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ORIGINAL ARTICLE

Highly specific responses to queen pheromone in three *Lasius* ant species

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Abstract Queen pheromones mediate the reproductive division of labor in social insect colonies and provide novel opportunities for investigating the evolution of animal communication. Previous work found that queens in the ant genus *Lasius* produce several 3-methylalkanes in greater relative amounts than workers do. At least one of these (3-MeC_{31}) is a queen pheromone that regulates worker sterility in two *Lasius* species, although there are indications that other 3-methylalkanes might also function as queen pheromones. Here, we presented workers from three *Lasius* species with four different 3-methylalkanes, and measured the effect on worker ovary development. In all three species, only 3-MeC_{31} showed clear evidence of inhibiting worker fecundity. Our results suggest that worker ants can discriminate homologous hydrocarbons that differ in chain length and only treat specific homologs as queen pheromones. These results provide insight into the conflicting selective pressures on cuticular hydrocarbons arising from their multiple roles in signaling and adaptation to the abiotic environment.

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Significance statement

Queen pheromones are chemical signals produced by queen social insects, which perform a variety of important regulatory functions in the colony. Few queen pheromones have been discovered so far, and it is unclear whether these pheromones are generally composed of one, few, or many different compounds. Here, we present queenless groups of workers, from three different ant species, with four different chemicals; these chemicals included one known queen pheromone, as well as three other structurally similar chemicals whose activity as pheromones is unknown. Contrary to expectations, we found that only one chemical, 3-MeC_{31}, elicited a response from workers, suggesting a specific response to a queen pheromone that is conserved across species. These results shed light on queen-worker communication, and on the potentially conflicting selection pressures shaping the diverse blend of hydrocarbons produced by ants.

Introduction

Biological signals are commonly defined as an adaptively evolved action or structure (e.g., a behavior, color patch, sound, odor, or construct such as a bower) that increases the sender's fitness by eliciting a specific response in one or more receivers, whose responses have also adaptively evolved (Grafen 1990; Maynard Smith and Harper 1995; Holman 2012). Signals therefore indicate past or on-going co-evolution, meaning that

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signaler-receiver interactions are a key to understanding why some traits evolve into signals but others do not.

Queen pheromones are chemical signals produced by queen social insects, which elicit behavioral and/or physiological responses in other colony members. They are also called "fertility signals", a term which has the advantage of indicating that these chemicals are not unique to queens, but are instead a characteristic of fertile individuals (see Oi et al. 2015). All known queen pheromones except that of the honey bee (Butler et al. 1962) were identified only recently, offering novel opportunities to test predictions from signaling theory. For example, ants, wasps, and bumblebees were shown to possess queen pheromones that form part of the cuticular hydrocarbon (CHC) profile (Smith et al. 2009, 2012; Holman et al. 2010b, 2013a; Van Oystaeyen et al. 2014), which is a layer of non-volatile hydrophobic compounds covering the body surface of most insects. CHC-based queen pheromones are hypothesized to represent ritualized signals that evolved from pre-existing chemical cues associated with fecundity that were present in the solitary ancestors of all social insects (Van Oystaeyen et al. 2014). CHCs likely provided primitive social insects with a reliable means of identifying and responding to reproductives, such that CHCs both facilitated and were altered by the evolution of sociality (Holman 2014; Oi et al. 2015).

One type of CHCs, the 3-methylalkanes (i.e., saturated carbon chains with a methyl group attached to the third carbon atom), has been found to be a characteristic of queens and/or fertile females in at least 20 species of ants, 5 wasps, and a termite (reviewed in Van Oystaeyen et al. 2014). Additionally, synthetically produced 3-methylalkanes have been shown experimentally to negatively affect ovarian development in queenless groups of workers in three ant and one wasp species (Holman et al. 2010b, 2013a; Van Oystaeyen et al. 2014). Holman et al. (2013a) used phylogenetic comparative analyses to examine the evolution of putative queen pheromones in the ant genus *Lasius*, and found that 3-methylalkanes were more abundant in the CHC profiles of queens relative to workers in all 11 species surveyed. This provided circumstantial evidence that 3-methylalkanes might be queen pheromones throughout the genus. However, the chain length of the most abundant and most queen-specific 3-methylalkane differed across the genus. Some species' queens had primarily shorter-chain 3-methylalkanes such as 3-MeC_{27}, whereas others had large quantities of 3-methylalkanes with chain lengths of up to 35 carbons. These results suggest that different 3-methylalkanes might function as queen pheromones in different species.

Holman et al. (2013a) hypothesized that this evolutionary pattern might be explained by generalization of worker responses among certain hydrocarbons, such that all 3-methylalkanes might function equally well as queen pheromones. In support of this idea, there is experimental evidence that some ants respond similarly to hydrocarbons that differ in chain length, but not in methyl position, in the context of nestmate recognition (van Wilgenburg et al. 2010; Bos et al. 2012). Conversely, some species apparently can discriminate among homologous hydrocarbons with different chain lengths (Châline et al. 2005; Martin et al. 2008; van Wilgenburg et al. 2010). There is very little data on the discrimination of chain length in the context of queen pheromones. In a past experiment, 3-MeC_{29} had a slightly stronger effect than 3-MeC_{27} on worker ovarian development in the ant *Cataglyphis iberica* (binomial GLMM: $z = 2.00$, $n = 651$, $p = 0.045$; recalculated from the raw data of Van Oystaeyen et al. 2014), implying that chain length matters. Experiments examining behavioral responses to synthetic queen-like ant hydrocarbons have found mixed results: *Aphaenogaster cockerelli* responded differentially to the alkanes C_{25} and C_{29} (Smith et al. 2009), but *Odontomachus brunneus* did not discriminate between the alkenes (*Z*)-9-heptacosene and (*Z*)-9-nonacosene, only one of which appears to be correlated with fertility (Smith et al. 2012).

In the present study, we tested the physiological responses of three congeneric species (*Lasius niger*, *Lasius flavus*, and *Lasius lasioides*) to four 3-methylalkanes with varying carbon chain lengths. In previous studies of *L. niger* and *L. flavus*, queenless groups of workers continuously exposed to synthetic 3-MeC_{31} had less well-developed ovaries than controls; 3-MeC_{31} is the most abundant component of the CHC profile of *L. niger* and *L. flavus* queens, and is much less abundant in the CHC profile of workers (Holman et al. 2010b, 2013a). The queens of these two species also produce other 3-methylalkanes in smaller amounts (Holman et al. 2013a) (Fig. 1), but only the effect of 3-MeC_{31} has been investigated. By contrast, 3-MeC_{31} made up only a small portion of the CHC profile of *L. lasioides* queens, while the longer-chained 3-MeC_{33} and 3-MeC_{35} were much more abundant (Fig. 1). Thus, we hypothesized that this species may have evolved a new queen pheromone composed of one or more longer-chained 3-methylalkanes. We discuss our results in the context of the selective pressures and constraints shaping the evolution of hydrocarbon signals.

Methods

Ant collection and colony maintenance

L. niger and *L. flavus* were collected by excavating large colonies on Amager Fælled, Copenhagen, Denmark, on June 25, and June 22, 2014, respectively, while *L. lasioides* were collected from rotting tree stumps near Rethymno, Crete, on July 1, 2015 (five colonies per species). We preferentially collected workers found inside the nest and close to the brood, because these were hypothesized to be younger and more capable of reproduction. Soon after collection, all the workers collected from each colony (this varied between approximately 150–380

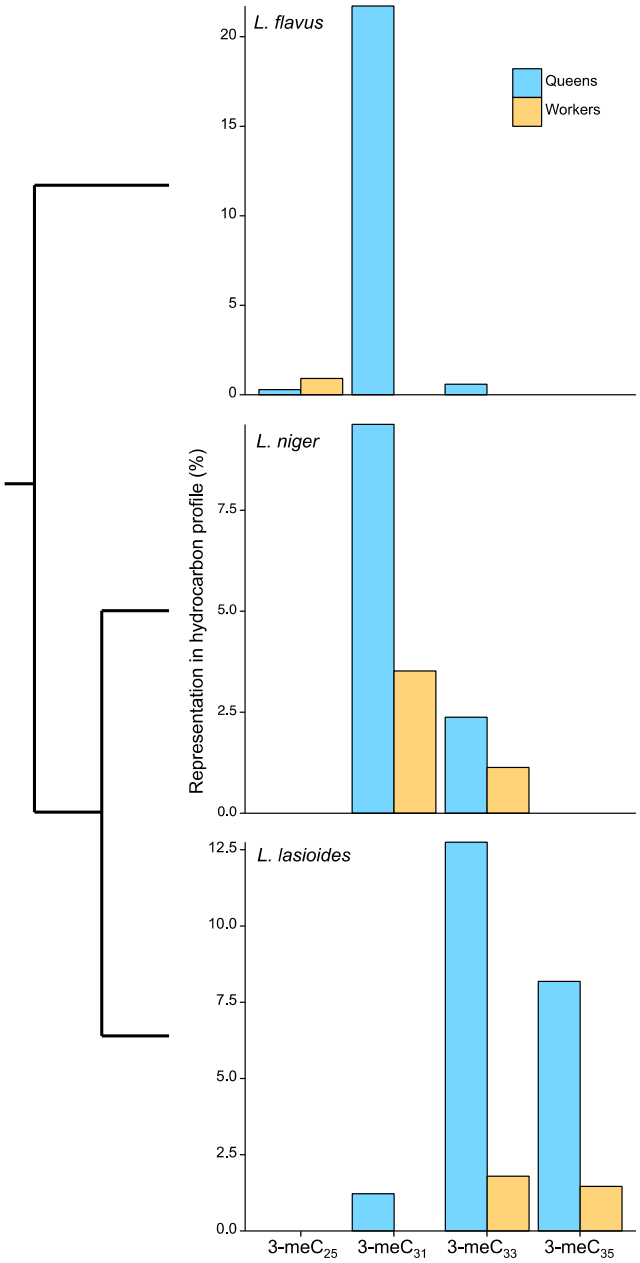

Fig. 1 The proportion of each of the four tested 3-methylalkanes in a sample of queens and workers from each of the three species. The data and phylogeny are from Holman et al. (2010a; 2013a); *missing bars* indicate that a chemical was not present at detectable levels. Note that most of the 3-methylalkanes are more abundant on queens than workers, and that the chain length of the most abundant 3-methylalkane differs between *Lasius lasioides* and the others

individuals, with an average around 300) were randomly and evenly divided among six nest boxes (8 × 6 × 5 cm) lined with plaster and provided with honey, water, and mealworms ad libitum; the nest boxes were kept in the dark at room temperature (approx. 21 °C). We thus used a "split-plot" experimental design, in which the 15 colonies (five per species) were each divided evenly among the 6 treatments, for a total of 90 nest boxes.

Hydrocarbon supplementation experiment

Every 24 h for 14 days, we added 10 μl of hydrocarbon solution to a 2 × 2-cm glass microscope coverslip in each nest box. In addition to a hexane-only control, we applied one of the synthetic hydrocarbons C_{31}, 3-MeC_{25}, 3-MeC_{31}, 3-MeC_{33}, or 3-MeC_{35}, dissolved in hexane at a concentration of 0.01 μg μl^{-1}, i.e., 0.1 μg of each hydrocarbon per nest per day. This mass is equivalent to approximately half the amount of 3-MeC_{31} on the cuticle of a *L. niger* queen in her first year (Holman et al. 2010a), though mature queens likely have larger amounts (Holman et al. 2013a). This dose is also the same as that used in previous experiments (Holman et al. 2010b, 2013a).

With the exception of C_{31} (purchased from Sigma-Aldrich), the hydrocarbons were synthesized as described in the online supplementary material. Our synthesis method produced a racemic mixture of each 3-methylalkane, i.e., one containing equal amounts of the (*R*)- and (*S*)-enantiomers. As in previous experiments (Holman et al. 2010b, 2013a), the unbranched alkane C_{31} acted as a control for the addition of a synthetic hydrocarbon to the nest. This compound is abundant on the cuticles of queen *L. niger* and *L. flavus*, has a chain length approximately in the middle of the range of chemicals presented, and was previously found to have no discernable effect on ovary activity in *L. niger* or *L. flavus* (Holman et al. 2010b, 2013a).

After 14 days, ants were freeze-killed for ovary dissections. We scored ovaries as active if they contained at least one fully developed egg (i.e., an egg that was subjectively scored as being of approximately the size of an egg that has been laid); otherwise, they were scored as inactive. Two of 90 nest boxes were lost before completing the experiment. Ovary dissections were performed blind to treatment and colony of origin, preventing observer bias (Holman et al. 2015). We used ovary dissections as a measure of worker fecundity because small groups of *Lasius* workers do not produce offspring in the laboratory (personal observation), though *L. niger* workers do reproduce in the wild (Fjerdingstad et al. 2002). This approach assumes that chemicals that modulate worker reproduction in the wild would also influence the frequency of worker ovarian activation in the laboratory. We also note that many of the eggs we observed may have been nonviable. Khila and Abouheif (2008) found that approximately 97 % of *L. niger* workers' eggs were nonviable, though in the conger *L. alienus*, only 18 % were nonviable (*L. flavus* and *L. lasioides* are unstudied).

The ovary activation data for each species were analyzed with generalized linear mixed models (GLMM) with binomial errors and colony as a random factor, using the *glmer* function in the lme4 package for R. Differences between treatments were assessed using Wald's tests. Effect sizes (log odds ratio) were obtained by running the *summary* function on the output

of *glmer*, and the associated 95 % confidence limits were estimated as the standard error multiplied by 1.96 (Nakagawa and Cuthill 2007) (Fig. 2 lower panel).

As a post hoc analysis (after discovering that only 3-MeC_{31} appeared to modulate fecundity), we also compared the effect of 3-MeC_{31} to the pooled data from the other five treatments using a GLMM. This analysis provides a more precise measure of the effect of 3-MeC_{31} on fecundity, assuming the hypothesis that only 3-MeC_{31} has any effect is correct.

Results

In *L. niger* workers ($n = 714$), the previously identified queen pheromone 3-MeC_{31} significantly reduced the frequency of ovary activation relative to all the other treatments except 3-MeC_{35} (Fig. 1; Table S1). All the other treatments did not differ significantly from each other, with the exception that significantly fewer workers had active ovaries following treatment with 3-MeC_{35} compared to C_{31} (Fig. 2; Wald's test: $z = 2.02$, $p = 0.043$). The 3-MeC_{31} treatment had a significant effect on fecundity when contrasted with pooled data from all other treatments (Fig. 2; $z = 2.62$, $p = 0.0088$).

In *L. flavus* ($n = 1038$), workers in colonies treated with 3-MeC_{31} were significantly less likely to have active ovaries than those treated with C_{31}, 3-MeC_{25}, or 3-MeC_{35} (Fig. 2; Table S1). There was a nonsignificant trend for a negative effect of 3-MeC_{31} relative to the hexane control ($p = 0.069$). All other treatments were not significantly different from one another. Again, the 3-MeC_{31} treatment had a significant effect on fecundity when contrasted with pooled data from the other treatments (Fig. 2; $z = 2.14$, $p = 0.032$).

In *L. lasioides* ($n = 886$), 3-MeC_{31} significantly decreased the proportion of workers with active ovaries relative to the hexane control, 3-MeC_{25}, and 3-MeC_{33} (Fig. 2; Table S1). Unexpectedly, treatment with 3-MeC_{35} appeared to elevate the proportion of fertile workers relative to most other treatments (Fig. 2). As before, the 3-MeC_{31} treatment had a significant effect on fecundity when contrasted with pooled data from the other treatments (Fig. 2; $z = 2.84$, $p = 0.0044$).

The proportions of workers with developed ovaries varied substantially among species, being highest in *L. lasioides*, then *L. niger*, and then *L. flavus*. Across species, the inhibitory effect of 3-MeC_{31} was negatively correlated with the proportion of fertile workers (Fig. 2), though this result may be due to chance because we only have data from three species.

Discussion

Our results imply that 3-MeC_{31} is a queen pheromone that reduces the frequency of worker ovarian activation in all three species tested. Additionally, there was no consistent indication that other 3-methylalkanes had the same effect; for example, no treatments other than 3-MeC_{31} resulted in significantly less ovary activation than the controls. Thus, workers of all species appear to respond to 3-MeC_{31} specifically, rather than to 3-methylalkanes more generally. Contrary to our expectation, there was no evidence that *L. lasioides*, which has evolved longer-chained cuticular hydrocarbons than its congeners (Fig. 1), treats longer-chained 3-methylalkanes as queen pheromones (if anything, 3-MeC_{35} stimulated egg production in *L. lasioides*). It is also surprising that 3-MeC_{31} reproductively inhibited *L. lasioides* workers even though queens of this species produce only small amounts of 3-MeC_{31} (Holman et al. 2013a).

The mean chain lengths of the various families of hydrocarbons making up the CHC profile are generally highly correlated (e.g., Blomquist 2010; Gosden et al. 2012; Holman et al. 2013b), which is to be expected because the same elongase enzymes are used to lengthen the carbon chains of multiple types of hydrocarbons (Blomquist 2010). Therefore, evolutionary constraints might make it difficult for a species to continue producing the same amount of any particular compound (such as 3-MeC_{31}) in the face of selection to produce a longer- or shorter-chained CHC profile (e.g., to adapt to a new climate; Gibbs 2002; Kwan and Rundle 2010; van Wilgenburg et al. 2011). Holman et al. (2013a) hypothesized that workers might treat all 3-methylalkanes as queen pheromones, meaning that species could readily evolve a longer-chained CHC profile and still have effective queen-worker signaling. This hypothesis was incorrect: the present data suggest that 3-MeC_{31} inhibits worker reproduction while other 3-methylalkanes have little or no effect, even in *L. lasioides*. Thus, the present results imply that when a species evolves a longer- or shorter-chained CHC profile, it may sometimes reduce or cease production of its primary queen pheromone, causing worker fecundity to increase. Interestingly, *L. lasioides* had higher rates of worker ovary activation than its congeners, and this is the only species we tested in which queens have meagre amounts of the queen pheromone 3-MeC_{31}. Data on additional species are required to test whether this result is coincidence or indicates a wider trend whereby species that have lost the queen pheromone used by their relatives tend to have more fecund workers.

Signal theory predicts that workers might evolve to respond preferentially to hydrocarbon(s) that signal the presence of reproductive conspecifics most reliably (Holman 2012). Accordingly, several results suggest that 3-MeC_{31} signals caste and fecundity more reliably than other CHCs. 3-MeC_{31} correlated most closely with experimentally induced changes in queen fecundity in *L. niger* (Holman et al. 2010a), declined more than all other CHCs following immune challenge (Holman et al. 2010b), and increased the most as queens underwent reproductive maturation in *L. niger* (Holman et al. 2010a) and *L. flavus* (Holman et al. 2013a). Additionally, 3-MeC_{31} had the strongest genetic correlation with fecundity of all *L. niger* hydrocarbons, consistent with

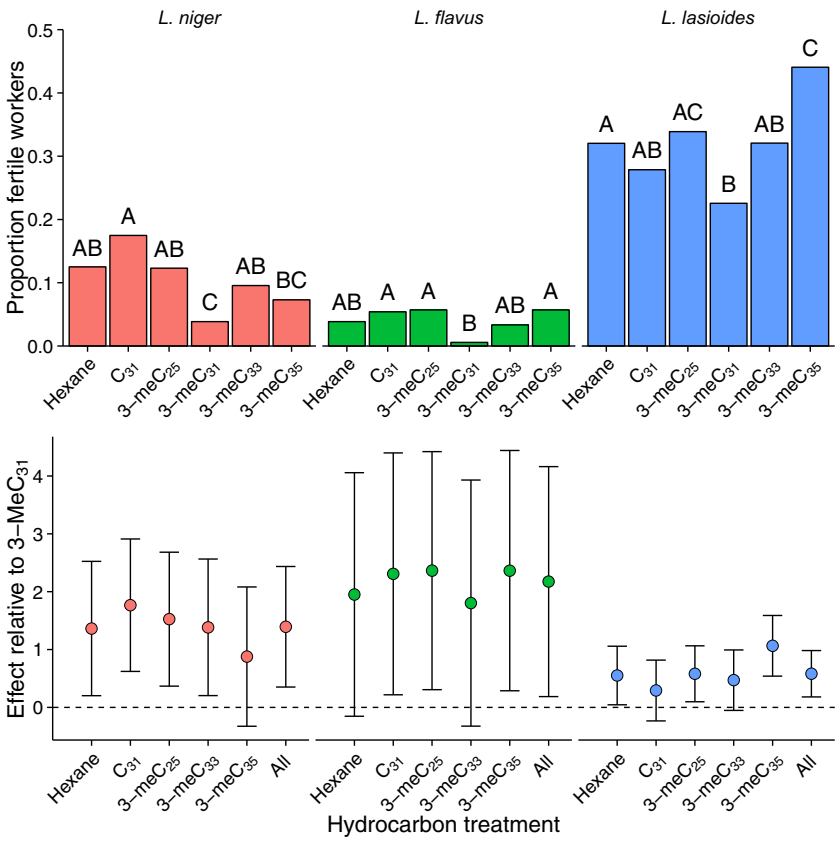

Fig. 2 The *top panel* shows the proportion of workers with active ovaries in each of the six treatment groups, and the *lower panel* shows the estimated effect sizes (log odds ratios ± 95 % confidence limits) for each of the hydrocarbon treatments on the frequency of workers with activated ovaries, relative to 3-MeC_{31}. The effect is positive for all treatments in all species, and often differs significantly from zero, suggesting that ovary activation was less frequent in colony fragments treated with 3-MeC_{31} relative to the other treatments. Data points labeled "*All*" are from contrasts of the 3-MeC_{31} group with pooled data from all the other treatments. The effect sizes were estimated from the binomial generalized linear mixed models described in Table S1, which accounted for the effect of colony. Bars sharing a letter are not significantly different ($\alpha = 0.05$)

strong mechanistic links between this chemical and egg laying, relative to other hydrocarbons (Holman et al. 2013b). Lastly, the amount of 3-MeC_{31} in the queen CHC profile was more affected than other CHCs by experimental treatment with juvenile hormone, which regulates fecundity (Holman 2012). It is not yet known whether patterns like these also apply to *L. lasioides*: queens of this species produce abundant 3-MeC_{33} and 3-MeC_{35}, but minimal 3-MeC_{31}, and correlations between hydrocarbons and fecundity have yet to be studied.

Several questions remain regarding *Lasius* queen pheromones. For example, it is still unclear why 3-methylalkanes are queen pheromones in multiple *Lasius* species (and other social insects; Van Oystaeyen et al. 2014) while other classes of hydrocarbons apparently are not (as evidenced by the fact that other compounds do not consistently correlate with caste and fecundity in Lasius; Holman et al. 2013a). 3-Methylalkanes are placed onto eggs in large quantities by queens (Holman et al. 2010b), which might simply reflect the role of these chemicals in signaling. However, it is also possible that 3-methylalkanes were used in egg production prior to their use as queen pheromones, and later evolved into signals of fecundity (Holman 2012). We hypothesize that queens might place hydrocarbons onto their eggs to help protect eggs and young larvae against desiccation, as has been found in nonsocial insects (Fan et al. 2002). If certain classes of hydrocarbons were used preferentially to coat eggs (e.g., hydrocarbons with the correct viscosity and permeability for this purpose; Gibbs 2002), then these hydrocarbons might be more abundant on the cuticles of fertile females than nonfertile ones, and might thus be pre-adapted to evolve into queen signals. One could test this hypothesis by examining whether particular classes of hydrocarbons are preferentially found on the eggs of solitary Hymenoptera. If solitary Hymenoptera coat their eggs with the same hydrocarbons that are used as queen pheromones in social species (e.g., 3-methylalkanes), this would suggest that queen pheromones evolved from compounds that were associated with eggs in the earliest social insects.

Lastly, we note that 3-methylalkanes can exist in two enantiomeric forms, and the available data suggest that most insects produce only the (*R*)-enantiomers of monomethylalkanes (Bello et al. 2015). Discrimination between enantiomers has been observed in ants (Sharma et al. 2015) and nonsocial insects (Silk et al. 2011; Ablard et al. 2012; Kühbandner et al. 2013). However, given that workers would not normally encounter the unnatural enantiomer, it seems unlikely that it would interfere with the signaling function of the natural enantiomer when presented as a racemic mixture. This supposition was borne out in our study: synthetic 3-MeC_{31} affected worker fecundity even though we used racemic mixtures of enantiomers as our test substances. It remains to be tested whether both the natural and unnatural enantiomers of 3-MeC_{31} function as queen pheromones.

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