Title: A context-dependent alarm signal in the ant *Temnothorax rugatulus*

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Abstract

Because collective cognition emerges from local signaling among group members, deciphering communication systems is critical to understanding underlying mechanisms. Alarm signals are widespread in the social insects and can elicit a variety of behavioral responses to danger, but the functional plasticity of these signals has not been well studied. Here we report an alarm pheromone in the ant *Temnothorax rugatulus* that elicits two different behaviors depending on context. When an ant was tethered inside an unfamiliar nest site and unable to move freely, she released a pheromone from her mandibular gland that signaled other ants to reject this nest as a potential new home, presumably to avoid potential danger. When the same pheromone was presented near the ants' home nest, they were instead attracted to it, presumably to respond to a threat to the colony. We used coupled gas chromatography/mass spectrometry to identify candidate compounds from the mandibular gland and tested each one in a nest choice bioassay. We found that 2,5-dimethylpyrazine was sufficient to induce rejection of a marked new nest and also to attract ants when released at the home nest. This is the first detailed investigation of chemical communication in the leptothoracine ants. We discuss the possibility that this pheromone's deterrent function can improve an emigrating colony's nest site selection performance.

Introduction

In many taxa, from slime molds to humans, groups cooperatively process information to achieve collective cognition (Couzin, 2009; Marshall and Franks, 2009). By distributing the burden of cognition across many individuals, groups can assess their environment and make consensus decisions, oftentimes more rapidly and accurately than a solitary animal could do (Biro et al., 2006; Sasaki et al., 2013; Ward et al., 2011). Collective cognition emerges in non-obvious ways from a complex network of local interactions among group members. Understanding this process requires decoding the specialized signals that group members exchange in these interactions (Sumpter, 2010). Communication systems, and the group behavior they underlie, have reached especially great diversity and complexity in the eusocial insects (Hölldobler and Wilson, 2009; Seeley, 1989; Wheeler, 1912). Extensive study of the ants and bees has revealed much about the physical nature and information content of signals, and how they contribute to emergent colony properties (Franks, 1989; Hirsh and Gordon, 2001; Marshall et al., 2009; Passino and Seeley, 2006; Pratt, 2005; Seeley and Buhrman, 2001; Seeley, 1997; Visscher, 2007).

Most of this work has concerned recruitment signals used by successful foragers or nest site scouts, but another fundamental type of communication is alarm signaling. In social insects, defensive behavior is closely connected with alarm signals that either recruit nest mates to combat a potential danger or warn them to stay away (Blum, 1969; Crewe and Fletcher, 1974; Maschwitz, 1964). Besides some early reports (Goetsch, 1953), the first thorough study of chemical alarm communication in ants was on the pharaoh ant *Monomorium pharaonis* (Sudd, 1957). Workers of this species reacted with escape behavior when a nestmate was crushed nearby. The first experimental investigations of the

anatomical origin and chemical nature of alarm communication by Wilson (1958) on the harvester ant *Pogonomyrmex badius* and by Butenandt et al. (1959) on the leafcutter ant *Atta sexdens* further showed that the worker ants of these species discharge a strong-smelling substance from the mandibular gland when they perceive a threat. The pheromone of *P. badius* was identified as 4-methyl-3-heptanone (McGurk et al., 1966), which was also later identified as the active component in the alarm pheromone of *A. sexdens* (Blum, 1969). In numerous subsequent investigations, various exocrine glands have been determined to be the sources of alarm pheromones (Buschinger and Maschwitz, 1984) and many compounds have been identified (Blum, 1985; Hölldobler and Wilson, 1990; Parry and Morgan, 1979; Van Meer and Alonso, 1998).

In some ant species alarm pheromones have been recognized as multi-component signals, whereby individual constituents of the blend of glandular secretions have different diffusion rates and accordingly elicit different behavioral responses in receivers (Bradshaw et al., 1975; Bradshaw et al., 1979; Fujiwara-Tsujii et al., 2006; Hölldobler and Wilson, 2009). The response behavior can also vary in different groups and castes of societies, and in time and space (Hölldobler, 1977). Although this functional plasticity was first recognized 50 years ago (Maschwitz, 1964), little attention has been given to specifying how social and environmental contexts, particularly those associated with collective information processing, affect behavioral responses to alarm pheromones in ants.

The present study reports the first analysis of context-specific functions of a hitherto unknown alarm pheromone in the myrmicine ant *Temnothorax rugatulus*. Ants of this genus form small colonies typically comprising 150-250 workers. They usually live in small cavities, such as acorns and rock crevices, whose fragility requires frequent emigrations to

new homes. They organize these moves using recruitment by tandem running and carrying of nestmates (Möglich, 1978), and they show remarkable abilities to collectively choose a single optimal nest among multiple options (Franks et al., 2002; Mallon et al., 2001; Pratt and Sumpter, 2006; Pratt et al., 2002; Sasaki et al., 2013). The role of chemical communication in *Temnothorax* societies is poorly known, other than that tandem run leaders discharge secretions from the poison gland that function as a recruitment signal (Möglich et al., 1974). In addition, indirect evidence suggests that nest site scouts of *T. albipennis* may place distinctive marks on undesirable nests that enhance the ability of colonies to collectively choose the best available site (Franks et al., 2007; Stroeymeyt et al., 2011; Stroeymeyt et al., 2014). However, the nature and origin of any such negative signal remains unknown. In preliminary observations, we noted that *T. rugatulus* colonies seemed reluctant to move into candidate nest sites in which some of their nestmates had been tethered to the nest wall. We set out to test whether these tethered ants released a pheromone that discouraged other ants from choosing the site and, if so, to determine the signal's anatomical source and its chemical identity. We further examined whether and how this signal functions outside the context of collective nest site selection.

Results

Experiment 1: Tethered ants emit a deterrent signal

We tested if tethering ants in an unfamiliar nest site caused them to release a pheromone that signals other ants to reject the nest during colony migration. Colonies were given a binary choice between a nest with five tethered ants and an empty nest. The results showed that colonies have a strong preference for the empty nest (2-tailed binomial test: *P*

= 0.008) (Figure 1A). This pattern remained consistent even when the tethered ants were removed from the nest before a migration started (2-tailed binomial test: $P = 0.016$) (Figure 1B). These results suggested that tethered ants released a pheromone that signals to other ants to reject the nest site. Video recordings showed that the tethered ants repeatedly opened their mandibles very wide while facing toward the nest floor. This suggests that this behavior is associated with release of a pheromone from their mandibular glands (Supplemental movie 1). The mandible opening can also indicate aggressive behavior. Based on our observational experience, however, the mandible flaring is typically faster and aimed at an "enemy" target during aggressive behavior. In the context of marking, on the other hand, mandible gaping is often slow and widely opened and pointed to the ground. Obviously, releasing an aversive pheromone or an alarm pheromone are parts of the same behavioral syndrome closely related to aggressive behavior.

Experiment 2: The signal originates in the head

If the pheromone originates in the mandibular gland, we predicted that marking a nest with crushed heads, thus releasing the pheromone, would cause ants to reject it. When presented with a binary choice between a nest with 5 crushed heads and a nest with 5 crushed alitrunks, colonies showed a strong preference for the alitrunk nest (2-tailed binomial test: $P < 0.001$) (Figure 2A). When gasters were used instead of alitrunks, the gaster nest was significantly preferred over the head nest (2-tailed binomial test: $P < 0.001$) (Figure 2B).

These results suggest that the ants rejected the nest that contained heads, but it might instead be the case that they were attracted to alitrunks and gasters. To exclude this possibility, we also tested a binary choice between a nest with 5 alitrunks or 5 gasters and an empty nest. Colonies showed no preference for either alitrunks (8 in empty, 4 in alitrunk, 3 split decisions; 2-tailed binomial test: $P = 0.38$) or gasters (7 in empty, 3 in gaster, 5 split decisions; 2-tailed binomial test: $P = 0.34$).

Experiment 3: The signal is present in solvent extracts of the head

After the results of Experiment 2 indicated the head as the source of the signal, we next tested whether the same effect could be produced by chemical extracts of the heads. Given a binary choice between a nest with a hexane extract of the head and a nest treated with only hexane, colonies strongly preferred the hexane-treated nest (2-tailed binomial test: $P < 0.001$) (Figure 3A). This pattern remained consistent even when migrations started 14h after chemical compounds were applied to the papers (2-tailed binomial test: $P = 0.049$) (Figure 3B). These results indicate that chemical compounds from heads signaled ants to reject the nest, and this effect persisted for at least 14 h.

Experiment 4: The mandibular gland contains multiple compounds

Coupled gas chromatography/mass spectrometry (GC/MS) was used to identify compounds in ant heads. The GC/MS analyses of volatile compounds collected from dissected mandibular glands by solid phrase microextraction (SPME) revealed the presence of several substances. To distinguish glandular compounds from contaminants, we compared these results to pararell analyses of empty vials (Figure 4) and found three compounds that were clearly derived from the mandibular glands: 2,5-dimethylpyrazine

(DMP, 1), benzyl alcohol (2), and 2-phenethyl alcohol (4). Because it is extremely difficult to dissect the mandibular glands of these tiny ants without risking some contamination with secretions from the postpharyngeal gland or other sources, we cannot be certain whether several other compounds, such as nonanal (3), undecanal (7) and geranyl acetone (8) are part of the mandibular gland secretions. We therefore also conducted either full bioassay series (for nonanal and decanal) or pilot tests (for geranyl acetone and undecanal) with these compounds. None of these compounds elicited any detectable behavioral responses from test ants, and so no extended bioassay series were carried out with these substances. It is also worth noting that some of these components such as the aldehydes are common contaminants (see Figure 4), for example from human skin odors, although this is clearly not the case for the compounds 2,5-dimethylpyrazine, benzyl alcohol, and 2-phenethyl alcohol.

Experiment 5: 2-5-dimethylpyrazine induces rejection of a nest site

We tested a series of binary choices between a nest with hexane solutions of one of eight compounds identified in Experiment 4 and a nest treated only with hexane. Ants were significantly more likely to choose the hexane-treated nest only when the other nest had DMP (2-tailed binomial test: $P < 0.01$). They also tended to reject the nonanal nest (2-tailed binomial test: $P = 0.10$). When the solutions of nonanal and DMP were diluted to 5 ppm, the effect disappeared for nonanal (2-tailed binomial test: $P = 1$), but not for DMP (2-tailed binomial test: $P < 0.01$). Surprisingly, ants rejected the nest with DMP even when it was as low as 0.5 ppm (approximately 2.5 ng of DMP on each filter paper) (2-tailed binomial test: $P < 0.01$). However, because we were unable to measure the actual amount of DMP in the mandibular gland, it is uncertain if this tiny dose is at or above the biologically relevant

amount. Furthermore, the effect of 5 ppm DMP (approximately 25 ng) seemed to persist even after 14 h (2-tailed binomial test: $P < 0.01$), consistent with the results of extracted heads in Experiment 3. Table 1 shows the summary of these tests. The long-lasting effect of DMP (which is quite volatile) inside test nests is possibly due to the fact that these nests are relatively closed entities so that the applied DMP dissipates slowly, and residues of the compound can still be detected by the ants after 14 h.

We further tested if the ants were sensitive to the dose of DMP by presenting a choice between a nest with 5 ppm and a nest with 0.5 ppm DMP. The results suggested that the ants rejected the nest with the higher dose of DMP (2-tailed binomial test: $P = 0.07$) (Figure 5) and thus could distinguish different DMP doses, at least between 5 ppm and 0.5 ppm.

Experiment 6: The signal induces attraction to the entrance when released at the home nest

Once we identified 2,5-dimethylpyrazine (DMP) as the signal responsible for nest rejection, we tested if it would elicit a different behavior in another context. When a head was crushed and presented near the home nest entrance, it attracted significantly more ants than did the controls (Figure 6). Alternatively, when the head was presented to ants away from their home nest, it was more often rejected than the controls (Table 2). Our preliminary test showed that dissected mandibular glands elicited responses similar to those elicited by the head in both contexts. Furthermore, crushed heads from which the mandibular glands had been removed did not elicit these behaviors, indicating that the mandibular gland was the source of the pheromone.

Presentation of DMP elicited the same patterns of responses as the intact head: it attracted ants that were in a home nest (Figure 6) but repelled them when they were away from

193 home (Table 2), confirming that DMP is the semiochemical mediating these behaviors.
194 Surprisingly, a very low dose of DMP (0.5 ppm) still elicited these behaviors (Figure 6).

195 **Discussion**

196 Chemical alarm signals are ubiquitous in the Formicidae. They are found even in
197 phylogenetically less derived subfamilies, such as the Ponerinae and Myrmeciinae, that
198 typically do not employ mass communication (Billen and Morgan, 1998; Duffield and Blum,
199 1973; Duffield et al., 1976; Hölldobler and Taylor, 1983; Longhurst et al., 1978; Wheeler
200 and Blum, 1973). Nevertheless, for many ant species no records yet exist as to whether
201 alarm pheromones are used. The closely related myrmicine genera *Leptothorax* and
202 *Temnothorax* belong to this group. It has even been suggested that alarm pheromones
203 might be absent in species like these that have very small colony sizes, because a massive
204 group defense is unlikely (Maschwitz, 1964).

205 Our present study is the first demonstration and in-depth investigation of alarm
206 communication in the genus *Temnothorax* (formerly *Leptothorax*). Chemical analyses
207 combined with behavioral bioassays identified 2,5-dimethylpyrazine as an alarm
208 pheromone. Pyrazines have been previously reported as alarm pheromones in other ant
209 species. For example, 2-ethyl-3,5-dimethylpyrazine and 2,5-dimethyl-3-isopentylpyrazine
210 have been reported to be at least part of an alarm pheromone in the ponerine species
211 *Odontomachus brunneus* and *Odontomachus hastatus*, respectively (Longhurst et al., 1978;
212 Wheeler and Blum, 1973). Among the myrmicine ants, only the fire ant, *Solenopsis invicta*,
213 has previously been shown to use a pyrazine as an alarm pheromone, specifically 2-ethyl-
214 3,6-dimethylpyrazine originating in the mandibular glands of workers, males, and female

sexuals (Vander Meer et al., 2010). 2,5-Dimethylpyrazine, identified here as an alarm pheromone, is also known in other myrmicine species. However, it is typically used as a trail pheromone originating from the poison gland (Billen and Morgan, 1998). To our knowledge this is the first report of its function as an alarm pheromone originating in the mandibular gland.

Alarm pheromones may have different behavioral effects on different recipients. For example, in some ant species young workers respond to alarm pheromones by retreating into the nest, whereas older workers move out and exhibit aggressive behavior (Maschwitz, 1964; also see Hölldobler 1977). Reactions may also vary among different species. In the harvester ant genus *Pogonomyrmex*, which have large colonies, old workers are attracted to low concentrations of their alarm pheromone, 4-methy-3-heptanone. At high concentrations, they either show aggressive behavior or they perform digging behavior in an attempt to rescue a buried nestmate (Wilson, 1958, Wilson and Bossert, 1963). Species with small colonies, on the other hand, may react very differently. For example, workers of the ponerine ant *Hypoponera opacior* frantically evacuate the area when nestmates release the alarm signal 2,5-dimethyl-3-isopentylpyrazine from their mandibular glands (Duffield et al., 1976).

Although the diversity of behaviors elicited by alarm pheromones is well appreciated, little attention has been given to the context specificity of responses. In the first thorough research on this topic, Maschwitz (1964) showed that, for some hymenopteran species, alarm signals release aggressive behavior when discharged close to the nest, but escape behavior when emitted far from the nest. In the subsequent 50 years, there has been little further investigation of context-specific responses. Our findings are consistent with the

pattern Maschwitz described. When *Temnothorax* workers perceived the alarm pheromone in the arena far from their nest, they exhibited escape behavior. In contrast, when the alarm signal was instead presented at the nest entrance, a large number of workers inside the nest moved towards the nest entrance. Video recordings of pilot tests suggest that these workers then attempted to close the nest entrance (Supplemental video 2), behavior that was not seen on exposure to a hexane control. This is consistent with previous findings that they use soil and debris to reduce entrance size or even to close it entirely for defensive purposes (Aleksiev et al., 2007). These observations are preliminary, and further investigation will be required to show if the compound actually elicits entrance-closing behavior.

The importance of positive feedback to collective decision making has been extensively investigated (Camazine et al., 2003; Jeanson et al., 2012; Sumpter, 2010; Sumpter and Pratt, 2009), but the role of negative feedback has, until recently, been less appreciated. Honeybee foragers have been found to use a form of vibrational communication–the stop signal–to suppress recruitment to a food source where they had been briefly trapped, perhaps to reduce the colony's exposure to dangerous areas (Nieh, 2010). Our study similarly showed that *Temnothorax* workers tethered within a site release a signal that induces their nestmates to avoid moving there. This effect can be considered altruistic because it does not lead to rescue of the signaler, but instead helps the colony as a whole to avoid danger (Blum, 1985).

Negative signals may also contribute to the speed or accuracy of a colony's collective decision-making. Many species rely on positive feedback from mass recruitment to concentrate foraging forces on the best available food source (Hölldobler and Wilson, 2009;

261 Seeley, 1995; Sumpter, 2010). In a few species, evidence suggests that scouts apply
262 repellent pheromones to deter nestmates from foraging in areas of low-quality food (Giurfa
263 and Núñez, 1992; Robinson et al., 2005; Robinson et al., 2008; Stout et al., 1998).
264 Theoretical models predict that such repellent signals can prevent the strong positive
265 feedback of mass recruitment from locking a colony into a suboptimal choice (Giurfa and
266 Núñez, 1992; Robinson et al., 2005; Robinson et al., 2008; Stout et al., 1998). However,
267 none of these proposed pheromones have been identified. A much clearer example of
268 negative signaling in the context of decision-making was recently found in honeybees
269 (Seeley et al., 2012). The stop signal, noted above for its use by foragers, is also used by
270 nest site scouts during a colony's collective choice of a new nest site. Successful scouts, in
271 addition to recruiting to the site they have found, use stop signals to inhibit recruitment to
272 competing sites. This may serve to speed the attainment of consensus on a single site, and
273 may also enhance the colony's ability to optimize the tradeoff between decision speed and
274 accuracy. Indeed, the role of these signals in nest site choice is remarkably similar to
275 inhibitory pathways in analogous decision-making systems in the primate brain
276 (Hofstadter, 1999; Passino et al., 2008; Seeley and Buhrman, 2001; Visscher, 2007). In both
277 systems, populations (of either neurons or ants) accumulate evidence for competing
278 options; a decision is made for whichever population first crosses a threshold (of either
279 neural activity or ant numbers). Models suggest that mutual inhibition between the
280 populations allows them to make a statistically optimal tradeoff between decision speed
281 and accuracy (Marshall et al., 2009).

282 Emigrating *Temnothorax* colonies follow a remarkably similar nest choice strategy, but the
283 potential role of inhibition for their decisions remains uncertain. Indirect evidence

indicates that *T. albipennis* leave a deterrent signal in low-quality nests during emigrations (Franks et al., 2007; Stroeymeyt et al., 2014; Stroeymeyt et al., 2011). The nature of this signal has not been determined, but it may be the same as the alarm pheromone that we have identified in *T. rugatulus*. In both cases, unlike other reported negative pheromones (Giurfa and Núñez, 1992; Robinson et al., 2005; Robinson et al., 2008; Stout et al., 1998), the signal does not actually repel ants from entering a marked nest, but instead reduces the colony's probability of moving to the nest (Stroeymeyt et al., 2014; personal observation). The signal could accomplish this by altering the behavior of a scout that enters a marked nest, perhaps causing her to refrain from recruiting other ants to the nest. We speculate that *Temnothorax* ants may use 2,5-dimethylpyrazine as an integral part of their decision-making strategy. However, testing this idea must await detailed observations on whether and how scouts emit and respond to this signal during colony emigration.

Materials and methods

Nest designs

We evaluated pheromone effects in the context of nest site selection experiments carried out in laboratory arenas. Each candidate nest was made from a balsa wood slat (2.4 mm thick) sandwiched between glass microscope slides (50 x 75 mm). A circular cavity (38 mm diameter) was cut through the middle of the slat, and a round entrance hole (∅ = 2 mm) was drilled through the center of the glass roof (Figure 7). The entrance of the home nest was either a hole (∅ = 3.2 mm) on the center of the roof or a slit (2 mm) was cut out of the side of the nest (Sasaki et al., 2013). Balsa slats were made fresh for each experiment and never reused. Glass slides were reused after washing in a commercial dishwasher. The

walls of experimental arenas were coated with Fluon to prevent the ants from escaping. Before each experiment, the experimental arena was cleaned with ethanol to remove any chemical marks that the ants may have left.

Subjects

A total 126 colonies of *Temnothorax rugatulus* were used. Each colony was used only once in each experiment except for Experiment 5. Colonies were collected in the Pinal Mountains near Globe, Arizona. All had at least one queen, with worker populations ranging from 121 to 280 and brood populations ranging from 18 to approximately 300. Each colony was housed in a nest like those described above. Nests were kept in a plastic box (11 cm x 11 cm) with Fluon-coated walls. Each box was provided with a water-filled plastic tube capped with cotton and an agar-based diet that was refreshed weekly (Bhatkar and Whitcomb, 1970; Sasaki et al., 2013).

Experiment 1: Do tethered ants release a pheromone?

Ants were tethered with a string of silk (Part # 7.091, Louet North America, Prescott, ONT, Canada; www.louet.com) tied around the petiole using a knot tyer (Haight, 2012). The length of each string was approximately 2 cm with one side fastened with adhesive tape between the floor glass and the balsa sheet. Five worker ants from the same colony were tethered in the same nest, equidistant from each other (Figure 7).

Colonies were given a binary choice between a nest with tethered ants and a nest that had five strings but no ants. These two target nests were first placed adjacent to one another against one wall of the test arena (Figure 8). The home nest containing the colony from which the tethered ants were taken was then placed against the center of the wall opposite

to the location of the target nests. Finally, the roof of the home nest was removed to induce migration.

The colony's choice was assayed by recording the site occupied 12 h after inducing the migration. In every trial, all ants moved entirely from the home nest to one of the target sites. If one site contained more than 90% of colony members, including all queens and brood items, we designated that as the colony's choice. If this criterion was not achieved, the choice was recorded as a "split" decision.

To exclude the possibility that ants avoided the nest as a result of direct contacts with the tethered ants, we also conducted another experiment, in which tethered ants were absent during the migration. The procedure was identical to the one described above except that the tethered ants were left in the nest for 3 h and then removed immediately before the migration was induced.

To closely observe the behavior of ants releasing pheromone, we additionally filmed tethered ants using a high-resolution camera (Canon EOS Rebel T2i; www.usa.canon.com) with a macro lens (Canon MP-E 65 mm f/2.8 1-5x macro lens). The ants were tethered in the same way described above.

Experiment 2: Does the pheromone come from the head?

We freeze-killed five worker ants from the same colony and used fine forceps to separate each ant's head and gaster from its alitrunk. We then placed five heads in a nest, equidistant from one another, and crushed them with a wooden applicator stick to release any potential pheromones. We similarly crushed either five alitrunks or gasters in another nest. The colony from which the crushed ants were taken was then induced to choose

between these nests, as in Experiment 1 (Figure 8). To test if the effect of alarm pheromone would persist over time, we repeated the experiment, except that the emigration was induced 14 h after crushing the body parts. The colony's choice was assayed by recording the site occupied 12 h after inducing the emigration using the same criteria as in Experiment 1.

Experiment 3: Is the pheromone present in a chemical extract of heads?

Twenty heads from the same colony were placed in 100 μl hexane and crushed with a wooden applicator stick. After 3 h, we used a glass syringe (www.hamiltoncompany.com) to apply 5 μl of this solution to a small filter paper (approximately 1 cm x 1 cm), which was then placed in a standard nest (Figure 7). Another nest received a similar filter paper marked with 5 μl of pure hexane. The colony from which the ants were taken was then induced to choose between these nests, as in Experiment 1 (Figure 8). The colony's choice was assayed by recording the site occupied 12 h after inducing the emigration using the same criteria as in experiment 1.

Experiment 4: Identification of substances in the mandibular gland

Ants were freeze-killed and shipped to UC Riverside on dry ice. After thawing, the ants were decapitated, and groups of about 50 heads were transferred to 1.5 ml glass vials. The heads were crushed with a flat-bottomed glass rod, and the top of the vial was tightly covered with aluminum foil. A polydimethylsiloxane SPME (solid-phase microextraction) fiber was cleaned by thermal desorption in a GC injector port at 250 ºC for 5 min, and after cooling, the fiber was inserted into the covered vial and left exposed to the headspace volatiles for 45 min. The loaded fiber was then thermally desorbed in the injector port of

372 the GC/MS for 30 sec in splitless mode, with an injector temperature of 250ºC. The GC was
373 fitted with a 30 m × 0.25 mm ID DB-5 column (J&W Scientific, Folsom CA, USA), and was
374 temperature programmed from 10ºC for 1 min, then 10º/min to 280ºC, hold 20 min.
375 Analyses were conducted with an 6890N GC interfaced to a 5975C mass selective detector
376 (Agilent Technologies, Wilmington DE, USA), with electron impact ionization (70 eV).
377 Compounds were tentatively identified by matches with the NIS mass spectral database,
378 and identifications were confirmed by matching mass spectra and retention times with
379 those of authentic standards. Analogous analyses were conducted on the crushed bodies
380 minus the heads. Authentic standards were purchased from Aldrich Chemical Co.
381 (Milwaukee, WI, USA).

382 To confirm that compounds found in the volatiles from the crushed heads were from the
383 mandibular glands, about 35 glands were dissected from the heads of freeze-killed workers
384 (Figure 9) and placed in a 1 ml tapered glass screw-cap vial with a Teflon septum. The
385 septum was punctured with a needle, and the SPME fiber was inserted through the hole to
386 collect volatiles. The volatiles were then analyzed as described above. The analyses were
387 replicated with two sets of dissected glands.

388 *Experiment 5: Testing candidate chemical compounds*

389 All eight compounds identified from the mandibular gland were first diluted to 50 ppm in
390 hexane, or even lower if a 50 ppm dilution elicited an effect. As in Experiment 3, we applied
391 5 μl of one of these solutions to a small filter paper and placed it in the standard nest
392 (Figure 7). We also applied 5 μl of hexane to a filter paper and placed it in another identical

393 nest. A colony was then induced to choose between these nests, as in Experiment 1 (Figure
394 8).

395 The colony's choice was assayed by recording the site occupied 12 h after inducing the
396 emigration using the same criteria as in experiment 1. A total of 69 colonies were used, and
397 all were used three or four times, but no colony experienced the same compound more
398 than once. At least 10 days elapsed between experiments on a given colony, to avoid any
399 influence of previous migrations on the current migration (Langridge et al., 2004;
400 Langridge et al., 2008).

401 *Experiment 6: Does the pheromone elicit different behaviors in different contexts?*

402 We crushed a head with a wooden applicator stick or applied DMP (2 μl of 5ppm [10.0 ng],
403 0.5ppm [1.0 ng], 0.1ppm [200 pg] or 0.05ppm [100 pg] solution) to a stick. The stick was
404 then slowly presented near the ant's home nest. The reaction was measured by counting
405 how many ants within the home nest moved towards the nest entrance (i.e. 1 cm mark
406 from the entrance was placed on the computer screen and a number of ants who
407 completely crossed this line was counted). We did not count ants that were already by the
408 entrance when the stick was introduced. The order of the tests was randomized, and at
409 least 45 min elapsed between tests. The DMP was purchased from Sigma Aldrich Co. (St
410 Louis, MO, USA).

411 To confirm that the source of the pheromone was the mandibular gland, we also presented
412 a dissected mandibular gland and a head from which the mandibular gland had been
413 removed. Finally, we presented an untreated stick and a stick treated with hexane as
414 controls.

We further tested how the ants responded to the same alarm pheromone when they were not in the home nest. Similar to the previous test, a head or DMP was first applied to a stick. We then slowly presented the stick to ants that were at least 10cm away from their home nest. Their reaction was categorized as either avoidance (walking away from the stick) or attraction (walking towards the stick). The order of the tests was randomized, and each ant was tested only once.

Statistical analysis

We tested nest site preferences using a 2-tailed binomial test in Experiments 1, 2, 3 and 5. Split colonies were not included in the analyses. A 2-tailed t-test was used for investigating attraction released by DMP inside the home nest, and a χ^2 test of partial independence was used for investigating avoidance of DMP away from the home nest in Experiment 6. The statistical package R (v. 2.9.0) was used for all analyses.

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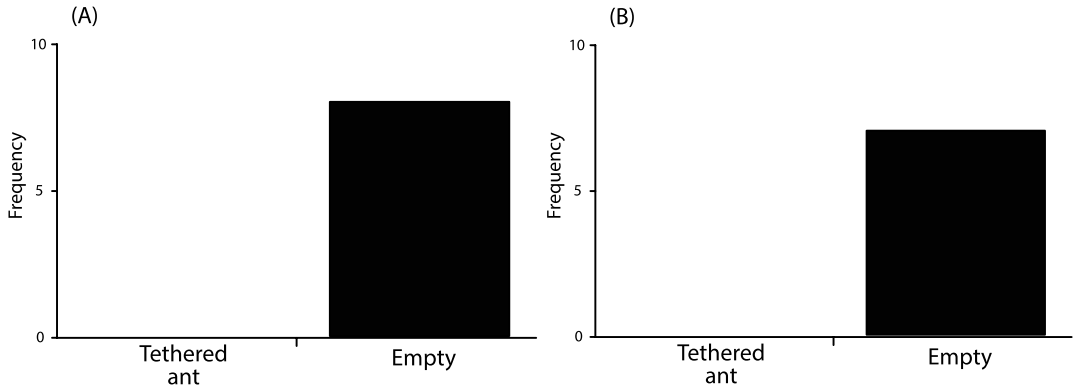


Figure 1. Results of a binary choice between a nest with tethered ants and an empty nest. All colonies chose the empty nest (A), even when the tethered ants had been removed before the migration started (B). Colonies did not split between the nests.

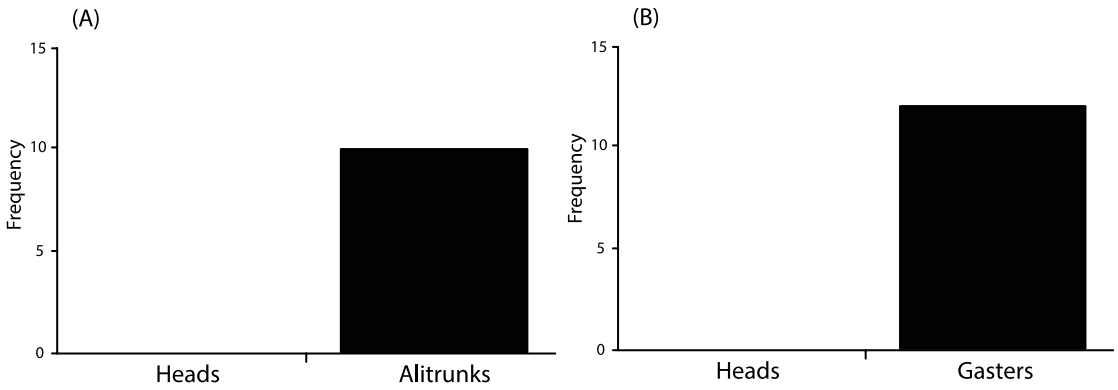


Figure 2. Results of a binary choice between a nest with heads and a nest with either alitrunks or gasters. All colonies chose the alitrunk nest (A) or the gaster nest (B) over the head nest. Colonies did not split between the nests.

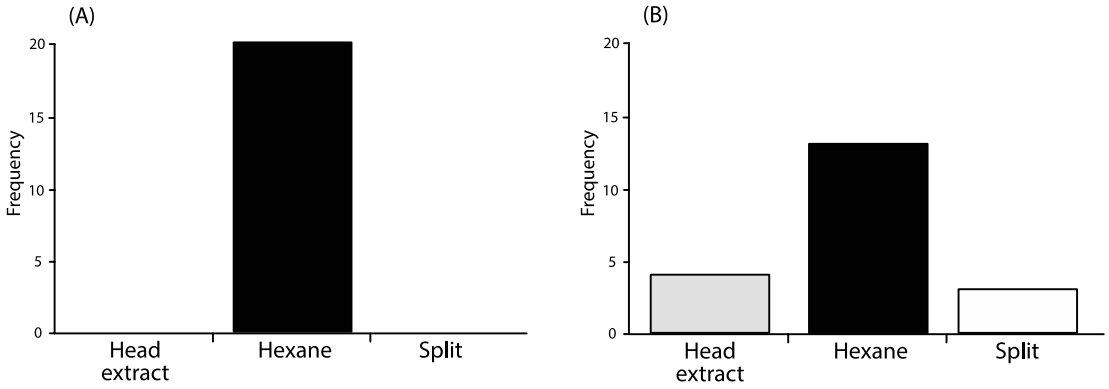


Figure 3. Results of a binary choice between a nest with a hexane extract of heads and a nest treated with hexane only. All colonies chose the hexane-treated nest (A). Even when migrations started 14h after chemical compounds were applied, colonies were still significantly more likely to choose the hexane nest (B) (2-tailed binomial test: $P = 0.049$).

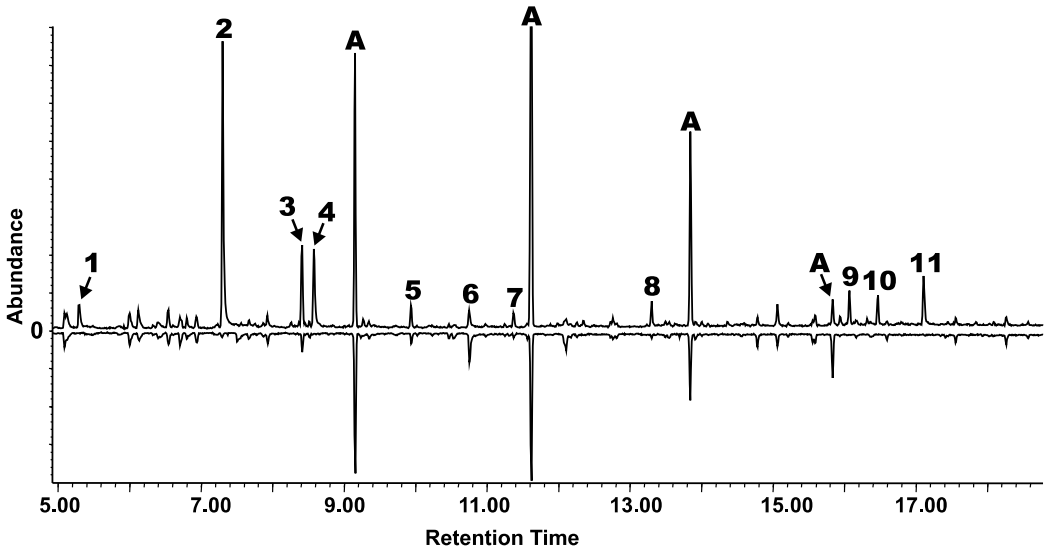


Figure 4. Total ion chromatograms from an SPME collection of volatiles from 25 crushed mandibular glands in a 1.5 ml closed vial (top), and control SPME collection from an empty vial. Peak identification: 1) 2,5-dimethylpyrazine; 2) benzyl alcohol; 3) nonanal; 4) 2-phenethyl alcohol; 5) decanal; 6) nonanoic acid; 7) undecanal; 8) geranyl acetone; 9) unknown; 10) unknown; 11) unknown. Peaks marked with an A are artifacts from the SPME device.

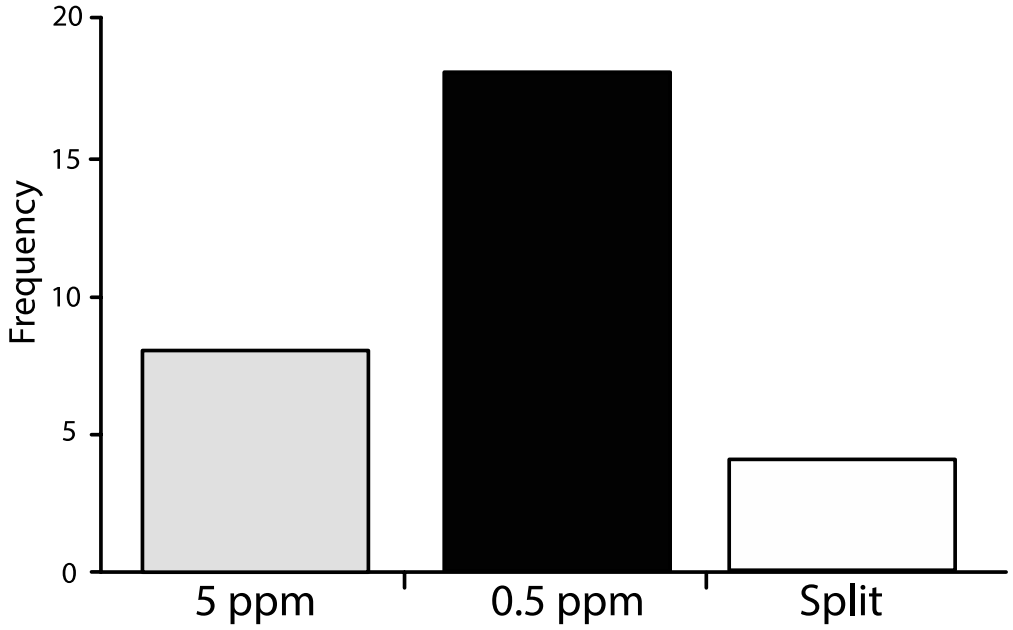


Figure 5. Results of a binary choice between nests with different concentrations (5 ppm [25.0 ng] and 0.5 ppm [2.5 ng]) of DMP. There was a trend towards colonies choosing the 0.5 ppm nest over the 5 ppm nest (2-tailed binomial test: $P = 0.07$).

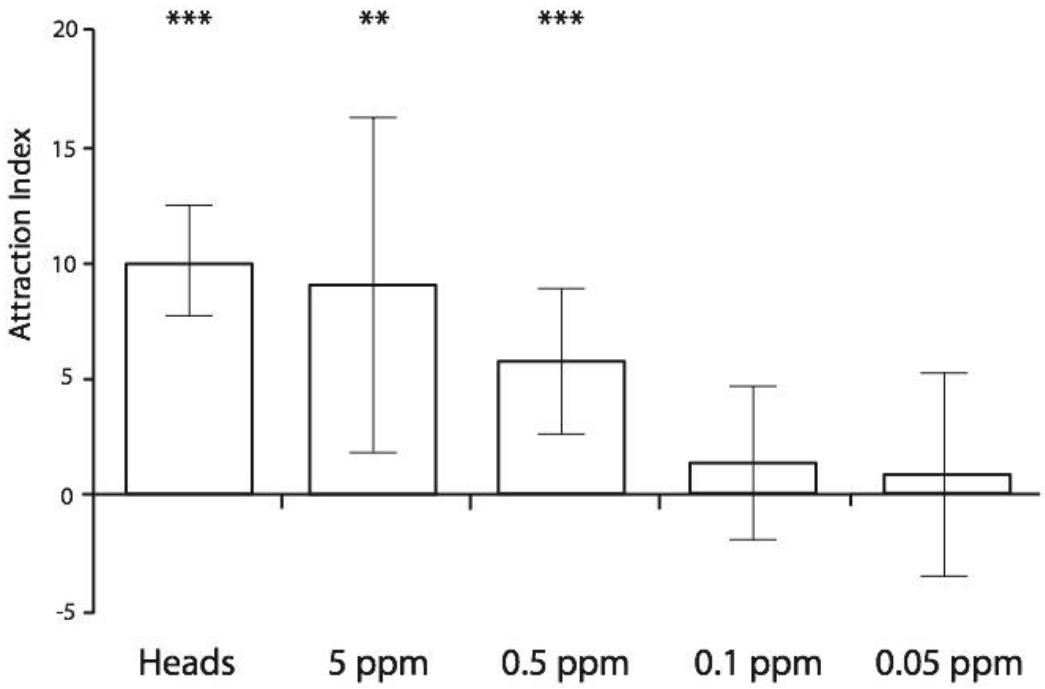


Figure 6. Number of ants attracted to crushed heads and different concentrations of DMP when presented in the home nest. Y-axis is the attraction index, calculated as the number of ants attracted to DMP minus the number attracted to a hexane control. DMP significantly attracted ants when the concentration was higher than 0.5 ppm. ** $P < 0.01$; *** $P < 0.001$.

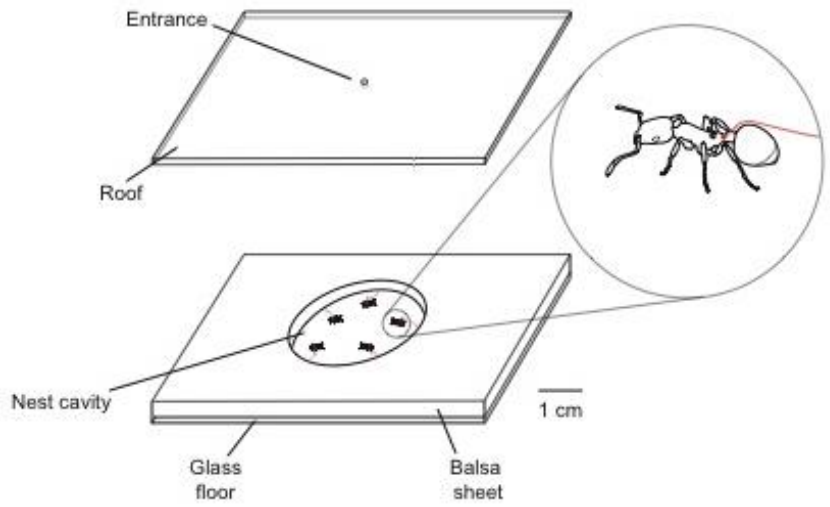


Figure 7. Nest design and ant tethering. Nests were constructed from a balsa wood slat with a circular hole drilled through its center. The roof and floor of the nest were made of glass microscope slides. An entrance hole was drilled through the center of the roof. In Experiment 1, five ants were tethered within the nest cavity using a silk thread that was wrapped around the petiole (see enlarged image at right). The strings are shown thicker than their actual size for better visualization.

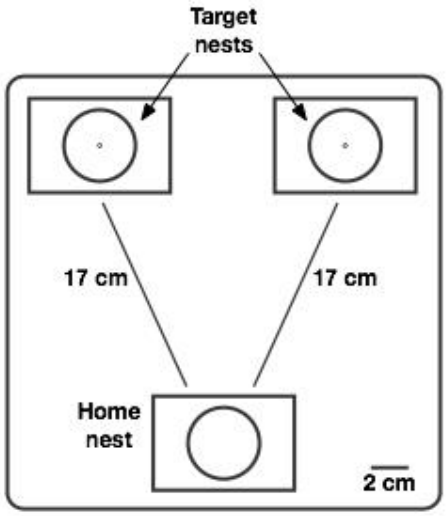
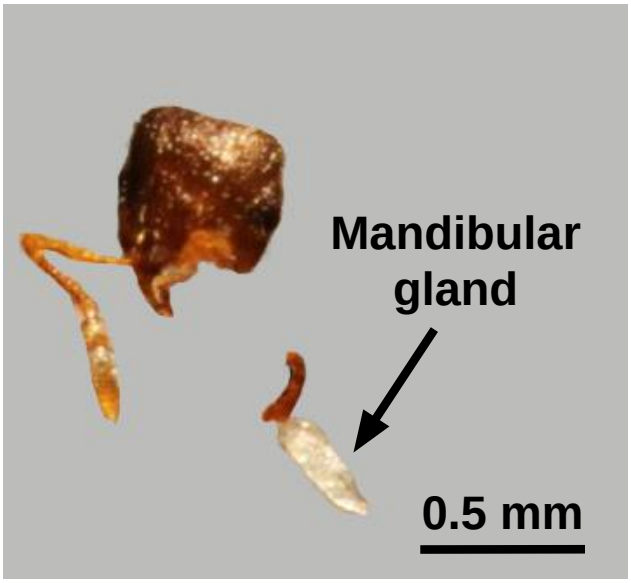


Figure 8. Experimental arena for nest choice tests. Colonies initially lived in the home nest, from which the roof was removed to induce migration. Colonies were allowed to choose between two target nests, which were identical in design but contained different materials (see text for details). The arena size was 20 cm x 20 cm and 1 cm in height.



519
520 Figure 9. Dissected mandibular gland. The gland was removed by carefully pulling a
521 mandible with fine forceps.

Table 1. A series of binary nest choice bioassays evaluating candidate alarm pheromones. One nest always was treated with hexane as a control; the other nest was treated with one of the chemical compounds that were identified in the head in Experiment 4. DMP was the only chemical that clearly elicited rejection responses from test ants.

Experimental design			Choice			
Chemical compound	Concentration	Induction of emigration	Test compound	Hexane control	Split	
Benzaldehyde	50 ppm	immediately	13	19	8	$P = 0.38$
Benzyl acetate	50 ppm	immediately	6	6	7	$P = 1$
Benzyl alcohol	50 ppm	immediately	7	10	3	$P = 0.63$
2-Phenylethanol	50 ppm	immediately	5	7	4	$P = 0.77$
Nonanal	50 ppm	immediately	5	13	2	$P = 0.10$
Nonanal	5 ppm	immediately	10	9	1	$P = 1$
Decanal	50 ppm	immediately	6	11	3	$P = 0.33$
2,5-Dimethylpyrazine (DMP)	50 ppm	immediately	2	18	0	$P < 0.01$
DMP	5 ppm	immediately	3	16	1	$P < 0.01$
DMP	1 ppm	immediately	8	21	1	$P = 0.02$
DMP	0.5 ppm	immediately	2	18	0	$P < 0.01$
DMP	0.1 ppm	immediately	10	8	2	$P = 0.81$
DMP	5 ppm	after 14 h	2	13	5	$P < 0.01$

Table 2. Effects of crushed heads and DMP on ants far from the home nest. Ant behavior was categorized as either "avoidance" or "attraction". When ants were far from their home nest, they were more likely to avoid DMP than hexane as long as the concentration of DMP was higher than 0.1 ppm. All statistical comparisons are to the hexane controls.

Chemical compound	Avoidance	Attraction	χ^2	*P*
Hexane	4	6		
Heads	10	0	8.57	0.003
5 ppm DMP	9	1	5.49	0.019
0.5 ppm DMP	10	0	8.57	0.003
0.1 ppm DMP	9	1	5.49	0.019
0.05 ppm DMP	3	7	0.22	0.64

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