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Spatial and temporal variation in pheromone composition of ant foraging trails

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Many social insects use pheromones to communicate and coordinate their activities. Investigation of intraspecific differences in pheromone use is a new area of social insect research. For example, interindividual variation in alarm pheromone content has been found in physical castes of polymorphic ants. Many ant species use multiple trail pheromones. Here we present novel research into trail pheromone variations between behavioral subcastes of pharaoh ants, *Monomorium pharaonis*. *Monomorium pharaonis* is attracted to trail pheromones found in its poison glands (monomorines) and Dufour's glands (faranal). We show that the most abundant monomorines, I (M1) and III (M3), can be readily detected in pheromone trails. A behaviorally distinct subcaste known as "pathfinder" foragers can relocate long-lived pheromone trails. Chemical analysis showed that pathfinder foragers had low M3:M1 ratios (mean 3.09 ± 1.53, range 1.03–7.10). Nonpathfinder foragers had significantly greater M3:M1 ratios (38.3 ± 60.0, range 3.54–289). We found that M3:M1 ratio did not differ between foragers of different age but was correlated with behavioral subcaste at all ages. The relative abundance of M3:M1 on foraging trails ranged from 3.03–41.3 over time during pheromone trail build-up. M3:M1 ratio also varied spatially throughout trail networks, being lowest on trail sections closest to a food source (M3:M1 = 1.9–3.61) and highest near the nest (M3:M1 = 67–267). Our results indicate a functional role for differences in pheromone trail composition, whereby pathfinder foragers might preferentially mark sections of pheromone trail networks for future exploration. *Key words:* ant, foraging, *Monomorium pharaonis*, monomorine, pheromone trail, self-organization. *[Behav Ecol 18:444–450 (2007)]*

Pheromones are signaling chemicals used for communication between conspecifics that modulate the "pattern of behavior in another organism in an adaptive fashion" (Wilson 1970). Where there is a need to ensure privacy in communication, then most animals have evolved pheromones that are species specific. Privacy is probably of benefit in all circumstances excluding alarm responses. Species specificity is achieved either by evolving large unique molecules, such as peptide pheromones, or by combining a unique blend of simple components (Wyatt 2003). In insects, unique molecules are rare and multicomponent pheromones are commonplace. A new understanding of the molecular basis of odor coding in insects (Hallem et al. 2004) means we are well placed to advance research into pheromone systems with multiple components. A major goal is to elucidate the complementary roles played by multiple pheromones within a single species. Furthermore, if the relative proportion of pheromones is important as well as interaction effects, we need to know how variation is controlled at an individual level.

Social insects make widespread and often sophisticated use of a diverse range of pheromones to coordinate the activities of their nest mates in defense, foraging and reproduction (Holldobler and Wilson 1990). The foraging trails of ants are one of the most important and widely studied examples. Mechanistically, the pheromones used in ant foraging trails have been extensively studied as single pheromone systems, but most species actually produce trails containing multiple pheromones (Holldobler and Wilson 1990; Holldobler 1995). For example, the trails of *Solenopsis invicta* contain 6 pheromones with distinct roles in trail following (Vander Meer 1986; Vander Meer et al. 1990). Individual trail pheromones can vary in volatility (and stability) and also elicit different behavioral responses that are dependent on concentration, context, and their proportion in mixtures (Van Vorhis Key et al. 1981; Jones and Blum 1982; Holldobler and Wilson 1990).

Pharaoh ants (*Monomorium pharaonis*) use pheromone trail networks for orientation between the nest and foraging sites, and the trails also recruit additional foragers (Sudd 1960; Jackson et al. 2004). Recent research has demonstrated that *M. pharaonis* employs both short-lived and long-lived pheromones while foraging (Jeanson et al. 2003; Jackson et al. 2006). Trails contain pheromones secreted from both of the sting glands (poison gland and Dufour's gland), and trails are deposited on surfaces using the extruded stinger (Blum 1966; Ritter and Persoons 1975; Ritter, Bruggemann, Persoons, et al. 1977). In *M. pharaonis*, like all myrmicine ants, each sting gland opens separately through the stinger, and the glands "possess their own muscular control mechanism that allows independent discharge of secretion" (Billen 1987). Faranal, found in trace amounts in Dufour's glands (Ritter, Bruggemann-Rotgans, Verwiel, Persoons, et al. 1977, Ritter, Bruggemann-Rotgans, Verwiel, Talman, et al. 1977), was shown to elicit the greatest trail-following activity, being followed at concentrations as low as 10^{-12} g cm^{-1} (maximum activity at 10^{-9} g cm^{-1}). Alkaloidal monomorines isolated from poison gland extracts have equal trail-following activity to faranal at a concentration of 10^{-8} g cm^{-1}. However, monomorines are abundant in the poison gland reservoir (Ritter, Bruggemann, Persoons, et al. 1977). Furthermore, a synergistic mixture of monomorines I (M1 = 5-methyl-3-butyl-octahydroindolizidine, molecular weight [mw] = 195.3) and III (M3 = trans-2-pentyl-5 (5′-hexenyl)-pyrrolidine, mw = 223.4) was found to be 5–10 times more active than single monomorines in eliciting trail following

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(Ritter and Persoons 1976; Ritter, Bruggemann, Persoons, et al. 1977). This demonstrates the potential importance of monomorine blends in *M. pharaonis* trail communication. Chemical analysis of individual ants (Ritter, Bruggemann, Persoons, et al. 1977) found a mean M3:M1 ratio of 3:1, in the individual abdomens of a small sample of *M. pharaonis* workers. Subsequent research with synthetic blends showed that the most attractive synthetic pheromone trail mix was M3:M1 = 2.3:1 to 4:1, similar to that found in individual workers (Ritter and Stein 1978).

Caste-related variation in pheromone composition has been demonstrated in several ant species exhibiting physical caste polymorphism particularly in the case of alarm pheromones (e.g., Hughes et al. 2001). However, individual variation in pheromone content has never been demonstrated in behavioral subcastes of monomorphic ants. Jackson et al. (2006) have demonstrated a behavioral subcaste (pathfinders) in *M. pharaonis* foragers, which have a particular role in long-lived trail exploration and reestablishment. Approximately, 18% of all foragers were found to be pathfinder ants, characterized by low antennal position and the ability to always detect and follow trails that were produced as much as 48 h previously. In contrast, most ants walked with their antennae held above the substrate and could not detect or follow these old trails. The development of a simple behavioral bioassay for pathfinders prompted us to investigate the possibility that there could be variability in relative pheromone abundance between individuals within *M. pharaonis*. In addition, we hypothesized that any differences in pheromone proportion between ants might also be detectable in the pheromone trails, and this would provide further insight into the role of pathfinders in the organization of trail networks.

The aim of this study was to investigate the relative abundance of the 2 major monomorines (M3 and M1) throughout the *M. pharaonis* trail network, both spatially and temporally. We then tested the hypothesis that the major monomorines found in *M. pharaonis* might vary in their relative abundance among distinct groups of foraging ants with different roles (pathfinders, nonpathfinders, and different age groups) if they are important as multicomponent trail pheromones.

MATERIALS AND METHODS

Study organism

Study colonies of *M. pharaonis* contained 1200–2500 workers, brood of all stages and multiple queens (12–50), and were housed in wooden nest-boxes (11 × 8 × 2 cm) held within a large plastic foraging box (45 × 30 × 15 cm) in a climate controlled room (24 ± 2 °C, relative humidity = 30%, 12:12 h light:dark). Colonies were given fresh water ad libitum in glass tubes sealed with cotton wool and fed with mealworm (*Tenebrio*) larvae, sugar syrup, and dried egg yolk. In *M. pharaonis*, manipulation of colony size is simple due to the absence of nest mate recognition (Holldobler and Wilson 1990). Frequent splitting and combining of the colonies meant that the 6 colonies used were very similar in genetic composition.

Trail chemistry—analysis at timed intervals

Workers were allowed to forage along a narrow corridor to a sugar syrup feeder via a bridge, as in Figure 1 (after Jackson et al. 2006). A colony's foraging box was temporarily linked to a foraging platform (140 × 70cm) by a drawbridge. Part of the area was covered with 2 A4 (29.5 × 21.0 cm) sheets of eucalyptus chlorine-free paper photocopied with a 2-mm grid. Two parallel polycarbonate strips (60 × 4 × 0.5 cm), internally coated with Fluon to prevent ants from climbing them, were placed to give a 4-mm wide corridor leading to a syrup feeder within an enclosure of Fluon-coated plastic pipe (8 cm diameter). Ants crossing the drawbridge were guided into the corridor by 2 Fluon-coated plastic barriers. The floor of the corridor was covered with glass microscope slides (7.5 × 2.5 cm) previously washed in hexane. At timed intervals (5, 10, 15, 20, and 30 min), a slide was removed and replaced with a fresh slide. The central region of the slide, which had been accessible to ants, was immediately swabbed with hexane-moistened fused silica wool. Swab samples were then sealed into chromatography vials prior to storage (refrigerated at 4 ± 4 °C). This procedure was performed on 3 trails each produced by a different colony. Ant traffic passing the trail midpoint was counted in both directions at 5-min intervals. Swab samples were chemically analyzed using the technique outlined below.

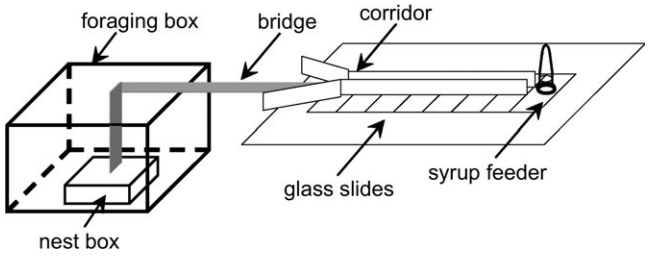


Figure 1
Experimental apparatus for production of constrained pheromone trails (after Jackson et al. 2006). Ants accessed the corridor leading to the sugar syrup feeder after crossing a bridge from the foraging box. The polycarbonate corridor constrained foraging ants so as to form a straight trail to the feeder on the glass slides.

Smoked glass trail network—chemical analysis

We investigated straight trails using the apparatus in Figure 1, but we also required some insight into what happens in a complete trail network. *Monomorium pharaonis* colonies naturally produce branching networks of trails while exploring the environment, one or many sections of which may lead to food sources (Jackson et al. 2004). This network of trails can persist for 2–3 days without ants walking on it but much longer if trails are reused. If sections of the trail network leading to food are differentially marked, this could enable foragers to relocate food sources on subsequent days.

We allowed a colony to form a trail network on a smoked glass surface, which enables the trail network to be visualized because ant activity wears away the soot (Hangartner 1969a, 1969b). The ant colony was linked to a foraging area, as in Figure 1, where the paper and corridor were removed, to be replaced with a sheet of toughened glass (39.2 × 27.6 cm). The glass sheet had been held over a wax candle flame until it was coated with a fine layer of soot. A single sucrose syrup feeder was placed on the glass, and the ants were allowed to forage for 1.5 h. The glass sheet was then cleared of ants and hexane-moistened swab samples were taken from trail sections close to the nest, close to the feeder, and also from branch points in the network that did or did not lead to food. The 8 sampling points are shown in Figure 4. Swab samples were placed in chromatography vials and analyzed using liquid chromatograph–time-of-flight mass spectrometer (LCT-MS) (see chemical analyses, below). We repeated this analysis with a further 2 colonies.

Trail-finding bioassay for pathfinders

To perform pathfinding bioassays, we produced straight foraging trails in known positions on sheets of paper, using the

apparatus shown in Figure 1 (Jackson et al. 2006). We counted total ant traffic passing the midpoint of the corridor in both directions. Once 2000 ants had been counted, all ants were shaken from the paper. The paper was then stored, away from ants but exposed to the air and light, in the ant room. The next day ants were taken from the foraging box floor (these ants were thus foragers, not ants performing within-nest tasks) and placed in empty plastic boxes prior to being tested. Individual ants were then carefully transferred, on a 4 × 2-cm section of thin plastic, to a separate testing box containing a 20 × 10-cm section of the paper on which a pheromone trail had been established the previous day. The ant was guided, using sections of plastic as barriers, to within 4 cm of the trail and allowed to cross the trail at approximately 90°. If, on crossing the trail, the ant followed the trail without deviation for at least 8 cm the ant was considered to have successfully located the old trail. This procedure was repeated on 5 occasions per ant. After each test, the ant was removed from the trail and replaced at a distance of 4 cm from the trail. Ants scoring 4 or 5 successes out of 5 trials were designated as "pathfinders," whereas those scoring 0–3 were designated as "nonpathfinders." In practice, 96% of nonpathfinders scored zero successes in the 5 trials (Jackson et al. 2006).

Production of uniform age cohorts

We investigated the pathfinding ability of worker ants in 6 age groups to determine whether variation in the abundance of monomorines in individual ants was correlated with age and (or) pathfinding ability. Maturation from egg to adult worker takes 36 days and workers typically live for no more than 70 days after eclosion. *Monomorium pharaonis* workers are small with a mean body length of approximately 2 mm. They also have a very oily cuticle that prevents paint markings from adhering longer than 2 days.

As an alternative to individual marking, we produced worker cohorts of known age by placing queens with brood piles containing eggs, larvae, and an abundance of dark pupae (close to eclosion) into nest-boxes. No workers were initially present in these nest-boxes. Food (mealworms, dried liver, and sugar syrup) and water were supplied. Workers were allowed to eclose from pupae for a fixed period (2, 7, and 10 days). These age groups were thus limited in age to <2, <7, and <10 days. Samples of these "young," or callow, workers were tested for pathfinding ability (using trail-finding bioassay, above). Only workers foraging outside the nest-box were tested to ensure that they were foragers. Ants were prepared for monomorine content analysis by placing each individual in a separate chromatography vial with 200 µl of hexane. Individual ants were stored in hexane for a minimum of 1 day prior to chemical analysis.

Similarly "old" cohorts of ants were produced by transferring approximately 50 worker ants into nest-boxes containing 2–10 queens and brood piles. Ants were then maintained for a fixed "ageing" period (28, 40, or 60 days). These ants were thus limited in age to >28, >40, and >60 days because we did not know the initial age of workers. During the ageing period, all dark pupae were regularly removed from the brood piles to prevent young ants from eclosing. On completion of the limited ageing period, individual ants were tested for pathfinding ability and monomorine content following the same procedure as previously described for young ants.

Chemical analysis procedure

Hexane was evaporated from samples before adding 100 µl of 50:50 acetonitrile:water, followed by vortex mixing for 1 min and standing for 10 min prior to analysis. Chemical analysis of samples was performed using a Waters Micromass LCT-MS. The instrument was operating under the following conditions: capillary, 3100 V; sample cone, 25 V; RF lens, 100 V; extraction cone, 2 V; desolvation temperature, 150 °C; source temperature, 120 °C; desolvation gas flow, 400 $dm^3\ h^{-1}$; nebulizer gas flow, 100 $dm^3\ h^{-1}$. Samples were directly infused into the MS (no column separation) using a Hamilton 250-µl syringe and a Razel (Stamford, CT) Syringe Pump. Sample infusion was at a constant rate of 10 µl min^{-1}, and all data were acquired in electrospray positive mode. Three replicates per sample were each run for 1 min. Each replicate data set was the summation of all scans within 1 min. Spectra were collected over the mass range 50–800 KDa using 0.5 s scan time and 0.1 s interscan delay time. All scans were corrected using Micromass Lockspray Technology, which enables automated accurate mass measurements to be collected by the use of an internal reference. We used leucine enkephalin (mass 556.2771 in positive mode) as the internal reference at a concentration of 5 µg ml^{-1}. The reference was infused into the MS using a syringe pump as for a sample and monitored every 5 s during a 1-min acquisition period.

Peak identification was by reference to published mass spectral data for whole ants and separate gland analyses previously performed on *M. pharaonis* (Talman et al. 1974; Ritter, Bruggemann, Persoons, et al. 1977). The relative abundance of M3 when compared with M1 was calculated as a ratio for each analysis, by reference to total ion counts. The absolute amount of monomorines present was not investigated in this study because ants could vary in their total pheromone content if they had recently been engaged in pheromone laying activity. Our study was concerned with detecting relative amounts of pheromone deposited because there is good evidence that synergistic blends of *M. pharaonis* monomorines can have different effects on behavior (Ritter, Bruggemann, Persoons, et al. 1977).

RESULTS

Trail chemistry: temporal effects in constrained straight trails

Results for the 3 straight trails we analyzed are shown in Figure 2. Total forager traffic after 30 min for the 3 trails, each produced by a different similar-sized colony, was similar (1589, 1347, and 1356 ants passing trail midpoint). The relative abundance of the 2 main monomorines (M3:M1) was variable throughout test periods, but all trails were characterized by a trend of increasing M3:M1 ratio rising to a peak (13.6, 41.3, and 31.5, respectively) followed by an abrupt drop. The time at which this peak occurred varied, but at the end of each 30-min test period, we found that all 3 trails had stabilized to a similar M3:M1 ratio (5.87, 3.03, and 4.11). Final M3:M1 ratios are in a similar range to that found for individual ants by Ritter, Bruggemann, Persoons, et al. (1977), but during the early part of the test period, preceding stabilization, the ratio was as high as 41.3. We performed an analysis of covariance (ANCOVA) analysis and the test for homogeneity of regression shows (ANCOVA; degrees of freedom [df] = 2, df error = 9, $F = 0.35$, $P = 0.741$) that the response (M3:M1 ratio) of the 3 colonies was not significantly different with increased traffic. Significantly, the statistical test also shows that variation in M3:M1 ratio cannot be attributed to trail traffic alone (ANCOVA; df = 2, df error = 9, $F = 0.65$, $P = 0.540$). This is important because in a homogeneous population, we would expect traffic to be the main factor. Our results thus strongly suggest a heterogeneous population as the source of variability. Variability in relative trail pheromone abundance is presumably caused by relative differences in the deposition of M3 and M1. Variable deposition must arise from differences in

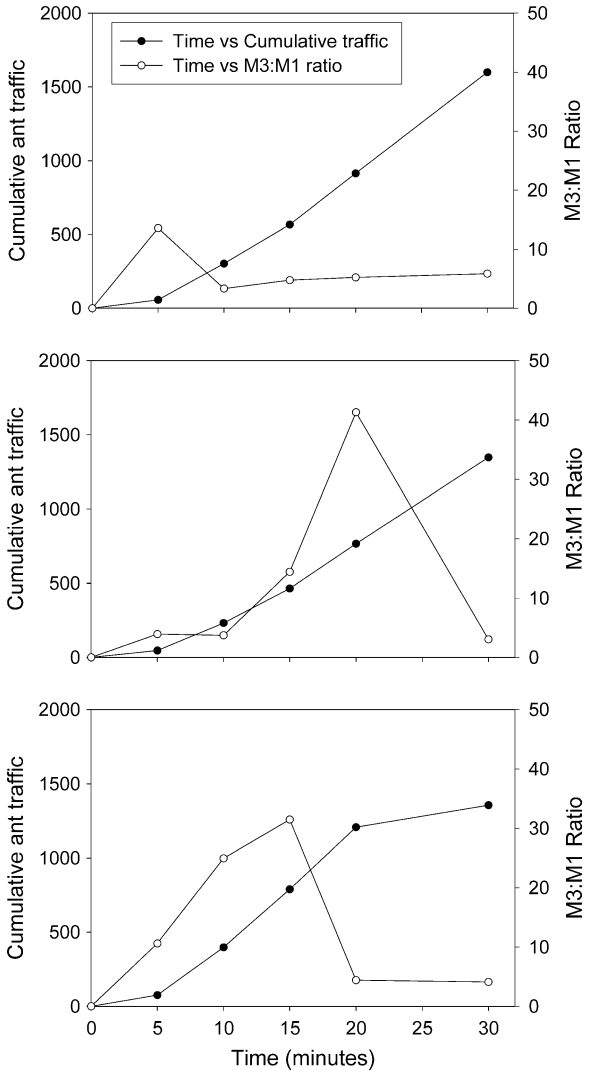
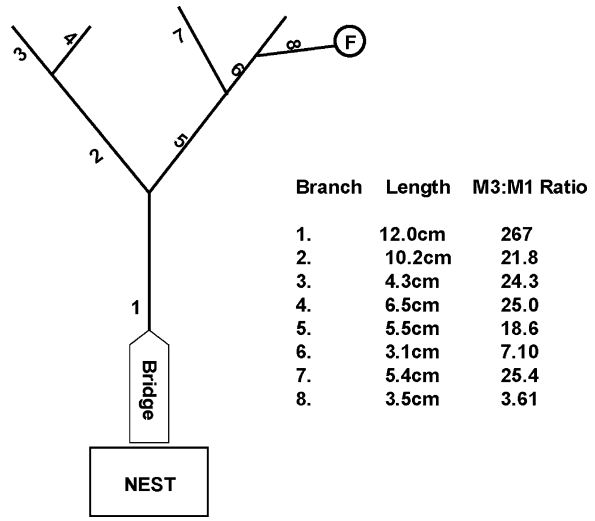


Figure 2
Chemical analysis of the buildup of 3 *Monomorium pharaonis* pheromone trails (using LCT-MS). Filled circles show the cumulative amount of traffic passing the trail midpoint, whereas empty circles denote the ratio of M3 to M1 detected in the trail (sampled from 2.5 cm on a glass slide).

the chemical composition of ants laying the trail because the 2 monomorines both originate in the poison gland.

Trail chemistry: spatial effects in unconstrained trail networks

Our analyses of unconstrained trail networks showed that sections of trail located closest to food had lower M3:M1 ratios. In the example presented in Figure 3 (trial 1), it can be seen that the section leading immediately to food (branch 8) had the lowest ratio in the network (3.61), whereas the adjoining section leading to the nest (branch 6) had the second lowest (7.10). The first section of trail encountered when leaving the bridge (branch 1) had the highest ratio (267), whereas all other branches tested had similar, intermediate, ratios ranging from 18.6 to 25.4. Thus, to reach a food site, an ant could simply walk down a gradient of decreasing M3:M1 ratio. In the other 2 trail networks, an identical trend was found: in trial 2 the M3:M1 ratio on trail sections leading to a food site was 133, 16.2, 4.21, and 2.25 and in trial 3 (fewer branches)



Branch	Length	M3:M1 Ratio
1.	12.0cm	267
2.	10.2cm	21.8
3.	4.3cm	24.3
4.	6.5cm	25.0
5.	5.5cm	18.6
6.	3.1cm	7.10
7.	5.4cm	25.4
8.	3.5cm	3.61

Figure 3
Schematic of an unconstrained foraging trail network formed on smoked glass showing the sampling points, branch lengths, and the M3:M1 ratio on that branch. A 2.5-cm length of trail on each branch was sampled from the smoked glass surface after ants had been foraging for 1.5 h to a syrup feeder, F. Ant traffic crossing bridge in either direction = 4300.

the ratio was 67, 9.6, and 1.9. Statistical analysis showed that there was no significant difference between the 3 regressions (ANCOVA test for homogeneity of regression; df = 2, df error = 9, $F = 0.60$, $P = 0.584$) and that distance from food source was significantly correlated with M3:M1 ratio ($r^2 = 0.580$).

Age: pathfinding and pheromone variation

Table 1 shows that there was no significant correlation between pathfinding score and age (Spearman's rank correlation coefficient: $r = 0.050$, $P = 0.691$). Comparison of the pathfinding assay results for the 6 same-age cohorts (<2, <7, <10, >28, >40, and >60 days old) showed no significant difference in the proportion of ants capable of pathfinding in the 6 age classes ($\chi^2 = 1.952$, df = 5, $P = 0.856$). There was no correlation between age and the M3:M1 ratio of individual ants (Spearman's rank correlation coefficient: $r = 0.013$, $P = 0.920$). We also found no significant differences between the M3:M1 ratio of young (all ants <10 days old: mean ratio = 24.7 ± 55.5, $n = 31$) and old ants (all ants >28 days old: mean ratio = 34.1 ± 53.4, $n = 34$) when divided into these 2 main age categories (*t*-test: $t = -0.696$, df = 63, $P = 0.489$).

Individual ant chemistry and pathfinding

Figure 4 summarizes the chemical analysis of all individual ants tested (all ages) and shows that pathfinder ants form a discrete cluster because of their lower M3:M1 ratio. We found a significant correlation between low M3:M1 ratio and high pathfinding score (Pearson's correlation coefficient: $r = 0.262$, $P = 0.035$). Remarkably, the M3:M1 ratio varied by more than 2 orders of magnitude among nonpathfinders (1.41–288) but by less than 1 order of magnitude in pathfinders (1.03–7.18). We found a highly significant difference between the low M3:M1 ratios of pathfinders (3.09 ± 1.53, $n = 16$) and the high ratios of nonpathfinder (38.3 ± 60.0, $n = 49$) ants (*t*-test: $t = 4.096$, df = 48, $P = <0.001$; Levene's test for equality of variances: $F = 7.939$, $P = 0.006$).

Our results show that the 2 behavioral classes of *M. pharaonis* foragers are chemically distinct. When separated into 2 main

Table 1

Ratio of M3:M1 for ants in 6 age cohorts separated into 2 behavioral classes, pathfinders and nonpathfinders, using the trail relocation bioassay

Age group	*n* (ants tested)	Pathfinders M3:M1 ratio	Nonpathfinders M3:M1 ratio	Pathfinders, %
<2	5	1.03	23.39 ± 11.65	20.00
<7	7	4.29	15.98 ± 9.20	14.29
<10	19	2.44 ± 0.34	40.75 ± 77.31	26.32
>28	19	3.52 ± 2.49	56.45 ± 73.27	21.05
>40	10	3.03 ± 0.27	29.78 ± 20.83	40.00
>60	5	5.75	25.76 ± 10.92	20.00
All <10	31	2.50 ± 0.99	31.14 ± 61.86	20.20
All >28	34	3.55 ± 1.76	45.08 ± 58.67	27.02

Pathfinders refers to those ants scoring successes from 5 attempts in the trail-finding bioassay. Nonpathfinders scored zero successes in the bioassay.

age cohorts (young = <10 days, old = >28 days), we found that there was no significant difference between the M3:M1 ratios of pathfinding ants (young ants—2.50 ± 0.99, $n = 7$; old ants—3.55 ± 1.76, $n = 9$; *t*-test: $t = -1.40$, df = 14, $P = 0.183$). There was also no significant difference between the M3:M1 ratios of nonpathfinding ants in the 2 age cohorts (young ants—31.1 ± 61.9, $n = 24$; old ants—45.1 ± 58.7, $n = 25$; *t*-test: $t = -0.809$, df = 47, $P = 0.422$). Our results show that pathfinding ants are distinct from nonpathfinders at all ages, both behaviorally and in their production of monomorine trail pheromones.

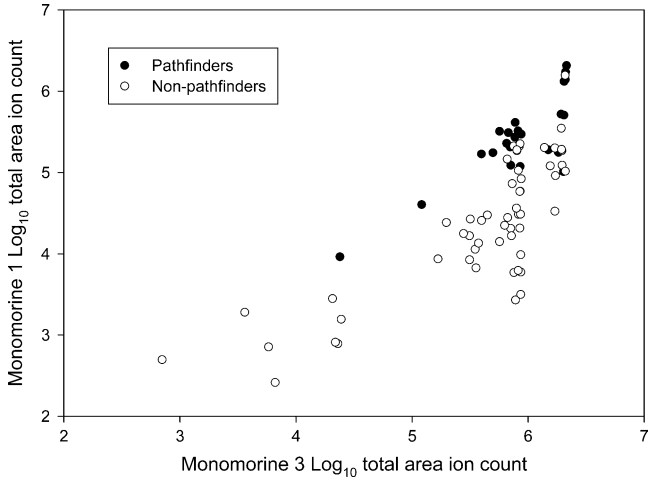


Figure 4
Results of chemical analysis performed on hexane extracts of whole individual ants. Workers were separated into 2 classes using a behavioral (trail relocation) bioassay as pathfinders (score 4 or 5) or nonpathfinders (score 0–3). Mean total ion counts (peak areas) for M3 and M1 are shown for extracts of whole *Monomorium pharaonis* foraging workers ($n = 82$). The proportion of M1 relative to M3 was significantly higher in pathfinder ants. Mean ratio of M3:M1 in pathfinders was 3.09 (standard deviation [SD] = 1.528, $n = 16$), whereas in nonpathfinders the mean was an order of magnitude greater at 38.25 (SD = 60.03, $n = 49$).

DISCUSSION

Analysis of *M. pharaonis* pheromone trails, constrained by straight corridors, showed that there is great variability in monomorine composition of pheromone trails, over time. All 3 trails we analyzed showed the same pattern, initially building to a high M3:M1 ratio (ca., 30) followed by an abrupt fall in ratio and stabilization in the range M3:M1 = 4–5. The fall in ratio is probably caused by an increased deposition of M1 relative to M3. Variation in deposition of these 2 pheromones cannot be achieved by a single ant regulating its pheromone deposition because both must be released simultaneously from a single storage source, the poison gland reservoir (Billen 1987). Thus, the variation in the ratio of the major monomorines on pheromone trails must be caused by variation in deposition by ants with different stored compositions.

We found evidence for spatial as well as temporal variation in the monomorine content of unconstrained natural trail networks formed on smoked glass. Trail sections nearest the food source had lower M3:M1 ratios than sections of the trail network nearest to the nest. Our analysis shows that trail network branches leading immediately to food have received more marking by ants with M1 relative to M3, when compared with other trail sections not leading to food and those at long distance from food. These results suggest a sophisticated use of pheromone blends in *M. pharaonis* trails with differential marking signposting trail branches leading to food. We then showed that individual variations in trail pheromone composition were also linked to known behavioral differences in *M. pharaonis* foragers.

Previous research has demonstrated clear behavioral differences in response to trail pheromones among *M. pharaonis* foragers (Robinson et al. 2005; Jackson et al. 2006). *Monomorium pharaonis* foraging trails contain both short lived (20 min), long lived (up to 3 days), and negative pheromone components that facilitate foraging to ephemeral and persistent food sources (Blum 1966; Ritter et al. 1973; Jeanson et al. 2003; Robinson et al. 2005; Jackson et al. 2006). Only pathfinding foragers can detect and follow long-lived trails. We hypothesized that the predicted variations in monomorine content between individual foragers may be more pronounced in pathfinders because they are a readily identifiable forager subcaste with a particular role in trail network exploration. Furthermore, monomorines are highly stable, low volatility compounds, which retain their activity even after being refluxed for 24 h in chloroform (Blum 1966). Monomorines are good candidates for long-lived trail pheromones. Subtle communication of information using monomorine blends would be of much greater importance to pathfinders, if monomorines are indeed long-lived trail pheromones.

Our behavioral study of pathfinders from cohorts of known age showed that pathfinders always represented approximately 20% of foragers, irrespective of age. This proportion replicates that found in a previous study, where the effect of age was not investigated (Jackson et al. 2006). The absence of a correlation between pathfinding and age strongly suggests that this behavior is not associated with age polyethism.

Chemical analysis of individual foragers showed no significant correlation between M3:M1 ratio and age. However, a highly significant difference in mean M3:M1 ratio was found between pathfinding and nonpathfinding ants. Pathfinding ants had a low M3:M1 ratio of 3.09, whereas nonpathfinders had a much higher ratio of 38.3. The relatively high proportion of M1 in pathfinders suggests that pathfinders are the probable source of a low M3:M1 ratio when it occurs on trails. Low ratios were found on constrained trails after 10–30 min of foraging to a syrup feeder and also in those parts of an unconstrained trail network that were closest to food.

Our data strongly suggest that pathfinder ants are markers of trails that are profitable for long periods. Based on the constrained trail study, we can also say that nonpathfinders actively mark trails during the early stages of foraging on a new food source because ratios were high in this period.

However, the contribution of pathfinders must become much greater, as the foraging process continues, because the ratio drops abruptly. We suggest 3 possible hypotheses explaining how this drop-in ratio might occur. The first is that pathfinders may continue laying pheromones after a trail is established, whereas nonpathfinders desist from laying trail pheromones once it is established. Such a mechanism could be possible with differing behavioral response thresholds (to trail pheromone concentration) in the 2 classes of forager. Second, pathfinder ants may only become active in trail maintenance later in a foraging bout. The available evidence contradicts this second hypothesis because pathfinders are found at all times outside the nest when other foragers are present (Jackson et al. 2006). Finally, it is possible that pathfinders contribute to the trail more frequently, or with greater amounts, than nonpathfinders. The first and last hypotheses are not mutually exclusive and would both mean that pathfinder ants are specialists in all aspects of trail production, maintenance, detection, and reestablishment. Specialization to this degree would mean there is a remarkable division of labor in pheromone trails by pharaoh ants. We speculate that the presence of pathfinders could increase the efficiency of foraging trails by allowing the remaining 80% of foragers to concentrate on walking more rapidly between food and nest. Ants laying trail would be moving more slowly than those not doing so, but if only a minority of ants continue laying trail (or lay trail more frequently), then the speed of resource returns would be increased, along with a decreased predation risk.

Monomorines are already known to serve important roles as venoms and repellents against insect competitors (Jones and Blum 1982; Bacos et al. 1988). Monomorines also have proved trail-following activity, but it was found that they were most active as mixtures. The optimum blend of synthetic monomorines for trail following was M3:M1 = 2.3:1 to 4:1 (Ritter, Bruggemann, Persoons, et al. 1977). Our study shows that variation in the mixture of the 2 major monomorines found in *M. pharaonis* could be used to convey information that facilitates search in an established trail network on subsequent days. We suggest that the monomorines are good candidates for long-lived trail components, and it is probable that the pathfinders lay pheromone blends that they themselves will be utilizing for information on subsequent days. In an unconstrained branching network of natural trails, the branches that have most recently proved rewarding would be distinguishable by their low M3:M1 ratio. Remarkably, the variations in the monomorine content of individual foragers could allow them to deposit information in trails for the specific use of their own subcaste, the pathfinders. This mechanism would not exist solely for selfish reasons but could aid overall colony function in the division of foraging labor into explorers (trail marking and pathfinding) and exploiters.

Our study shows how a specific blend of pheromones arises on the trails of *M. pharaonis* and how the information it contains might be exploited by a specialized behavioral caste. Multicomponent pheromone systems are common in social insects, but one researcher notes that "the identification of these (pheromone trail) components has mostly outpaced an understanding of their function" (Holldobler 1995). Pheromone blends have been found in the trails of other ant species. In *Tetramorium caespitum*, for example, a blend of 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine in a ratio of 3:7 elicited maximum trail-following activity (Jones and Blum 1982). This is the blend typically found in all *T. caespitum* individuals examined and requires no further explanation. In the African termite *Schedorhinotermes lamanianus*, pheromones from the labial glands and sternal glands modulate recruitment during food exploitation (Reinhard and Kaib 1995). A volatile pheromone from the sternal gland can inhibit the behavioral response to the nonvolatile pheromone from the labial gland in dose-dependent manner. At high doses, termites are repelled from gnawing aggregations and established ones disperse. The interaction between the 2 pheromones dynamically regulates food exploitation. In our current study of *M. pharaonis*, we have shown a novel use of pheromone blends that contrasts greatly with previous work. Our study suggests that variation in pheromone blends on the trail can potentially convey spatial and temporal foraging information.

The absence of variation in individual M3:M1 ratio with age, and the significantly lower ratio that is characteristic of pathfinders, suggests that pathfinder ants assume this role early in their development and also shows that there is a physiological component to pathfinding. In a semiquantitative study of *Solenopsis invicta* it was shown that workers only synthesize venom during the period immediately after eclosion until 15 days old, with negligible synthesis after this date (Haight and Tschinkel 2003). Similarly in the honeybee (*Apis mellifera*) venom synthesis peaks within 14 days and declines to nil thereafter as the venom gland degenerates (Owen 1978; Owen and Pfaff 1995). In contrast three uneclosed *M. pharaonis* dark pupae we qualitatively analyzed already contained monomorines demonstrating that venom synthesis had occurred before eclosion (M3:M1 ratios of 231, 585 420). Monomorines have multiple roles in *M. pharaonis* serving as venoms, trail pheromones and repellents. The requirement of monomorines as trail pheromones may make them essential for foraging. We always found newly eclosed ants foraging in the ageing trials we conducted. Foragers are unlikely to switch rapidly to the pathfinding role because the enzymes required in the M1 biosynthetic pathway must first be synthesized. It is interesting that *M. pharaonis* queens have an M3:M1 ratio of approximately 30.0 (Ritter, Bruggemann, Persoons, et al. 1977), ten times that found in pathfinders but approximately the same as the mean ratio found in nonpathfinder ants.

Differences between worker castes have been found in the alarm pheromones of *Oecophylla longinoda* (Bradshaw et al. 1979) and several *Atta* species (Hughes et al. 2001). However, these differences were associated with worker polymorphism, which can readily be envisaged as influencing gland development as well as physical size. The variation in individual trail pheromone composition we have identified in pathfinder ants of *M. pharaonis* is in a monomorphic species and, therefore, particularly novel. Behavioral differences between physically identical *M. pharaonis* foragers are coupled to variations in individual pheromone composition. We suggest that the behavioral and pheromonal specializations of pathfinders, and nonpathfinders, might divide the foraging force efficiently in the exploration and exploitation of foraging trail networks. Localized differences in trail composition may optimize search by focusing attention on those branches "known" by pathfinders to previously lead to food. The highly sophisticated foraging system employed by *M. pharaonis* demonstrates novel aspects of division of labor in insect societies and provides fresh insight into the complexity of chemical communication. We anticipate that a fuller elucidation of the roles of other trail pheromones, particularly faranal, in *M. pharaonis* will help us to understand how this fascinating communication system functions. Our findings lend weight to the claim that ant pheromone trails are the most elaborate of all the forms of chemical communication employed by social insects (Wilson 1971).

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