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Animal behaviour

A social insect fertility signal is dependent on chemical context

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Identifying group members and individuals' status within a group are fundamental tasks in animal societies. For ants, this information is coded in the cuticular hydrocarbon profile. We manipulated profiles of the ant *Odontomachus brunneus* to examine whether the releaser and primer effects of fertility signals are dependent on chemical context. Fertility status is signalled through increased abundance of (*Z*)-9-nonacosene ($Z9:C_{29}$). Across the ant's distribution, populations have distinct hydrocarbon profiles but the fertility signal is conserved. Foreign queens and fertility-signal-treated workers from the same population, sharing a similar chemical background, elicited releaser effects from workers, whereas queens and fertility-signal-treated workers from different populations did not. $Z9:C_{29}$ presented without chemical background did not elicit releaser effects. A primer-effect experiment found that $Z9:C_{29}$, presented without a chemical background, did not inhibit worker reproduction. Our results demonstrate that a familiar chemical background is necessary for appropriate responses to fertility signals.

1. Introduction

Social behaviour depends on identifying group members who benefit from mutual cooperation [1,2]. In large societies where unfamiliar individuals encounter one another, phenotypic 'tags' can be used to indicate relatedness or group membership [3]. For example, in human evolution, hunter–gatherer bands may have used tags such as shared accents of identifiable vocalizations or proto-languages [4,5]. To interpret group membership, the receiver of these signals must first recognize the vocalization and then the accent nested within. Like humans, social insects often form large societies, wherein individual-level recognition is not possible. Instead, colony members share a gestalt nestmate chemical profile [6,7]. This cuticular hydrocarbon profile consists of approximately 10–40 long-chained (C_{25}–C_{35}) hydrophobic lipids that coat the insect cuticle. Within this profile, a subset of compounds signal an individual's fertility status [8]. Social insects, therefore, compose model systems for studying nested signals of group membership and individual status.

Social insects are thought to first assess group membership, then make more detailed assessments related to division of labour or reproductive status ([9], but see [10]). This hypothesis remains largely untested owing to the small number of experimentally identified signals of caste or reproductive status. Experimental studies identifying the primer effect of queen hydrocarbon fertility signals in which a single compound is introduced to workers suggest that compounds without chemical background can mimic the presence of a queen by inhibiting worker ovarian development [11–13]. However, these compounds occur naturally only in the context of the entire chemical profile of an individual or an egg. By contrast, experimental studies of the releaser effect of fertility signals have supplemented the cuticular profile of non-reproductive workers with single compounds correlated with fertility [14,15]. These treatments evoke aggressive reactions (policing) from nestmate workers, inhibiting the potential reproductive efforts of their nestmates.

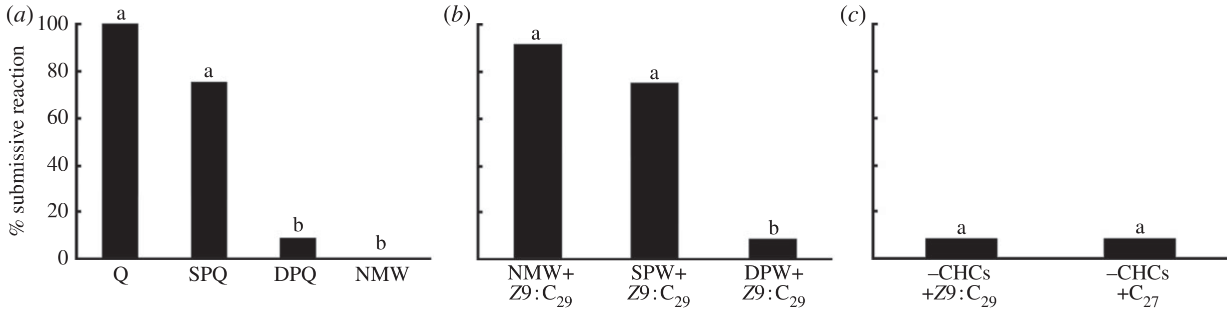

Figure 1. Releaser-effect experimental results showing submissive reactions of test workers to (*a*) queens, (*b*) workers treated with the fertility signalling compound *Z*9 : C_{29} and (*c*) workers stripped of their hydrocarbon profiles and treated with *Z*9 : C_{29} or C_{27}. (*a*: Cochran's *Q*-test, $Q = 24.64$, $p < 0.001$; same-population queen (SPQ) versus different population queen (DPQ) $p = 0.013$, SPQ versus nestmate worker (NMW) $p = 0.007$, nestmate queen (Q) versus DPQ $p = 0.002$, Q versus NMW $p = 0.008$, Q versus SPQ $p = 0.24$, DPQ versus NMW $p = 1$). (*b*: Cochran's *Q*-test, $Q = 15.27$, $p < 0.001$; NMW versus same-population worker (SPW) $p = 0.61$, SPW versus different population worker (DPW) $p = 0.013$, NMW versus DPW $p = 0.004$). (*c*: $z = 0$, $p = 1$). $N = 12$ for all groups.

Cuticular hydrocarbon fertility signals have been experimentally identified in only seven species [13]. Of these, the trap-jaw ant *Odontomachus brunneus* is an ideal system to study the effect of chemical background on signal perception. This species is distributed throughout the southeastern United States, and populations have specific qualitative and quantitative differences in cuticular hydrocarbon compound presence and abundances [16]. Nevertheless, different populations signal fertility using the same compound, (*Z*)-9-nonacosene (*Z*9 : C_{29}), which is relatively more abundant in the profile of reproductive queens and reproductive workers than in non-reproductives [15,16]. Population variation of the overall profile and the conserved fertility signal allow us to examine the influence of a group membership signal on the perception of a signal of individual status. We measured the releaser-effect reaction of workers to nestmate and non-nestmate queens of differing hydrocarbon profiles, non-reproductive nestmate and non-nestmate workers treated with *Z*9 : C_{29}, and *Z*9 : C_{29} without a hydrocarbon profile background. We also tested for primer effects by introducing *Z*9 : C_{29} to isolated groups of workers and monitoring their reproductive activity, relative to various controls.

2. Material and methods

Colonies were collected from three populations in Florida: Archbold Biological Station in Venus, West Palm Beach and Withlacoochee State Forest in Lecanto. (See the electronic supplementary material for expanded methods.)

(a) Releaser-effect experiments

Test workers originated from 12 Archbold colonies and were harnessed in a 5 × 5 cm paper restraint. Stimuli were presented to restrained workers by contacting their antennae and measuring antennal retraction. Antennal retraction is a releaser-response, a stereotypical submissive posture of *O. brunneus* worker display in proximity to a reproductive queen or worker [15–17]. Each worker was presented with a stimulus three times and the consensus reaction of that worker (submissive or non-submissive reaction in at least two of the three trials) was recorded. Experiment 1 exposed workers to nestmate queens, non-nestmate same-population queens, non-nestmate different population queens and nestmate non-reproductive workers as a control. Experiment 2 exposed workers to *Z*9 : C_{29}-treated (i) non-reproductive nestmate workers, (ii) non-reproductive non-nestmate workers from the same population and (iii) non-reproductive non-nestmate workers from a different population. Experiment 3 exposed workers to nestmate workers whose hydrocarbon profiles had been removed (through successive hexane washes) and which had been treated with *Z*9 : C_{29} or a hydrocarbon control (heptacosane, C_{27}). Thirty microlitres of a hydrocarbon working solution (0.125 mg ml^{-1} in hexane; 3.75 µg of hydrocarbon) were used for all experiments as per previous bioassays with this species [15]. Each experiment used a different set of workers, and stimuli were presented in random order. (See the electronic supplementary material for an additional releaser-effect experiment.)

Trials were video recorded, with videos given a coded title assuring that the data recorder was blind to the treatments. Data were analysed using Cochran's *Q*-test, and sign tests were used for pairwise comparisons with Holm–Bonferroni adjusted significance levels for pairwise comparisons. All statistics were performed using STATISTICA v. 7 software (StatSoft, USA).

(b) Primer-effect experiment

Thirteen colonies from the Archbold population were split into three equal-sized queenless groups (mean group size 21; min. 12, max. 34), which were housed in a single 60 × 15 mm Petri dish nest with a moistened dental plaster floor, within a 19 × 13.5 cm arena. Treatments consisted of adding *Z*9 : C_{29} or heptacosane (3.75 µg doses), or hexane (control) to glass coverslips. A single coverslip was added to each colony and replaced daily. Groups were fed sugar water ad libitum and 2–3 termites per day. Each nest was inspected daily for the presence of worker-laid eggs. Successful worker egg-laying was classified according to the date eggs first appeared and remained present in the nest for at least 48 h. This ensured that the eggs were not trophic (shared as food between nestmates) or policed (destroyed). All groups were given 45 days to lay eggs. The likelihood that worker groups successfully laid eggs was compared between treatments. For colonies in which all treatment groups laid eggs (8/13), the number of days until egg-laying was compared, within colonies, by a Friedman ANOVA test.

3. Results

(a) Releaser-effect experiments

A larger percentage of workers showed submissive reactions when they were presented with nestmate queens and non-nestmate queens from the same population than when presented queens from different populations or nestmate workers (figure 1*a*). Worker submissive reactions were more frequent in response to nestmate and foreign workers from the same population treated with the fertility signal, than to fertility-signal-treated workers from a different population

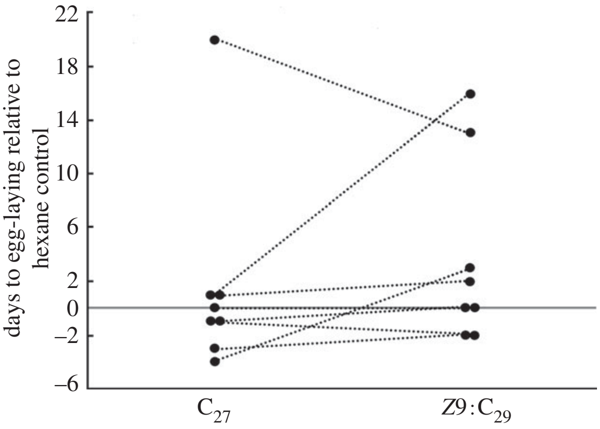

Figure 2. Tests of primer effects of the pure compounds C_{27} (hydrocarbon control) and $Z9:C_{29}$ showing days to egg-laying, relative to the hexane control group (zero). Dotted lines connect data from the same colonies, Friedman ANOVA $\chi^2 = 1.6$, $p = 0.46$.

(figure 1*b*). By contrast, submissive reactions of workers to nestmates stripped of hydrocarbons and then treated with $Z9:C_{29}$ were not different from the responses displayed to the C_{27}-treated controls (figure 1*c*; electronic supplementary material contains similar results from an additional experiment with isolated compounds).

(b) Primer-effect experiment

Treatment type did not influence the probability that queenless worker groups would lay eggs (number of groups egg-laying per treatment out of 13 possible: $Z9:C_{29} = 10$, $C_{27} = 12$, hexane = 10; Cochran *Q*-test $Q = 4$, $p = 0.13$). Across the entire experiment, the average number of days until worker egg-laying was 23 (min. = 10, max. = 44). Survival of initial worker populations up to the point of egg-laying averaged 98% (min. = 87%, max. = 100%). A total of eight of 13 colonies had all worker groups lay eggs and were therefore directly comparable across all treatments. There was no effect of treatment on differences in days until worker egg-laying (figure 2).

4. Discussion

Our results indicate that the fertility signal of *O. brunneus* requires a familiar or near-nestmate chemical background to be perceived properly as a fertility signal. This result contrasts with other primer-effect experiments, wherein a compound presented without the normal chemical background inhibited worker ovarian development [11–13].

$Z9:C_{29}$ is present on the cuticles of non-reproductive *O. brunneus* workers and brood [16,18]. The numbers of workers and brood vary seasonally and throughout the colony life cycle [19]. Thus, assessing queen fertility by the total amount of fertility signal present in the colony is unreliable. Instead, encountering a complete hydrocarbon profile in which the fertility signalling compound is present in a relative abundance unique to reproductive individuals is likely to be a more reliable indicator of queen presence and fertility.

Our results are in agreement with the hypothesis of hierarchical assessments of hydrocarbon profiles, wherein recognition of nestmate or near-nestmate signals precedes caste and task-specific recognition [9]. Additional evidence of the importance of a chemical background comes from social parasites that invade colonies to usurp the role of the queen. For example, the bumblebee, *Bombus terrestris*, can be parasitized by congeners *B. vestalis* and *B. bohemicus*, whose hydrocarbon profiles mimic the complete chemical profile of the host queen and suppress host worker ovarian development [20,21]. If a single compound were sufficient for mimicking a queen, social parasites would not need to replicate or obtain more complete profiles and an individual with a profile lacking nestmate signals but displaying the correct fertility signals would be an omnipotent parasite of the host species. However, resistance to parasitism in ants and wasps is thought to evolve by increasing the diversity and complexity of the nestmate recognition signals coded in the cuticular profiles, supporting the notion that a complex chemical background is advantageous because it inhibits mimicry [22].

Interpreting nested signals is not a challenge unique to social organisms. For example, a male parasitoid wasp chooses mates based on one or a small subset of female-specific cuticular hydrocarbons. Bioassays have shown that courtship behaviour is not elicited by single, sex-specific compounds, but rather requires other non-sex-specific compounds as a chemical background [23]. Further research in other systems examining context-specific signals of individual status will broaden our understanding of the evolution of cooperation.

Data accessibility. All data are available in the electronic supplementary material.

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Author contributions. A.A.S. designed and performed the experiments, analysed data and wrote the manuscript. J.G.M. synthesized the chemicals and wrote the manuscript. A.V.S. designed the experiments, funded the project and wrote the manuscript.

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Conflict of interests. The authors have no competing interests.

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EXPANDED METHODS

Ants

Mature *O. brunneus* colonies were collected from three locations in Florida: Archbold Biological Station, Pine Jog Environmental Education Center in West Palm Beach, and Withlacoochee State Forest in Lecanto. In the laboratory, individual colonies were housed in two interconnected 60×15mm Petri dishes with plaster-lined bottoms that were kept moist. Colonies received a constant supply of water and 20% sugar-water solution, and were fed 2 days a week on live termites and freeze-killed crickets. All colonies were kept at an average temperature of 27°C. Colonies were used in experiments after being held in laboratory conditions for at least 3 months.

Releaser experiment 1: queens

Test workers from twelve Archbold colonies were harnessed in a restraint fashioned from a 5cm × 5cm square of paper. A radial slit was made through the paper square, ending in a small hole at the center. The petiole (constriction between the thorax and the second abdominal segment) of the test ant was placed in the hole, resulting in the workers legs, thorax, and head on one side of the paper, with the abdomen on the other (Supplementary video). Cold-anesthetized egg-laying queens were then presented to the restrained workers by contacting the antennae of the restrained worker with the queen, and measuring antennal retraction by the restrained worker. Antennal retraction is a stereotypical submissive posture that *O. brunneus* workers display when in proximity to a reproductive queen or worker [1-2]. Antennal retraction consists of pulling the antennae away from the encountered individual so that the antennal scape is perpendicular to the long axis of the head and the antennae are held back and retracted away from the individual (Supplementary video).

Each worker was presented with a queen three times and the consensus reaction of that worker (submissive or non-submissive reaction ≥ 2 of the 3 trials) was counted. Each worker was presented with each type of queen (nestmate queen, non-nestmate same population queen, non-nestmate different population queen, non-reproductive nestmate worker control). Same population queens came from other colonies collected from Archbold, whereas different population queens came from colonies collected from West Palm Beach. All trials were video recorded. The videos were given a coded title that assured that the data recorder was blind to the treatments and identity of individuals. The data were analyzed using Cochran's Q test, and sign tests were used to test pairwise differences. Holm-Bonferroni adjusted significance levels were used for the pairwise comparisons. All statistics were performed using the software package STATISTICA 7 (StatSoft, Tulsa, OK, USA).

Releaser experiment 2: workers treated with a fertility signal

The experimental protocol above was used to test reactions of workers to other workers treated with the hydrocarbon fertility signal (*Z*)-9-nonacosene (*Z*9:C_{29}). Nestmate workers, non-nestmate same-population (Archbold) workers, and non-nestmate different population (West Palm Beach) workers were presented to Archbold workers from twelve colonies. Data were recorded and analyzed as stated above.

Synthetic *Z*9:C_{29} used for treatments was synthesized using previously published methods [3-4] with 1mg diluted in 8ml of hexane. For hydrocarbon treatments of workers, 30µl of the hydrocarbon working solutions (3.75µg of hydrocarbon) were dropped onto the surface of deionized water in a 10 ml glass beaker. The hexane was allowed to evaporate, leaving a thin hydrocarbon film on the surface of the water. Before treatment, the ants were temporarily immobilized by 30s exposures to freezing temperatures. Before the ants reanimated, they were dropped onto the surface of the water with the hydrocarbon films and swirled, thereby transferring the hydrocarbons onto the surface of their cuticle [2, 5]. Treatments resulted in ∼17 % increase in the relative abundance of the test compounds on the cuticle, as revealed by solid-phase microextraction sampling of treated individuals [2, 6].

Releaser experiment 3: fertility signal on nestmate workers stripped of their cuticular hydrocarbon profiles

The experimental protocol for experiment 1 was used to test worker reactions to nestmate workers stripped of their hydrocarbon profile and treated with *Z*9:C_{29}**,** compared to a hydrocarbon control of pure heptacosane (C_{27}). Workers (n=12) from Archbold and Withlacoochee populations were used. Nestmate workers were freeze-killed and successively soaked for five minutes in three glass vials containing 200µl of hexane. The workers were then treated with the test compounds (as above). The removal and compound additions were verified by an additional hexane-extraction of the treated worker (Supplemental Figure 1).

Supplemental releaser experiment: fertility signal without background chemical context

The experimental protocol for experiment 1 was used again to test worker reactions to *Z*9:C_{29} in isolation, compared to a hydrocarbon control of pure heptacosane (C_{27}). Workers (n=12) from Archbold, West Palm Beach, and Withlacoochee were presented with a glass rod (6cm diameter) to which the test compounds had been transferred. The solutions of compounds (equivalent concentration and amount as above) were pipetted directly onto the tip of the glass rod, where the solvent evaporated leaving only the test compounds. Data were recorded as stated above and a sign test was used to compare the reactions of test individuals to the two compounds.

Primer experiment

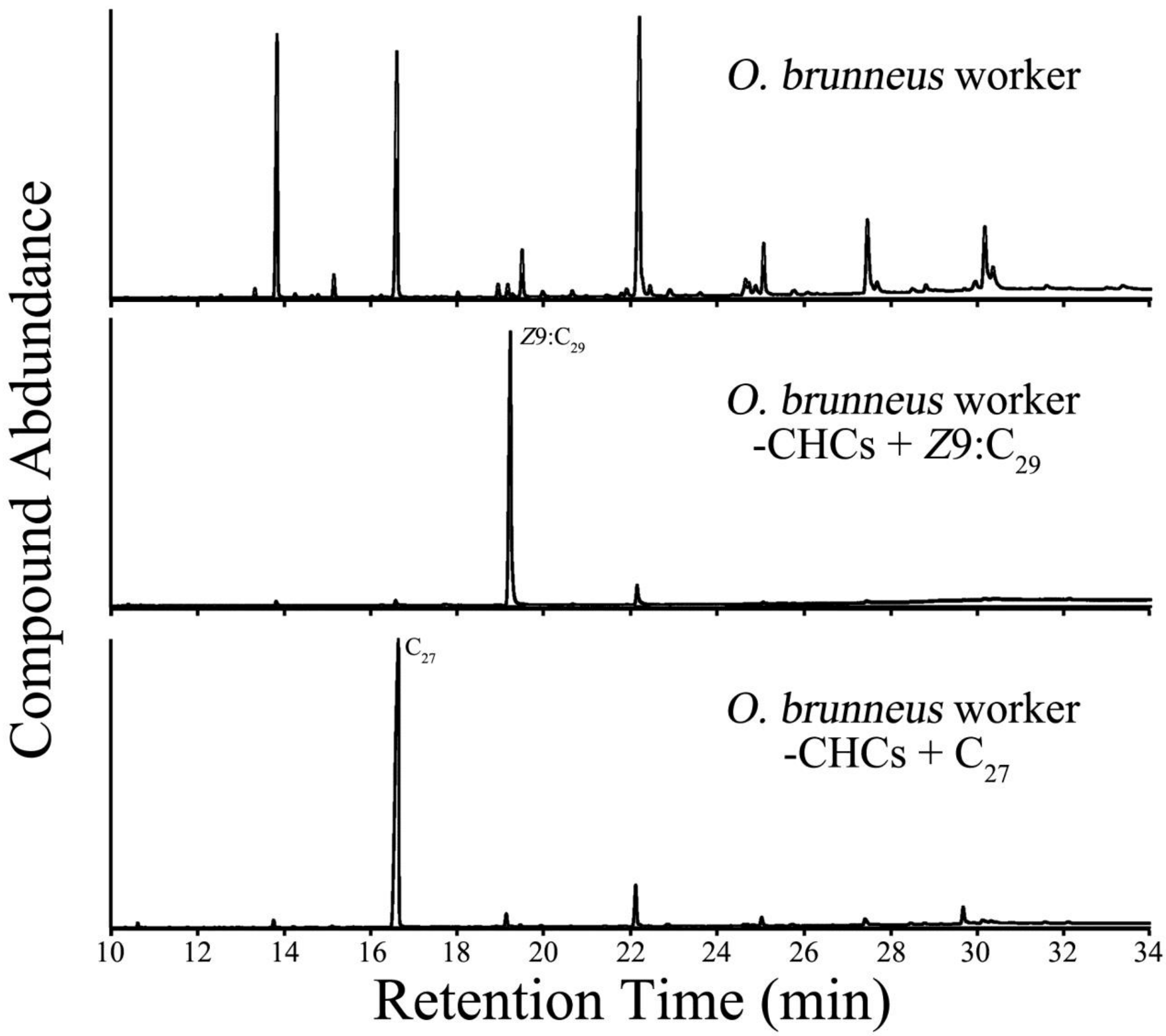
Thirteen colonies from the Archbold population were split into three equal-sized queenless groups (mean group size 21; min 12, max 34) that were housed in a single 60×15mm Petri dish nest with a moistened dental plaster floor, within a 19×13.5cm arena (Supplemental Figure 2). Treatments consisted of adding *Z*9:C_{29} or heptacosane (concentrations in hexane as above), or hexane (control) to glass cover slips. A single cover slip was added to each colony (Supplemental Figure 2) and replaced daily. Cover slip placement consisted of lifting the lid of the Petri dish nest, removing the previous cover slip and placing the new slip on the nest chamber floor. Worker groups had a constant supply of sugar water and were given 2-3 live termites as prey every day. Each nest was inspected daily for the presence of worker-laid eggs. Successful worker egg-laying was classified according to the date eggs first appeared and remained present in the nest for at least 48 hours. This ensured that the eggs were not trophic (shared as food between nestmates) or policed (destroyed). All groups were given a maximum of 45 days to lay eggs. The likelihood that worker groups successfully laid eggs was compared between treatments. For colonies in which all treatment groups laid eggs (8/13) the number of days until egg laying was compared, within colonies, by a Friedman ANOVA test.

SUPPLEMENTAL RESULTS

Supplemental releaser experiment: fertility signal without background chemical context

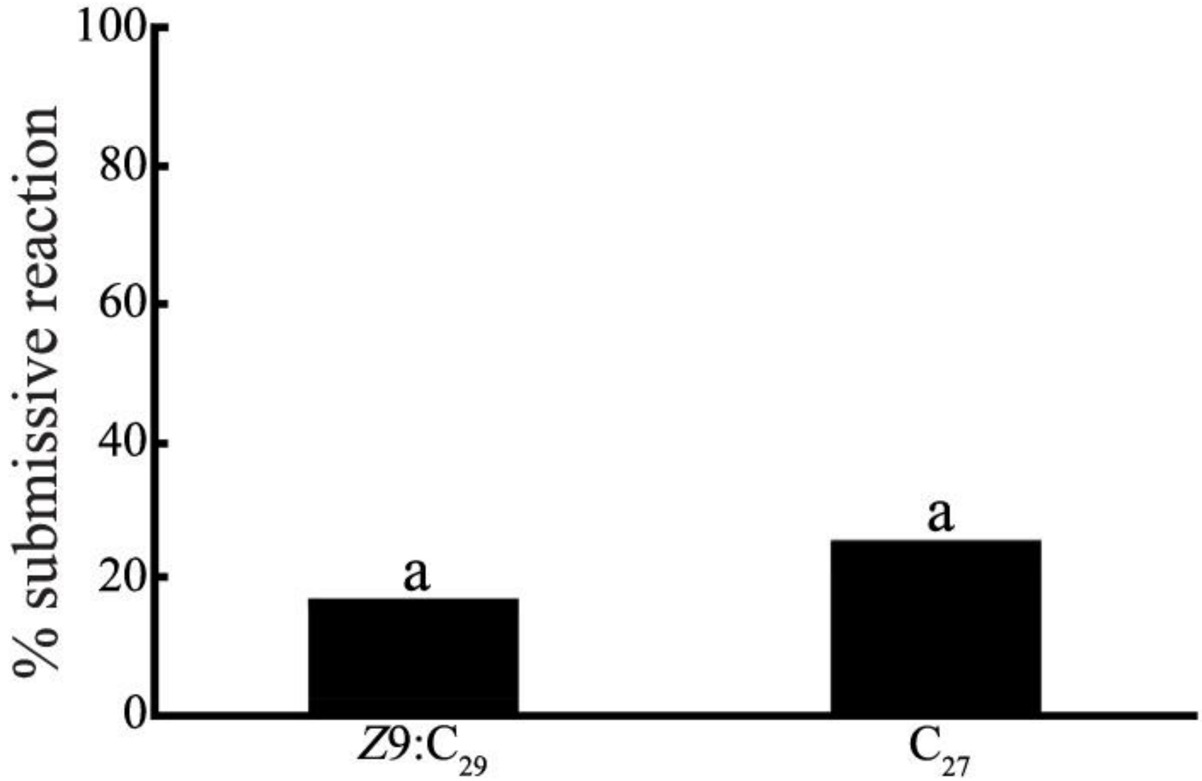
When the fertility signaling compound *Z*9:C_{29} coated on a glass rod was presented to workers, only a small percentage of workers displayed a submissive reaction, that was not significantly different from the response displayed to a C_{27} hydrocarbon control (Supplemental Figure 3).

SUPPLEMENTAL FIGURES

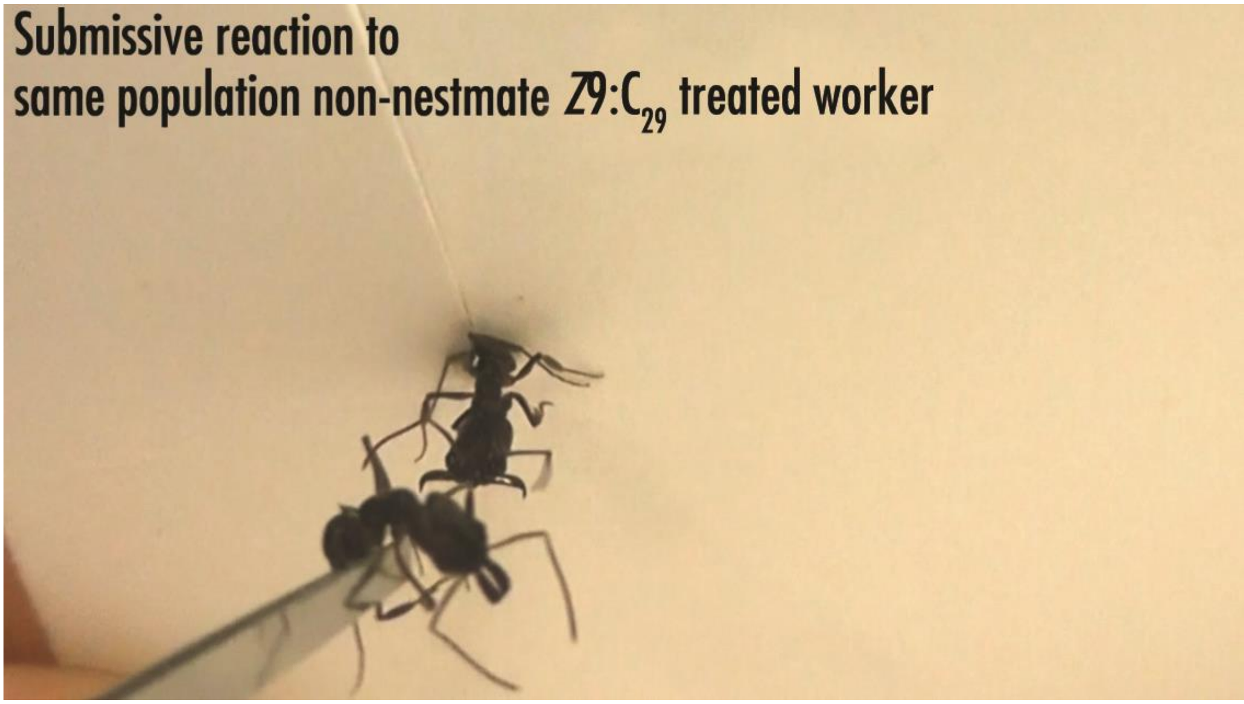


Supplemental Figure 1: Hexane extract of an *O. brunneus* worker from the Archbold population (top) compared to hexane extracts of workers from the same population treated with the test compounds *Z*9:C_{29} (middle) and C_{27} (bottom) after three successive hexane washes to remove the background hydrocarbon profile.

Supplemental Figure 2: Primer experiment experimental setup. a) The glass coverslip onto which the chemical treatments we placed (outlined). b) The nest chamber with a moist dental plaster floor. c) Tube of sugar water constantly available to foragers. d) Foraging arena into which termites were introduced as food.



Supplemental Figure 3: Releaser-effect experimental results showing submissive reactions of test workers to the fertility signaling compound *Z*9:C_{29} and a C_{27} control presented on a glass rod. **(**$z = 0$, $p = 1$).



Supplementary video (screenshot): Restrained test workers are presented with cold-anesthetized non-nestmates. A queen and fertility signal treated worker from the same population as the test worker evoke submissive reactions (antennal retraction). A fertility signal treated worker from a different population does not evoke a submissive reaction.

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