3 mmol) was added dropwise. The resulting solution was stirred 5 minutes at -78°C, then warmed to 0°C and stirred for 1 hour. The reaction was then quenched by addition of saturated aqueous NH_4Cl, extracted three times with hexane, and the combined hexane extracts were dried and concentrated. The residual 1-undecyne was removed by Kugelrohr distillation (oven temp. 50°C, 0.1 mm Hg), and the residue was purified by vacuum flash chromatography, eluting with hexane, yielding the diyne as a viscous oil (0.38 g, 43%). ^{1}H NMR ($CDCl_3$): δ 2.13-2.16 (m, 8H, H5, H8, H16, H19), 1.45-1.53 (m, 8H, H 4, H9, H15, H20), 1.28-1.42 (m, 24H, H2, H10-14, H21-26), 1.12-1.2 (m, 1H, H3), 0.86-0.91 (m, 9H, H1, H27, methyl). EI-MS, *m/z* (abundance): 357 (M^+-29, 3), 329 (2), 315 (2), 301 (7), 287 (3), 273 (3), 259 (5), 245 (3), 233 (1), 217 (3), 203 (2), 189 (7), 175 (8), 161 (7), 149 (6), 147 (9), 135 (12), 133 (9), 121 (20), 109 (26), 95 (59), 81 (69), 67 (80), 55 (81), 43 (76), 41 (100).

Synthesis of 3-methylheptacosane

A mixture of 3-methylheptacosa-6,17-diyne (0.33 g, 0.68 mmol) and 5% Pd on carbon (100 mg) in 10 ml hexane was stirred under hydrogen for 3 hours. The mixture was then filtered through a plug of Celite, rinsing with hexane, and concentrated to colorless oil, which crystallized to a white solid on standing at room temperature. ^{1}H NMR ($CDCl_3$): δ 1.05-1.35 (m, 49H), 0.84-0.91 (m, 9H). EI-MS, *m/z* (abundance): 365 (M^+-29,7), 337 (1), 336 (1), 253 (1), 225 (1), 211 (1), 197 (1), 183 (2), 169 (2), 155 (3), 141 (3), 127 (4), 113 (5), 99 (9), 85 (26), 71 (48), 57 (100), 43 (80). The spectra were in accord with those previously reported (*53*).

Synthesis of 3-methylnonacosa-6,17-diyne

This compound was made by coupling of 1-tridecyne with the triflate of 14-methylhexadec-10-yn-1-ol, as described above for 3-methylheptacosa-6,17-diyne, in 72% yield. ^{1}H NMR ($CDCl_3$): δ 2.13-2.16 (m, 8H, H5, H8, H16, H19), 1.44-1.53 (m, 8H, H 4, H9, H15, H20), 1.12-1.2 (m, 1H, H3), 1.27-1.42 (m, 28H, H2, H10-14, H21-28), 0.86-0.91 (m, 9H, H1, H29, methyl). EI-MS, *m/z* (abundance): 385 (M^+-29, 4), 357 (2), 329 (6), 315 (2), 273 (3), 259 (5), 203 (3), 189 (9), 175 (9), 161 (8), 147 (9), 135 (12), 133 (10), 121 (19), 109 (28), 95 (58), 81 (68), 67 (81), 55 (81), 43 (93), 41 (100).

Synthesis of 3-methylnonacosane

This compound was made by reduction of 3-methylnonacosa-6,17-diyne, as described above for 3-methylheptacos-6,17-diyne, in quantitative yield. ^{1}H NMR ($CDCl_3$): δ 1.05-1.35 (m, 53H), 0.84-0.91 (m, 9H). EI-MS, *m/z* (abundance): 393 (M^+-29,), 365 (1), 364 (1), 253 (1), 211 (1), 197 (1), 183 (1), 169 (2), 155 (2), 141 (3), 127 (4), 113 (7), 99 (10), 85 (27), 71 (43), 57 (100), 43 (79).

The spectra were in accord with those previously reported (*53*).

TABLES

Table S1. Compounds showing maximal queen-caste specificity in *Vespula vulgaris* and *Cataglyphis iberica* (Cohen's *d* effect size > 2). Compounds used in bioassays are highlighted in red. One queen equivalent (QE) was calculated as the average amount of the compound present on the queen's body surface (in µg). In our bioassays, two queen equivalents of each test compound were added daily to the test colonies.

Species	Rank	Compound	Relative peak areas (mean ± *SD*)			Effect size (Cohen's *d*)
			mother queens (*N*=13)	virgin queens (*N*=17)	workers (*N*= 49)	
Vespula vulgaris	**1**	***n*-C27**	30.00 ± 3.75	12.62 ± 1.67	7.37 ± 2.26	8.62
	2	11,17-, 13,17-, 15,19-diMeC31	1.98 ± 0.43	3.46 ± 2.37	0.38 ± 0.13	7.12
	3	**3-MeC29**	3.30 ± 0.94	2.53 ± 1.92	0.41 ± 0.15	6.55
	4	***n*-C29**	6.46 ± 1.53	5.13 ± 1.16	1.20 ± 0.57	6.16
	5	***n*-C28**	2.22 ± 0.63	1.34 ± 0.35	0.53 ± 0.16	5.35
	6	n-C26	4.43 ± 0.62	2.05 ± 0.36	2.39 ± 0.51	3.82
			mother queens (*N*=7)	virgin queens (*N*=9)	workers (*N*=18)	
Cataglyphis iberica	**1**	***n*-C29**	4.11 ± 1.46	1.30 ± 0.36	0.39 ± 0.24	4.80
	2	5-MeC29	2.00 ± 0.83	0.94 ± 0.28	0.16 ± 0.05	4.33
	3	**3-MeC29**	2.89 ± 1.20	1.73 ± 0.58	0.35 ± 0.13	4.08
	4	11,15-,13,17-diMeC29	3.67 ± 1.86	2.45 ± 1.73	0.30 ± 0.27	3.44
	5	5-MeC27	2.31 ± 1.28	1.01 ± 0.34	0.17 ± 0.11	3.23
	6	5-MeC25	1.64 ± 0.90	0.68 ± 0.39	0.21 ± 0.22	2.89
	7	***n*-C27**	1.68 ± 1.11	1.40 ± 0.76	0.26 ± 0.21	2.38
	8	**3-MeC27**	2.90 ± 2.17	2.18 ± 1.01	0.30 ± 0.19	2.32
	9	3,9-,3,11-diMeC27	3.48 ± 2.86	2.05 ± 1.30	0.32 ± 0.19	2.14
	10	*n*-C15	9.94 ± 7.63	1.80 ± 0.95	1.68 ± 1.04	2.07

Table S2. The effect of pheromone treatments on the proportion of workers with developed or regressed ovaries in the common wasp *Vespula vulgaris*, the buff-tailed bumblebee *Bombus terrestris,* and the Iberian ant *Cataglyphis iberica* as inferred from binomial generalized linear mixed models. Treatment effects were compared with queenless pentane-treated control colonies (intercept, first row). For each treatment level and each significant covariate, the model estimate, standard error, odds ratio, *z* value and *p* value are given. Treatment levels were compared with the pentane-only control using contrasts.

(a) *Vespula vulgaris* - effect on ovary development						
	Estimate	*SE*	Odds ratio	*z* value	*p* value	
(Queenless control)	-0.05	0.26		-0.17	0.86	
3-MeC$_{29}$	-0.82	0.13	0.44	-6.48	9.E-11	***
***n*-C$_{28}$**	-0.19	0.14	0.83	-1.39	0.17	
***n*-C$_{29}$**	-0.72	0.12	0.49	-6.26	4.E-10	***
***n*-C$_{27}$**	-0.71	0.12	0.49	-5.99	2.E-09	***
Colony size	0.00	0.00	1.00	-2.75	0.01	**
Number of males	0.00	0.00	1.00	-2.20	0.03	*
(b) *Vespula vulgaris* - effect on ovary regression						
	Estimate	*SE*	Odds ratio	*z* value	*p* value	
(Queenless control)	-3.85	0.44		-8.67	2.E-16	***
3-MeC$_{29}$	0.64	0.26	1.89	2.47	0.01	*
***n*-C$_{28}$**	0.73	0.26	2.08	2.86	0.004	**
***n*-C$_{29}$**	1.34	0.22	3.81	6.07	1.E-09	***
***n*-C$_{27}$**	0.69	0.25	1.99	2.73	0.007	**
(c) *Bombus terrestris* - effect on ovary development						
	Estimate	*SE*	Odds ratio	*z* value	*p* value	
(Queenless control)	-2.81	0.61		-4.57	5.E-06	***
Eicosyl oleate	0.03	0.36	1.03	0.08	0.93	
Docosyl oleate	-0.35	0.41	0.70	-0.86	0.39	
Tetracosyl oleate	0.30	0.37	1.35	0.83	0.41	
Hexacosyl oleate	0.38	0.33	1.46	1.15	0.25	
n-C$_{25}$	-0.37	0.40	0.69	-0.93	0.35	
Queenright control	-2.11	0.60	0.12	-3.51	0.0004	***
(d) *Bombus terrestris* - effect on ovary regression						
	Estimate	*SE*	Odds ratio	*z* value	*p* value	
(Queenless control)	-0.86	0.37		-2.31	0.02	*
Eicosyl oleate	0.21	0.28	1.24	0.76	0.45	
Docosyl oleate	-0.20	0.30	0.82	-0.66	0.51	

Tetracosyl oleate	-0.39	0.31	0.68	-1.26	0.21	
Hexacosyl oleate	0.12	0.28	1.12	0.41	0.68	
***n*-C_{25}**	0.86	0.28	2.36	3.05	0.002	**
Queenright control	1.10	0.26	3.02	4.21	3.E-05	***
Colony size	-0.01	0.00	0.99	-3.32	0.0009	***
(e) *Cataglyphis iberica* - effect on ovary development						
	Estimate	*SE*	Odds ratio	*z* value	*p* value	
(Queenless control)	0.10	0.70		0.14	0.89	
3-MeC_{27}	-0.68	0.43	0.50	-1.59	0.11	
3-MeC_{29}	-2.01	0.62	0.13	-3.27	0.001	**
***n*-C_{27}**	-1.88	0.58	0.15	-3.23	0.001	**
***n*-C_{29}**	-1.78	0.56	0.17	-3.16	0.002	**
Colony size	-0.05	0.02	0.95	-3.05	0.002	**
(f) *Cataglyphis iberica* - effect on ovary regression						
	Estimate	*SE*	Odds ratio	*z* value	*p* value	
(Queenless control)	-1.96	0.79		-2.50	0.01	*
3-MeC_{27}	0.62	0.62	1.87	1.01	0.31	
3-MeC_{29}	1.12	0.53	3.07	2.10	0.04	*
***n*-C_{27}**	1.49	0.53	4.42	2.83	0.005	**
***n*-C_{29}**	0.56	0.58	1.75	0.97	0.33	
Colony size	-0.04	0.02	0.96	-2.75	0.006	**

Table S3. Evidence for the identity of particular compounds or compound classes being used as queen or fertility signals in different groups of social insects (compound classes: FA=fatty acids, KA=keto acids, LA=linear alkanes, MA=methyl-alkanes, UH=unsaturated hydrocarbons, E=esters, L=lactones, SA=saturated alcohols, AA=aromatic alcohol, AL=aldehydes, T=terpenes, TA=terpene alcohols; type of evidence: C=correlational, BS=bioassays of specific compounds, BC=bioassays of particular compound classes, BE=bioassays of glandular or cuticular extracts). Species in which fertility-suppressing queen pheromones have been experimentally identified, as well as the compounds themselves, are highlighted in red. The species examined in the present study are underlined and in bold. Note that queen compounds that induced retinue behaviour in workers but which did not cause inhibition of worker ovary development, as well as species in which workers are obligately sterile such that queen pheromones obviously play no role in suppressing worker reproduction, are not listed.

Species	Identified queen pheromones or compounds overrepresented in queens or dominant egg-layers (source)	Compound classes	Type of evidence	References
HALICTINE BEES				
Lasioglossum malachurum	*n*-C_{21}, *n*-C_{23}, *n*-C_{27}, *n*-C_{29}, 7+9-$C_{23:1}$, 7+9-$C_{25:1}$, 7+9-$C_{27:1}$, 20-eicosanolide, 22-docosanolide, 24-tetracosanolide, ethyl eicosenoate, docosenoic acid, tetracosanoic acid (cuticle of nesting vs. virgin females)	FA, LA, UH, E, L	C	(*54*)
CORBICULATE BEES				
Apis mellifera[a]	(*E*)-9-oxo-2-decenoic acid (9-ODA) (queen mandibular gland), ethyl palmitate, methyl linoleate (queen mandibular gland and cuticle and salivary glands of young female larvae), $C_{23:1}$ to $C_{37:1}$ and $C_{31:2}$ to $C_{37:2}$ (specific isomers, only odd-chain) , palmitic acid, methyl palmitate, (*Z*)-9-octadecenoic acid (cuticle and tergal glands of mated queens), (*E*)-β-ocimene (mated, egg-laying queens and young female larvae)	KA, FA, UH, E, T	C, BS, BE	(*2, 16, 19, 22, 55-71*)
Bombus hypnorum	branched alkanes, alkenes, alkadienes, geranyl citronellol (cuticle of queens vs. workers)	MA, UH, TA	C	(*72*)
<u>***Bombus terrestris***</u>	*n*-C_{21} to *n*-C_{29} (only odd-chain), $C_{27:1}$, $C_{29:1}$, $C_{29:2}$, 9+11-MeC_{21}, decyl tetradecanoate, dodecyl dodecanoate, dodecyl hexadecenoate, dodecyl octadecenoate (queen mandibular gland of mated egg-laying queens vs. virgin non-laying queens), *n*-C_{25}, triacontenal, dotriacontenal, eicosyl oleate, docosyl oleate, tetracosyl oleate, hexacosyl oleate	LA, MA, UH, E, AL	C, BE, BC, BS	(*21, 73-78*); this study

	(cuticle of queens vs. nonreproductive workers), *n*-$C_{23:1}$ to *n*-$C_{31:1}$ (only odd-chain)			
Friesella schrottkyi	*n*-C_{21}, *n*-C_{23}, *n*-C_{25}, 11+5-MeC_{25}, *n*-C_{26}, *n*-C_{27}, 11-MeC_{27}, 11,15+5,17-diMeC_{27}, 11-MeC_{28}, *n*-C_{29}, 11+13-MeC_{29}, 13,15+5,19-diMeC_{29} (cuticle of egg-laying queens vs. workers)	LA, MA	C, BE	(*79, 80*)
Melipona bicolor	C_{12}OH, 5,11+5,19+11,13-diMeC_{25}, $C_{26:1}$, *n*-C_{26}, 11,13,15-triMeC_{27}, 5-MeC_{27}, *n*-C_{28}, 10+14+15-MeC_{28}, 11,13,15-triMeC_{29}, 5+15-MeC_{29}, *n*-C_{30}, $C_{31:1}$, *n*-C_{32} (cuticle of egg-laying queens vs. virgin, non-egg-laying queens and workers	LA, MA, UH, SA	C	(*81*)
Melipona scutellaris	*n*-C_{23}, stearyl acetate, arachidyl acetate, ethyl oleate (cuticle of virgin queens vs. workers)	LA, E	C	(*82*)
Schwarziana quadripunctata	*n*-C_{26}, *n*-C_{27} (cuticle of virgin queens vs. workers)	LA	C	(*83*)
STENOGASTRINE WASPS				
Eustenogaster fraterna	*n*-C_{23} (cuticle of egg-layers vs. non-egg-layers)	LA	C	(*84*)
Liostenogaster flavolineata	C_{20}OH, *n*-C_{23}, *n*-C_{24}, *n*-C_{25} (cuticle of egg-layers vs. non-egg-layers)	LA, SA	C	(*84*)
Liostenogaster vechti	$C_{31:1}$ (cuticle of egg-layers vs. non-egg-layers)	UH	C	(*84*)
Parischnogaster striatula	*n*-C_{26}, *n*-C_{27}, *n*-C_{28}, *n*-C_{29} (cuticle of egg-layers vs. non-egg-layers)	LA	C	(*84*)
POLISTINE AND VESPINE WASPS				
Dolichovespula maculata	$C_{27:1}$, $C_{29:1}$ (cuticle of queens vs. workers)	UH	C	(*85*)
Dolichovespula saxonica	*n*-C_{29}, *n*-C_{31}, 3-MeC_{29}, 3-MeC_{31} (cuticle of queens vs. workers)	LA, MA	C	(*86, 87*)
Polistes dominulus	*n*-C_{29}, *n*-C_{31}, 11+13-MeC_{27}, 5-MeC_{29} (cuticle and eggs of dominant foundresses vs. subordinate foundresses), 9-$C_{29:1}$, 9-$C_{31:1}$, $C_{33:2}$, $C_{35:2}$ (cuticle of dominant vs. subordinate foundresses), *n*-C_{31}, 2-MeC_{32}, *n*-C_{33}, 7+13+15+17-MeC_{33} (cuticle of foundresses vs. workers)	LA, MA, UH	C	(*88-91*)
Polistes gallicus	*n*-C_{30}, *x*-MeC_{31}, 11,17+9,19-diMeC_{31}, 3,13-diMeC_{31}, 13,15+ 9,11+11,21-diMeC_{33} (queen vs. worker Van der Vecht organ secretion)	LA, MA	C	(*92*)
Polistes metricus	*n*-C_{29}, 9+11+13+15-MeC_{29} (cuticle of queens vs. workers)	LA, MA	C	(*93*)
Polistes satan	*n*-C_{25}, *n*-C_{29} (cuticle of dominant egg-layers vs. subordinates)	LA	C	(*94*)
Ropalidia marginata	3+5-MeC_{29}, 3+7+9-MeC_{31}, 13+15+17-MeC_{33} (Dufour's gland of queens vs. workers)	MA	C, BE	(*95-97*)
Vespa crabro	*n*-C_{24}, *n*-C_{26}, *n*-C_{27}, 3-MeC_{27}, *n*-C_{29} (cuticle of queens vs. workers)	LA, MA	C	(*85, 98*)
Vespula maculifrons	*n*-C_{29}, 3-MeC_{29}, *n*-C_{31}, 3-MeC_{31} (cuticle of queens vs. workers)	LA, MA	C	(*85*)
Vespula squamosal	13+15-MeC_{29} (cuticle of queens vs. workers)	MA	C	(*85*)

Vespula vulgaris	*n*-C_{27}, *n*-C_{28}, *n*-C_{29}, 3-MeC_{27}, 3-MeC_{29}, 11,17-, 13,17-, 15,19-diMeC_{31} (cuticle of queens vs. workers)	LA, MA	C, BS	(*20*); this study
ANTS				
Aphaenogaster cockerelli	*n*-C_{25}, *n*-C_{23}, *n*-C_{26}, *n*-C_{27} (cuticle of queens vs. workers)	LA	C, BS	(*8, 99*)
Aphaenogaster senilis	3-MeC_{27}, 3,7+3,9-diMeC_{27}, 4,8+4,10-diMeC_{28} , 5-MeC_{29}, *n*-C_{30}, 10+12-MeC_{30} (postpharyngeal gland secretion of queens vs. workers), 3,11+3,9+3,7-diMeC_{29}, 3,11+3,9-diMeC_{31} (queen-laid vs. worker-laid eggs)	LA, MA	C	(*100, 101*)
Camponotus floridanus	9+11+13-MeC_{27}, 11,15-diMeC_{27}, 3-MeC_{27}, 9+11+13+15-MeC_{29}, 13,17+11,15+9,13-diMeC_{29}, 3-MeC_{29} (cuticle of queens vs. workers), *n*-C_{25}, *n*-C_{27}, 3-MeC_{27}, *n*-C_{28}, *n*-C_{29}, 3-MeC_{29} (queen-laid eggs vs. worker-laid eggs)	LA, MA	C	(*6, 102*)
Camponotus textor	*n*-C_{31}, *n*-C_{32}, *n*-C_{33}, *n*-C_{35} (cuticle of queens vs. workers)	LA	C	(*103*)
Camponotus vagus	4-MeC_{28}, *n*-C_{30}, 4-MeC_{30}, x,y-diMeC_{31} (cuticle of egg-laying vs. non-egg-laying queens)	LA, MA	C	(*104*)
Cardiocondyla obscurior	3-MeC_{25}, 11+13-MeC_{27}, 5-MeC_{27}, 3-MeC_{27}, and 12+14-MeC_{28} (cuticle of virgin queens vs. mated queens)	MA	C	(*105*)
Cataglyphis iberica	*n*-C_{27}, *n*-C_{29}, 3-MeC_{29}, 5-MeC_{25}, 5-MeC_{27}, 5-MeC_{29}, 11,15-,13,17-diMeC_{29} (cuticle of queens vs. workers)	LA, MA	C, BS	(*106*); this study
Crematogaster smithii	9-$C_{23:1}$, 9-$C_{27:1}$, $C_{29:1}$, *n*-C_{29}, *n*-C_{31} (cuticle of queens & intermorphs vs. workers)	LA, UH	C	(*107*)
Diacamma ceylonense	3+9+11+13-MeC_{25}, 3+5+7+9+11+13-MeC_{27}, *n*-C_{29} (cuticle of egg-layers vs. non-egg-layers)	LA, MA	C	(*108*)
Dinoponera quadriceps	9-$C_{31:1}$ (eggs of dominant egg-layers vs. subordinates), 9-$C_{31:1}$, 9-$C_{33:1}$ (cuticle of dominant egg-layers vs. subordinates)	UH	C	(*109-111*)
Ectatomma tuberculatum	*n*-C_{27}, 5-MeC_{27}, *n*-C_{28}, *n*-C_{29} (cuticle of queen with vs. without developed ovaries)	LA, MA	C	(*112*)
Formica fusca	$C_{23:1}$ to $C_{35:1}$ (only odd-chain), *n*-C_{30} to n-C_{35} (cuticle of queens vs. workers), 5-MeC_{25}, 5,13+9,13-diMeC_{25} (cuticle of more fecund queens vs. less fecund queens)	LA, MA, UH	C	(*113, 114*)
Gnamptogenys striatula	3-MeC_{29}, 3,15-diMeC_{29}, 3,*x*-diMeC_{31}, 4,*x*-diMeC_{32}, 3,*x*-diMeC_{33}, 4,*x*-diMeC_{34}, 3,*x*-diMeC_{35}, 3,11,15-triMeC_{35}, 3,*x*-diMeC_{37}, 3,11,15-triMeC_{37} (cuticle of queens vs. workers)	MA	C	(*115*)
Harpegnathos saltator	13,23-diMeC_{37}, $C_{33:1}$, $C_{35:1}$ (cuticle of mated egg-layers vs. workers)	MA, UH	C	(*116*)
Hypoponera opacior	*n*-C_{29}, 2+13-MeC_{30}, $C_{31:1}$ (cuticle of queens vs. workers)	LA, MA, UH	C	(*117*)
Lasius emarginatus	3-MeC_{29}, 3-MeC_{31}, 13,17+11,15+9,13+7,11-diMeC_{35} (cuticle of queens vs. workers)	MA	C	(*4*)
Lasius flavus	3-MeC_{31}, *n*-C_{29}, 3-MeC_{29}, *n*-C_{30}, $C_{31:1}$, *n*-C_{31} (cuticle of queens vs. workers)	LA, MA, UH	C, BS	(*4*)
Lasius fuliginosus	*n*-C_{25}, 5-MeC_{29}, 3-MeC_{29}, 3,7-diMeC_{29}, $C_{31:1}$, 15+13+11+9+MeC_{31}, 7-MeC_{31}, 5-MeC_{31}, 3-MeC_{31}, 3,7+3,9+3,11+diMeC_{31} (cuticle of queens vs. workers)	LA, MA, UH	C	(*4*)
Lasius grandis	*n*-C_{27}, 3-MeC_{27}, 8+6-MeC_{28}, 3-MeC_{29} (cuticle of queens vs. workers)	LA, MA	C	(*4*)

Lasius lasioides	3-MeC_{33}, 4-MeC_{34}, $C_{35:1}$, 5-MeC_{35}, 3-MeC_{35} (cuticle of queens vs. workers)	MA, UH	C	(*4*)
Lasius neglectus	*n*-C_{27}, *n*-C_{29}, 3-MeC_{29}, $C_{31:1}$, *n*-C_{31}, 3-MeC_{31}, *n*-C_{33}, 3-MeC_{33}, $C_{35:1}$ (cuticle of queens vs. workers)	LA, MA, UH	C	(*4*)
Lasius niger	3-MeC_{31}, *n*-C_{29}, *n*-C_{31}, $C_{33:1}$, $C_{31:1}$ (cuticle of queens vs. workers)	LA, MA, UH	C, BS	(*3, 118, 119*)
Lasius piliferus	n-C_{29}, 3-MeC_{29}, 3-MeC_{31} (cuticle of queens vs. workers)	LA, MA	C	(*4*)
Lasius platythorax	3-MeC_{29}, 3-MeC_{31}, 13+11+9+7-MeC_{33}, 11,15+9,13-diMeC_{33}, 3-MeC_{33}, 13+11+9+7-MeC_{37} (cuticle of queens vs. workers)	MA	C	(*4*)
Lasius psammophilus	$C_{29:1}$, 3-MeC_{29}, *n*-C_{31}, 3-MeC_{31} (cuticle of queens vs. workers)	LA, MA, UH	C	(*4*)
Lasius umbratus	$C_{25:1}$, *n*-C_{29}, 5-MeC_{29}, 3-MeC_{29}, *n*-C_{31}, 5-MeC_{31}, 3-MeC_{31} (cuticle of queens vs. workers)	LA, MA, UH	C	(*4*)
Leptothorax gredleri	n-C_{24}, n-C_{26}, n-C_{29} (cuticle of recently mated vs. unmated queens and cuticle of queens vs. workers)	LA	C	(*120*)
Linepithema humile	5-MeC_{27}, $C_{29:1}$, 5-MeC_{29}, 5-MeC_{30}, $C_{31:1}$, 5-MeC_{31}, 5-MeC_{32}, $C_{33:1}$, 5-MeC_{33}, 5-MeC_{34} (cuticle of mated, laying queens vs. unmated queens or workers)	MA, UH	C	(*121*)
Myrmecia gulosa	9-$C_{25:1}$, 3-MeC_{25} (cuticle of queens and reproductive workers vs. nonreproductive workers)	MA, UH	C	(*122*)
Odontomachus brunneus	(Z)-9-$C_{29:1}$, (Z)-9-$C_{31:1}$ (cuticle of reproductives including queens vs. sterile workers)	UH	C, BS	(*9*)
Pachycondyla inversa	3,9-diMeC_{25}, 3,11+5,11+11,15-diMeC_{27}, 3,11-diMeC_{29}, 5+7-MeC_{27} (cuticle of queens vs. workers), 3,11-diMeC_{27}, 3-MeC_{27}, *n*-C_{28}, *n*-C_{29}, 3-MeC_{29} (queen-laid vs. worker-laid eggs)	LA, MA	C	(*123-125*)
Pachycondyla verenae	$C_{23:1}$, $C_{25:1}$, $C_{27:1}$ (cuticle of queens vs. workers)	UH	C	(*126*)
Pachycondyla villosa	2-MeC_{26}, 9+11+13-MeC_{27}, 3-MeC_{27}, 9+11+13+15-MeC_{29} (cuticle of queens vs. workers)	MA	C	(*127*)
Platythyrea punctata	2+3+5+7+9+11-MeC_{23}, *n*-C_{25}, 2+7-MeC_{25} (cuticle of reproductives vs. nonreproductives)	LA, MA	C	(*128*)
Streblognathus peetersi	$C_{33:1}$, $C_{35:2}$, $C_{35:1}$, $C_{37:2}$, $C_{37:1}$, $C_{39:2}$ (cuticle of egg-layers vs. non-egg-layers)	UH	C	(*129, 130*)
Temnothorax affinis	9+11+13+15-MeC_{29}, 4-MeC_{32}, 9+11+13+15-MeC_{33}, *x,y*-diMeC_{33} (cuticle of queens vs. workers)	MA	C	(*37*)
Temnothorax crassispinus	3-MeC_{26}, 9+11+13-MeC_{27}, 11,15-diMeC_{27}, 8-MeC_{28}, *x,y*-diMeC_{29}, 3-MeC_{31}, *x,y*-diMeC_{33} (cuticle of queens vs. workers)	MA	C	(*37*)
Temnothorax lichtensteini	5+7-MeC_{29}, 3,*x*-diMeC_{29}, 4-MeC_{30}, 9+11+13+15-MeC_{33}, 3,*x*-diMeC_{33} (cuticle of queens vs. workers)	MA	C	(*37*)
Temnothorax nylanderi	3+4-MeC_{26}, 7-MeC_{27}, 11,15-diMeC_{27}, 3-MeC_{28}, 5,*x*-diMeC_{29} (cuticle of queens vs. workers)	MA	C	(*37*)
Temnothorax recedens	5-MeC_{27} , *n*-C_{31}, 3+9+11+13+15-MeC_{33} (cuticle of queens vs. workers)	LA, MA	C	(*37*)
Temnothorax unifasciatus	3,*x*-diMeC_{33} (cuticle queens vs. workers), *n*-C_{31} (queen-laid vs. worker-laid eggs)	LA, MA	C	(*37, 131*)

TERMITES				
Cryptotermes secundus	4-MeC_{27}, 4-MeC_{28}, $C_{29:1}$, 3+4-MeC_{29}, 4-MeC_{30}, $C_{31:1}$, *n*–C_{31}, 3-MeC_{31}, $C_{33:1}$, $C_{35:1}$, $C_{35:2}$ (cuticle of queens vs. workers)	LA, MA, UH	C	(*132*)
Nasutitermes takasagoensis	Phenylethanol (queen-specific volatile)	AA	C	(*133*)
Reticulitermes flavipes	9-$C_{25:1}$ (cuticle of supplementary reproductives vs. workers)	UH	C	(*134*)
Reticulitermes speratus	*n*-butyl-*n*-butyrate, 2-methyl-1-butanol (queen and egg volatiles)	E, SA	BS, BE	(*5, 135-137*)
Zootermopsis nevadensis	6,9-$C_{29:2}$, 6,9-$C_{31:2}$, 6,9,17-$C_{32:3}$, 6,9,17-$C_{33:3}$ (cuticle of queens and kings vs. workers)	UH	C	(*138*)

[a] Only the most thoroughly studied Western honeybee, *Apis mellifera*, is included here, although there is evidence that the queen pheromones in Asian honeybees are structurally similar to those of Western honeybees (*62, 139-142*).

Table S4. Data on the volatility of queen pheromones of social insects in terms of their ability to inhibit worker reproduction (type of evidence: CP=based on the known chemical properties of identified queen pheromones, SM and DM= based on experiments in which the inhibition of worker reproduction was measured after separating the queen from the workers with a single or double mesh).

Species	Volatility of active queen pheromone compounds	Type of evidence	References
CORBICULATE BEES			
Apis mellifera	volatile and non-volatile	CP, SM+DM	(*2, 19, 56, 67, 143-145*)
Bombus terrestris	non-volatile	CP, SM+DM	(*146, 147*); this study
Bombus lapidarius	non-volatile	DM	(*148*)
POLISTINE AND VESPINE WASPS			
Ropalidia marginata	non-volatile	SM	(*149*)
Vespula atropilosa	non-volatile	SM+DM	(*150*)
Vespula pennsylvanica	non-volatile	SM+DM	(*151*)
Vespula vulgaris	non-volatile	CP, SM+DM	(*151*); this study
ANTS			
Aphaenogaster cockerelli	non-volatile	CP	(*8*)
Aphaenogaster senilis	non-volatile	SM+DM	(*100*)
Aphaenogaster smythiesi	non-volatile	DM	(*152*)
Cataglyphis iberica	non-volatile	CP	this study
Diacamma sp.	non-volatile	SM	(*153*)
Gnamptogenys menadensis	non-volatile	SM	(*154*)
Harpegnathos saltator	non-volatile	DM	(*155*)
Lasius flavus	non-volatile	CP	(*4*)
Lasius niger	non-volatile	CP, SM+DM	(*3, 118*)
Leptothorax acervorum	non-volatile	SM	(*156*)
Myrmecia gulosa	non-volatile	DM	(*157*)
Odontomachus brunneus	non-volatile	CP	(*9*)
Ophthalmopone berthoudi	non-volatile	DM	(*158*)
Pachycondyla apicalis	non-volatile	DM	(*159*)
Pachycondyla inversa	non-volatile	SM	(*160*)
Temnothorax longispinosus	non-volatile	SM	(*161*)

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Table S5. Results of the maximum likelihood ancestral state reconstructions. Data show the likelihoods (in %) of different compound classes being used as ancestral fertility signals in different clades, assuming either a flat prior for the root states (a) or a root prior equal to the observed tip frequencies (b).

Clades	Saturated hydrocarbons	Unsaturated hydrocarbons	Esters	Saturated alcohols	Fatty acids	Keto acids	Aldehydes	Terpenes	Terpene alcohols
(a) Using a flat prior for the root states									
Corbiculate bees	83.41%	55.74%	45.51%	0.01%	14.71%	14.48%	0.01%	14.48%	0.01%
Stenogastrine wasps	99.56%	19.60%	0.03%	0.02%	0.01%	0.00%	0.00%	0.00%	0.00%
Polistine and vespine wasps	98.43%	43.47%	0.29%	0.05%	0.06%	0.01%	0.01%	0.01%	0.01%
Ants	81.43%	50.00%	3.05%	0.57%	0.59%	0.13%	0.13%	0.13%	0.13%
All clades	69.01%	50.00%	14.35%	1.42%	3.71%	0.31%	0.31%	0.31%	0.31%
(b) Using a root prior equal to the observed tip frequencies									
Corbiculate bees	97.79%	58.58%	82.23%	0.00%	49.79%	0.05%	0.00%	0.05%	0.00%
Stenogastrine wasps	99.91%	19.46%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Polistine and vespine wasps	99.72%	43.23%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Ants	93.64%	50.00%	1.24%	0.03%	0.14%	0.00%	0.00%	0.00%	0.00%
All clades	99.47%	29.12%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

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