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Sex Attractant Pheromones of Virgin Queens of Sympatric Slave-Making Ant Species in the Genus *Polyergus,* and their Possible Roles in Reproductive Isolation

Les Greenberg[1] · **Christine A. Johnson**[2] · **James C. Trager**[3] · **J. Steven McElfresh**[1] · **Joshua Rodstein**[4,5] · **Jocelyn G. Millar**[1,4]

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Abstract

Species of the ant genus *Polyergus* are social parasites that steal brood from colonies of their hosts in the closely related genus *Formica*. Upon emergence as adults in a mixed population, host *Formica* workers carry out all the normal worker functions within the *Polyergus* colony, including foraging, feeding, grooming, and rearing brood of the parasitic *Polyergus* ants. Some unmated *Polyergus* gynes (queens) run in the raiding columns of their colonies and attract males by releasing a pheromone from their mandibular glands. There are two *Polyergus* species groups in North America: an eastern *P. lucidus* group and a western *P. breviceps* group. One species of each of these groups, *P. lucidus* Mayr and *P. mexicanus* Emery, are sympatric in Missouri. In this study, we characterized the sex pheromones of virgin queens of two species of the *P. lucidus* group (*P. lucidus* sensu stricto and *P. sanwaldi*) and one species of the *P. breviceps* group (*P. mexicanus*), and compared these with the previously identified sex pheromone of *P. topoffi* of the *P. breviceps* group. We then used sex pheromone blends reconstructed from synthesized components of the two groups to test their efficacy at reproductively isolating these species. We found that methyl 6-methylsalicylate is conserved as the major component of the pheromone blends for both *Polyergus* species groups; however, methyl (*R*)-3-ethyl-4-methylpentanoate is the species-specific minor component produced by *P. lucidus* group queens, and (*R*)-3-ethyl-4-methylpentan-1-ol is the crucial minor component for *P. breviceps* group queens. The optimal ratio of the major and minor components for *P. lucidus* group queens was about 100:1 salicylate to ester. In concurrent field trials in Missouri, males of *P. lucidus* sensu stricto and *P. mexicanus* (a member of the *P. breviceps* group) were attracted almost exclusively to their particular blends of sex pheromone components. To our knowledge, this is the first example of a possible sex-pheromone-based reproductive isolating mechanism in ants.

Keywords Ant sex pheromone · Reproductive isolation · Sympatric species · Dulosis · 3-ethyl-4-methylpentanol · Methyl (*R*)-3-ethyl-4-methylpentanoate · Methyl 6-methylsalicylate

Introduction

Hybridization among closely related sympatric species is often prevented by pre- or post-zygotic mechanisms such as stereotyped reproductive behaviors, asynchronous temporal activity, ecological differences, mechanical barriers, various types of species-specific signals, or a combination thereof (Byrne and Anderson 1994; Mayr 1972). In the Insecta, species-specific pheromones are widely used to coordinate reproductive behaviors and minimize hybridization among congeners. Mate location in many insects is mediated primarily by volatile, long-range sex attractant pheromones, whereas larger, less volatile chemicals on the cuticle serve as contact

✉ Les Greenberg
Les.greenberg@ucr.edu

[1] Department of Entomology, University of California, Riverside, CA 92521, USA

[2] Division of Invertebrate Zoology, The American Museum of Natural History, New York, NY 10024, USA

[3] Shaw Nature Reserve, Missouri Botanical Garden, Gray Summit, MO 63039, USA

[4] Department of Chemistry, University of California, Riverside, CA 92521, USA

[5] Present address: Woodland Hills, CA 91365, USA

pheromones during the final stages of recognition once partners are in close proximity. For eusocial insects such as ants, there has been little progress in identifying either the source of female-produced sex pheromones or the active components. For example, it is known that extracts of the poison gland of *Xenomyrmex floridanus* Emery females attract male ants (Hölldobler 1971) but, until now, the identification of sex pheromones from females has been reported for only three species: *Formica lugubris* Zetterstedt (Walter et al. 1993) from Europe, *Polyergus topoffi* Trager [reported as *P. breviceps* (Greenberg et al. 2004, 2007)] from specimens collected in Arizona, and the European *P. rufescens* Latreille (Castracani et al. 2005, 2008; Grasso et al. 2003). The same sex pheromone was described for both of the above *Polyergus* species, demonstrating that components of the pheromone may be conserved within the genus. The specificity of the sex pheromone blend is likely to be important in the reproductive isolation of closely related species (Blum 1981), particularly species that are sympatric.

All *Polyergus* species are obligate social parasites that specialize in taking juveniles during raids on colonies of their *Formica* hosts. The *Formica* hosts then form the worker caste in the mixed colony, with the *Polyergus* parasites totally dependent on them for all normal worker functions. Young *Polyergus* queens establish new colonies by usurping nests of their host species (Mori et al. 2001; Topoff 1990). The newly-mated parasite queen invades a host nest and typically kills the resident queen. While doing so, the parasite queen acquires the cuticular hydrocarbon signature of the deceased host queen, so that host workers no longer recognize the usurper as foreign (Errard and D'Ettorre 1998; Johnson et al. 2001, 2002; Topoff and Zimmerli 1993). Some *Polyergus* virgin gynes attract males by releasing a pheromone from their mandibular (Topoff and Greenberg 1988) or intramandibular (Grasso et al. 2003, 2004) glands while running alongside nestmates during raids on the nests of host species. Alternatively, virgin queens fly from the natal nest, presumably to mate and disperse from the natal population and/or locate a host *Formica* colony [see summaries in Mori et al. 1994 and Trager 2013].

The genus *Polyergus* has a Holarctic distribution and occurs throughout much of the United States (Creighton 1950). *Polyergus* populations in the U.S. are fragmented, and parasitized host species differ among populations (e.g., King and Trager 2007). While the work described in this study was in progress, a major revision of the genus was published (Trager 2013). Before revision, the North American *Polyergus* were divided into the eastern *P. lucidus* Mayr and the western *P. breviceps* Emery. In the revision, *P. lucidus* is now described as a group consisting of 6 eastern species, whereas the former *P. breviceps* is now described as a group consisting of 5 western species. The European *P. rufescens* is considered part of a larger *rufescens-breviceps* group (See Table 1 for a list of the species referenced in this article). The two North American species groups overlap broadly from the base of the Rocky Mountains to the Mississippi River Valley. Thus, in the previous studies of *Polyergus* sex pheromones (Greenberg et al. 2004, 2007), the species/population from Arizona formerly referred to as *P. breviceps* has now been renamed as the new species *P. topoffi,* whereas the populations in Missouri, formerly also referred to as *P. breviceps*, have now been placed in Forel's species, *P. mexicanus* Forel (Trager 2013). One species of each group, *P. lucidus* Mayr of the *lucidus* group and *P. mexicanus* of the *breviceps* group, are sympatric and relatively abundant at some localities in Missouri, including Shaw Nature Reserve, where part of the fieldwork for this study was conducted.

Our objectives in this study were:

1. To identify queen-produced sex pheromones from the *P. lucidus* group.
2. To examine the attraction of *P. mexicanus* and *P. lucidus* males to reconstructed blends of their sex attractant pheromones in an area of sympatry in Missouri.

We were particularly interested in determining whether the pheromone blends might provide a reproductive isolating mechanism in areas where species from each of the two groups are sympatric.

Methods and Materials

Sample Collection

In July 2005, five alate *P. mexicanus* gynes of the *P. breviceps* group were collected at Shaw Nature Reserve, Gray Summit, Missouri. In August 2007, five alate *P. sanwaldi* Trager (*lucidus* group) gynes were collected from a single colony at Rocky Point, Long Island, New York. In September 2007, two additional alate gynes from a single

Table 1 *Polyergus* species referenced in this article

P. rufescens-breviceps group (Central to western North America and Europe)	*P. lucidus* group (Eastern to central North America)
P. topoffi Trager (NA)	*P. sanwaldi* Trager
P. mexicanus Forel (NA)	*P. lucidus* Mayr
P. vinosus Trager (NA)	*P. montivagus* Wheeler
P. rufescens Latreille (Europe)	

NA North America

P. lucidus sensu stricto colony were collected from that same site. Only low numbers of virgin queens of all species were available because *Polyergus* colonies occur in low abundance at most sites. Because it was known that queen pheromones of *Polyergus* spp. are produced from mandibular glands (Grasso et al. 2003; Greenberg et al. 2004; Topoff and Greenberg 1988), gynes were freeze-killed and decapitated with a razor blade, and individual heads were placed into glass vials with Teflon© lined screw caps, and shipped on dry ice to the University of California, Riverside, for analysis.

Collection and Analysis of Volatiles from Virgin Queen Heads

To characterize the constituents of glands within the heads of *P. mexicanus*, *P. sanwaldi*, and *P. lucidus,* individual gyne heads were warmed to room temperature in their vials and crushed with a glass rod. The vials were sealed with aluminum foil, and a solid phase microextraction fiber (SPME; polydimethylsiloxane, 100 μm, Supelco Inc., Bellafonte, PA, U.S.A.) was inserted into the vial for 30 min to adsorb the volatile compounds. Samples were analyzed on an Agilent 6890 gas chromatograph (GC, Agilent Technologies, Santa Clara, CA, U.S.A.) equipped with a DB5-MS capillary column (30 m × 0.25 mm ID, 0.25 μm film; J&W Scientific, Folsom, CA, U.S.A.) coupled to a 5975 mass selective detector (GC/MS; EI, 70 eV). The SPME fiber was inserted into the injection port of the GC and the volatile compounds desorbed for 30 s in splitless mode. The injector temperature was 250 °C and the oven temperature was programmed from 40 °C for 1 min, then increased to 280 °C at 10 °C.min^{-1}, and held for 10 min.

To determine the absolute configurations of the chiral components in the mandibular glands, SPME samples were analyzed on a Cyclodex-B column (30 m × 0.25 mm ID, 25 μm film; J&W Scientific) with a head pressure of 140 kPa. The injector and detector temperatures were 100 °C and 200 °C respectively; the oven was programmed from 30 °C for 1 min, increased to 65 °C at 15°.min^{-1} and held for 21.67 min, then increased to 240 °C at 10 °C.min^{-1}. Authentic standards of racemic and (*R*)-3-ethyl-4-methypentanol and methyl (*R*)-3-ethyl-4-methylpentanoate were analyzed under the same conditions.

Gyne head extracts of *P. sanwaldi* were further analyzed by gas chromatography coupled with electroantennogram detection (GC-EAD), using antennae of live male *P. sanwaldi*. The antennae were carefully removed from the head with forceps and a small fragment of the distal end of each antenna was sliced off using a razor blade. The base and distal tip of the antenna were mounted between glass capillary electrodes filled with saline (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, and 0.20 g $NaHCO_3$ in 1 l Milli-Q purified water), with an internal gold wire in each capillary for connection to the custom-built EAD amplifier. The effluent from the column was split using an 'X' cross with half of the sample shunted to the FID detector and the other half to the EAD. The portion directed to the EAD was diluted in a humidified air stream (200 ml.min^{-1}) directed over the antennal preparation. The GC was equipped with a DB-5 column as described above. GC and EAD signals were recorded simultaneously using PeakSimple software (SRI, Palo Alto, CA). Retention indices were calculated for unknowns and standards relative to a blend of straight-chain hydrocarbons.

Chemicals

Racemic 3-ethyl-4-methylpentan-1-ol and methyl 6-methylsalicylate (= methyl 2-hydroxy-6-methylbenzoate) were available from previous studies (Greenberg et al. 2004, 2007). Decanal, decanol, *m*-cresol, octyl butyrate, decyl acetate, decyl butyrate, and dodecyl acetate were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Dodecyl butyrate was made by esterification of dodecanol with butyryl chloride and pyridine in methylene chloride. The other required compounds were synthesized as described below.

Methyl (E)-4 methylpent-2-enoate (**1**) Triphenylphosphoranylidene acetate (5 g, 15 mmol; Aldrich Chemical Co.) and isobutyraldehyde (1.5 ml, 16 mmol; Aldrich) were added to a dry flask charged with methylene chloride (50 ml) and cooled to 0°C. After gradually warming to room temperature, the reaction was stirred overnight. Pentane (50 ml) was added, and the resulting slurry filtered to remove solids and then concentrated under reduced pressure. The crude product was Kugelrohr distilled (oven temp ~90 °C, 120 mm Hg), affording 1.02 g (8.0 mmol, 53%) of methyl (*E*)-4-methylpent-2-enoate **1**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.95 (dd, $J =$ 14.6, 6.7 Hz, 1H), 5.77 (dd, $J = 15.8$, 1.4 Hz, 1H), 3.73 (s, 3H), 2.46 (m, 1H), 1.06 (d, $J = 6.8$ Hz, 3H). ^{13}C NMR (101 MHz): δ 167.7, 156.0, 118.4, 51.6, 31.2, 21.4. MS: *m/z* (%): 128 (24), 113 (113), 97 (32), 81 (19), 69 (49), 53 (26), 41 (100). The ^{1}H and ^{13}C NMR spectra were consistent with those described previously (Wang et al. 2007).

Methyl (R)-(−)-3-ethyl-4-methylpentanoate (**2**). This compound was made by a stereoselective conjugate addition (Wang et al. 2007). Thus, (*R*)-(+)-2,2′-bis(di-*p*-tolylphosphino)-1,1′-binaphthyl (80 mg, 0.10 mmol; Alfa Aesar, Ward Hill, MA) and CuI (15 mg, 0.07 mmol) were added to a dry three-neck flask charged with *t*-BuOMe (14 ml) under argon. After stirring overnight, the mixture was cooled to −20 °C and EtMgBr (7 ml, 3 M solution in Et_2O, 21 mmol) was added dropwise. After stirring for 15 min, a solution of **1** (0.9 g, 7 mmol) in *t*-BuOMe (3.5 ml) was added via a syringe pump over 1 h while the temperature was maintained at or slightly below −20 °C. After stirring at −20 °C for another 2 h, the reaction mixture was quenched with MeOH (7 ml) and then diluted with saturated aqueous NH_4Cl. The mixture was extracted with Et_2O (4 × 75 ml) and the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by vacuum flash chromatography (hexane/EtOAc 95:5), then Kugelrohr distilled (oven temp. ~105 °C, 120 mmHg), yielding 0.795 g (76%) of **2**. Enantiomeric purity (93% ee) was determined by GC analysis on the chiral stationary phase Cyclodex B column as described above. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.67 (s, 3H), 2.29 (dd, $J = 14.8$, 5.9 Hz, 1H), 2.16 (dd, $J = 15.0$, 7.6 Hz, 1H), 1.65–1.77 (m, 2H), 1.20–1.42 (m, 2H), 0.87 (m, 9H). ^{13}C NMR (101 MHz): δ 174.9, 51.6, 42.7, 35.8, 29.6, 23.9, 19.7, 18.7, 11.9. MS: *m/z* (%): 143 (trace), 127 (6), 115 (8), 101 (4), 85 (26), 74 (100), 55 (36), 43 (64). The ^{1}H and ^{13}C NMR spectra were consistent with those described previously (Wang et al. 2007).

Racemic methyl 3-ethyl-4-methylpentanoate. A mixture of methyl 4-methyl-2-pentenoate (1.28 g, 10 mmol; Alfa Aesar) and CuI (190 mg, 1 mmol) in 5 ml dry THF was cooled to −30 °C and 12 ml of 1 M EtMgBr in THF was added over 1 h. The resulting blue-black slurry was stirred an additional 1.5 h between −20 and − 10 °C, at which point all the starting material had been consumed. The mixture was quenched by addition of saturated aqueous NH_4Cl, and was extracted twice with pentane. The combined pentane extracts were washed with brine, dried over anhydrous Na_2SO_4, concentrated by rotary evaporation without heating, and the residue was purified by vacuum flash chromatography on silica gel in a 60 ml sintered glass funnel, eluting sequentially with 50 ml aliquots of: pentane, 2 × 2% ether in pentane, 4 × 5% ether in pentane, and 2 × 10% ether in pentane. Fractions 5 and 6 contained the bulk of the desired product, and were combined and Kugelrohr distilled, yielding 340 mg of racemic methyl 3-ethyl-4-methylpentanoate. The GC retention time and mass spectrum matched those of the (*R*)-enantiomer described above.

Field Trials

Pheromone-baited traps were set out in late summer of 2013 at Shaw Nature Reserve in Gray Summit, MO (38.49°N, −90.82°W), where *P. mexicanus* and *P. lucidus* sensu stricto occur in sympatry. In NY, where only *P. lucidus* group species occur, traps were set out at Rocky Point State Pine Barrens Preserve, Long Island, NY, off of Whiskey Road (40.909153° N, −72.927743°W), and the Junction of highways 23 and 32, just outside Cairo, NY (42.308713°N, −74.005295°W. In MO, most males of both species were caught on sunny or partly cloudy days during August and September between 12:00–15:00 h, at temperatures between 30 and 34 °C. Each 8 × 8" Pherocon® AM sticky trap (Trécé Inc., Adair, OK, U.S.A.) was baited with a gray rubber septum (11 mm; West Pharmaceutical Services, Lititz, PA, U.S.A.) impregnated with 50 μl of a solution containing 100 μg of methyl 6-methylsalicylate and 17 μg of the alcohol and/or ester. These amounts were chosen based on the results of our previous studies with the *P. topoffi* pheromone, (Greenberg et al. 2004, 2007). The three lures contained: 1) methyl 6-methylsalicylate + the *P. lucidus* species-specific compound [methyl (*R*)-3-ethyl-4-methylpentanoate] + the *P. mexicanus* species-specific compound (racemic 3-ethyl-4-methylpentan-1-ol) (mixed lure), 2) methyl 6-methylsalicylate + racemic 3-ethyl-4-methylpentan-1-ol (*P. mexicanus* lure), or 3) methyl 6-methylsalicylate + methyl (*R*)-3-ethyl-4 methylpentanoate (*P. lucidus* lure). Each trap within a block of three traps was placed in random order ~3–5 m apart, and each replicate block was placed at various locations within the field sites. After 2 h, traps were collected and the numbers of trapped males counted. In Missouri, where the species are sympatric, 15 replicated blocks were completed, while in NY there were 6 replicated blocks.

Also, during 2013, to determine which ratio of methyl (*R*)-3-ethyl-4-methylpentanoate and methyl 6-methylsalicylate was most attractive to *P. lucidus* males, a series of 7 sticky traps with lures (each series comprising one randomized block), replicated six times, was tested at Shaw Nature Reserve in Missouri. Each rubber septum lure was impregnated with 100 μg of methyl 6-methylsalicylate and variable amounts of the ester according to the following ratios of salicylate to ester: 100:0, 100:1, 100:3.3, 100:10, 100:33, 100:100 and a solvent control. Within each block, each trap was placed in random order ~3 m apart from adjacent traps, with replicate blocks spread across the site.

Male ants in Missouri could readily be identified as either *P. mexicanus* or *P. lucidus* based on species-specific differences in the density of setae on the dorsal abdomen, with the lower density of setae in *P. lucidus* males resulting in a shinier cuticle. However, among the three New York *lucidus* species (*P. lucidus, P. sanwaldi*, and *P. montivagus* Wheeler) that could be present, no morphological characters to separate males have yet been elucidated. Thus, for trials in New York, we can only say that at least one species of the New York *P. lucidus* group responded to the pheromone blend.

Statistical Procedures

In both experiments, many traps had zero captures, making it difficult to normalize the data satisfactorily for analysis of variance. Therefore, in the first experiment, the count data were analyzed by 1-way and 2-way contingency tables. The 1-way tables tested whether the distribution of males of each species at the 3 tracking lures was random. The 2-way tables tested whether there was an interaction effect between species and the 3 lure types (in other words, did the ants have different preferences for the three lures?). For the blend ratios, differences in the number of *P. lucidus* males trapped were analyzed with the non-parametric Friedman's test. Multiple comparisons were done with Bonferroni corrections to determine which ratios were different. All analyses were carried out using Systat (2009).

Results

Identification of Volatiles from Heads of Gynes

GC/MS analysis of the volatiles collected from squashed heads of *P. mexicanus* gynes showed that the extracts contained methyl 6-methylsalicylate as the dominant component, and 3-ethyl-4-methylpentan-1-ol (Table 2), the same two compounds that had been previously described for *P. topoffi*, another member of the *P. breviceps* group (Greenberg et al. 2004).

Antennae of male *P. sanwaldi* of the *P. lucidus* group showed a single major EAD response to the most abundant compound in the SPME of crushed heads of *P. sanwaldi* gynes from Long Island (Fig. 1, peak 6). This compound was identified as methyl 6-methylsalicylate (= methyl 2-hydroxy-6-methylbenzoate) from comparison of its mass spectrum and retention time with those of an authentic standard. A second smaller response was elicited by a minor component (Fig. 1, peak 2). The mass spectrum of this compound (Fig. 2) was characterized by a base peak at *m/z* 74, diagnostic for a methyl ester with no alkyl substituents on the carbon α to the carbonyl. The molecular ion was not detectable, with the highest mass ion being *m/z* 143 (<1%), possibly arising from a compound with a molecular weight of 158 losing a methyl group. Fragments at *m/z* 127 and 115 suggested further losses of an ethyl group and a propyl or isopropyl group, respectively. On the basis of their enhanced abundance, these losses were probably from branch points. Given that 3-ethyl-4-methylpentanol was a pheromone component of *P. topoffi* and *P. rufescens*, a logical candidate for the unknown was the methyl ester of the analogous carboxylic acid, i.e., methyl 3-ethyl-4-methylpentanoate. This structure was shown to be correct by synthesis of an authentic standard and comparison of the retention time and mass spectrum of these with those of the insect-produced compound. The absolute configuration was determined to be (*R*) by stereoselective synthesis of a standard and comparison of the retention time of the insect-produced compound with those of the synthesized (*R*)-enantiomer (23.05 min) and the (*S*)-enantiomer (22.72 min) in a sample of the racemate.

From the GC/MS analyses, the ratio of methyl 3-ethyl-4-methylpentanoate to methyl 6-methylsalicylate was found to be 1.7 ± 0.8 to 100 (mean ± SD, $n = 5$). The extracts also contained variable amounts of a number of other compounds, including 3-ethyl-4-methylpentan-1-ol, decanol, *m*-cresol, decanal, octyl butyrate, decyl acetate,

Table 2 Relative amounts of compounds (mean ± SD) in headspace solid phase microextractions from squashed heads of queens from 4 *Polyergus* species

Peak number	Compound	*P. sanwaldi* $n = 5$ *P. lucidus* group	*P. lucidus* sensu stricto $n = 2$	*P. mexicanus* $n = 2$ *P. breviceps* group	*P. topoffi*[a] $n = 4$
1	3-Ethyl-4-methylpentanol	**0.11 ± 0.04**	**0.30 ± 0.21**	**3.10 ± 0.02**	**12.1 ± 2.3**
2	Methyl 3-ethyl-4-methyl pentanoate	**1.7 ± 0.8**	**2.1 ± 1.4**	**nd**	**0.76 ± 0.36**
3	*m*-Cresol	0.57 ± 0.07	0.24 ± 0.11	0.54 ± 0.11	0.61 ± 0.17
4	Decanal	0.40 ± 0.52	0.09 ± 0.11	nd	0.23 ± 0.20
5	Decanol	0.36 ± 0.64	nd	0.11 ± 0.15	2.1 ± 1.8
6	Methyl 6-methylsalicylate	**100**	**100**	**100**	**100**
7	Octyl butyrate	0.90 ± 0.85	nd	0.17 ± 0.02	5.0 ± 4.6
8	Decyl acetate	0.46 ± 0.84	nd	nd	1.7 1.8
9	Decyl propanoate	0.49 ± 0.42	nd	nd	nd
10	Decyl butyrate	5.0 ± 8.0	0.05 ± 0.04	0.55 ± 0.65	18.9 ± 14.0
11	Dodecyl acetate	0.19 ± 0.31	nd	nd	0.29 ± 0.25
12	Dodecyl butyrate	0.42 ± 0.82	nd	0.02 ± 0.03	0.40 ± 0.17

Peak numbers correspond to the peaks in Fig. 1. Values in bold face type highlight the key differences between the pheromone components of the species in the two species groups. *nd* not detected

[a] Unpublished data from previous studies of *P. topoffi*, described in Greenberg et al. (2004, 2007)

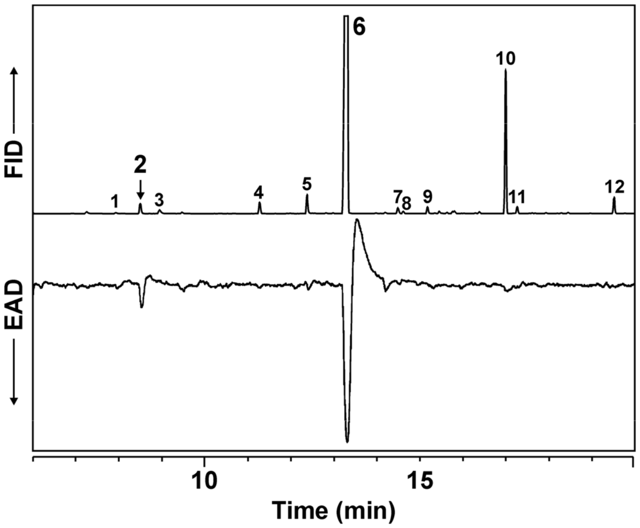


Fig. 1 Representative coupled gas chromatography-electroantennogram detection analysis of headspace volatiles from a squashed *Polyergus sanwaldi* queen head, detected by an antenna from a male *P. sanwaldi* from New York. Top trace: gas chromatogram; bottom, inverted trace, electroantennogram response. Peak identifications: 1 = 3-ethyl-4-methylpentanol, 2 = methyl 3-ethyl-4-methylpentanoate, 3 = *m*-cresol, 4 = decanal, 5 = decanol, 6 = methyl 6-methylsalicylate, 7 = octyl butyrate, 8 = decyl acetate, 9 = decyl propanoate, 10 = decyl butyrate, 11 = dodecyl acetate, 12 = dodecyl butyrate

decyl propanoate, decyl butyrate, dodecyl acetate, and dodecyl butyrate (Fig. 1, Table 2), but none of these compounds elicited EAD responses from antennae of male *P. sanwaldi*. The identities of these compounds were confirmed by matching their retention times and mass spectra with those of standards.

SPME analysis of volatiles from the heads of crushed *P. lucidus* sensu stricto gynes from New York showed that gynes also produced both methyl 6-methylsalicylate and methyl 3-ethyl-4-methylpentanoate. Table 2 summarizes the compounds found in extracts of volatiles from queens of *P. mexicanus, P. topoffi, P. sanwaldi,* and *P. lucidus*, while Fig. 3 shows the molecular structures of the key sex pheromone components.

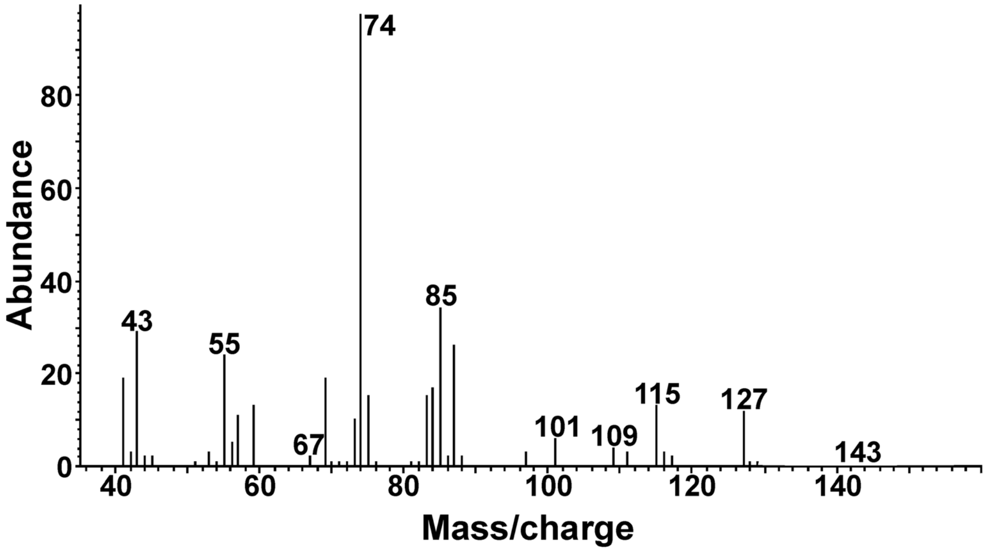
Pheromone Bioassays with Different Lure Blends

Both *P. lucidus* sensu stricto and *P. mexicanus* were present at the Missouri test site (J.C.T., pers. obs.). The larger colony size of *P. mexicanus* was reflected in the trap catches, with 91% of the male ants caught being *P. mexicanus* (Table 3). The results of the 2 × 2 chi-square analyses, testing whether there was an interaction between the two species and the 3 types of lure, were highly significant (Pearson chi-square = 1191, *df* = 2, $P < 0.001$). For the *P. mexicanus* data, the one-way chi-square test was highly significant (Pearson chi-square = 784, *df* = 2, $P < 0.001$), with males caught in large and equal numbers (Pearson chi-square = 3.3, *df* = 1, $P = 0.07$) on the traps baited with *P. mexicanus* and mixed lure types. For *P. lucidus*, the one-way chi-square test was also highly significant (Pearson chi-square = 112, *df* = 2, $P < 0.001$), with more males landing on traps baited with the *P. lucidus* lure than on the mixed lure (Pearson chi-square = 28.4, *df* = 1, $P < 0.001$), and only 2 males landing on the *P. mexicanus* lure.

At the New York site (Table 4), where at most only three *P. lucidus* species could be present, 20 males landed on traps baited with the mixed lures versus 49 on traps baited with the *P. lucidus* lures, with zero on traps baited with *P. mexicanus* lures. The one-way chi-square test was highly significant (Pearson chi-square = 52.8, *df* = 2, $P < 0.001$); more males landed on the *P. lucidus* lure than on the mixed lure (Pearson chi-square = 12.2, *df* = 1, $P < 0.001$).

Table 4 compares the *P. lucidus* data from New York and Missouri. Combining both locations, only 2 males out of 220 landed on the *P. mexicanus* lure. Comparing the number of *P. lucidus* males landing on the *P. lucidus* lure vs. the

Fig. 2 Electron impact ionization (70 eV) mass spectrum of methyl 3-ethyl-4-methylpentanoate in the volatiles collected from the squashed head of a *Polyergus sanwaldi* queen (*lucidus* group)



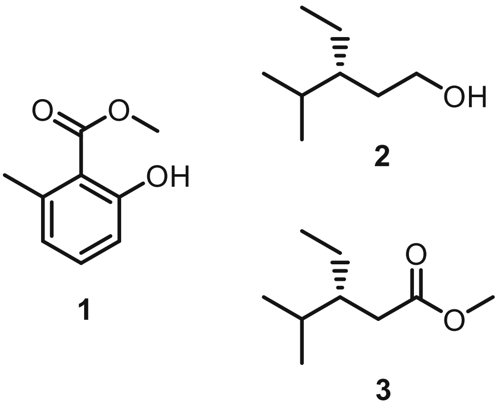


Fig. 3 Structures of the queen-produced sex attractant pheromone components. Methyl 6-methylsalicylate (compound 1) is the major component shared by species in both the *Polyergus breviceps* and *Polyergus lucidus* groups, whereas (*R*)-3-ethyl-4-methylpentan-1-ol (compound 2) is the crucial minor component for the *P. breviceps* group, and methyl (*R*)-3-ethyl-4-methylpentanoate (compound 3) is the crucial minor component for the *P. lucidus* group

numbers landing on the mixed lures at the two different sites showed that they were not different (*Pearson chi-square* = 0.9, *df* = 1, *P* = 0.9).

Besides the *Polyergus* males discussed in this manuscript, no other ants or insect species were attracted to any of the lures.

Bioassays of Ratio Blends

Preliminary trials with both pheromone components had shown that no *P. lucidus* males were attracted to lures containing only one component, i.e., methyl 6-methylsalicylate or methyl 3-ethyl-4-methylpentanoate (data not shown). Figure 4 shows responses of *P. lucidus* males to different ratios of the two components in Missouri. The Friedman's test was highly significant (test statistic = 25.6, *n* = 6 blocks, $P < 0.001$). The 100:1 and 100:3.3 ratios of methyl 6-methylsalicylate to methyl 3-ethyl-4-methylpentanoate were most attractive; however, as the amount of the latter compound increased further, trap catches decreased.

Table 3 Number of *Polyergus lucidus* and *Polyergus mexicanus* males landing on baited sticky traps in Missouri during concurrent trials in 2013

Species	Lure type			Total
	Mixed	*P. mexicanus*	*P. lucidus*	
P. lucidus	42 (27.8%)	2 (1.3%)	107 (70.9%)	151 (100%)
P. mexicanus	743 (47.7%)	815 (52.3%)	0 (0%)	1558 (100%)
Total	785	817	107	1709

Rows show counts and percentages. See text for a description of the lure composition

Pearson chi-square = 1191, *df* = 2, $P < 0.001$

Table 4 Comparison of number of *Polyergus lucidus* group males in New York and *Polyergus lucidus* males in Missouri landing on baited sticky traps during 2013

State	Lure type			Total
	Mixed	*P. mexicanus*	*P. lucidus*	
Missouri	42 (27.8%)	2 (1.3%)	107 (70.9%)	151 (100%)
New York	20 (29.0%)	0 (0%)	49 (71.0%)	69 (100%)
Total	62	2	156	220

Rows show ant counts and percentages

Pearson chi-square = 0.9, *df* = 1, *P* = 0.9, ns

Discussion

Here, we identified the queen-produced sex pheromones of *P. sanwaldi* and *P. lucidus* sensu stricto of the *lucidus* species group, and *P. mexicanus* of the *breviceps* species group, and demonstrated that in an area of sympatry in Missouri of *P. lucidus* and *P. mexicanus*, the males of each species preferentially go to the reconstructed blends of their respective queen's sex attractant pheromones.

Specifically, our analytical and bioassay data demonstrated that the sex attractant pheromone produced by

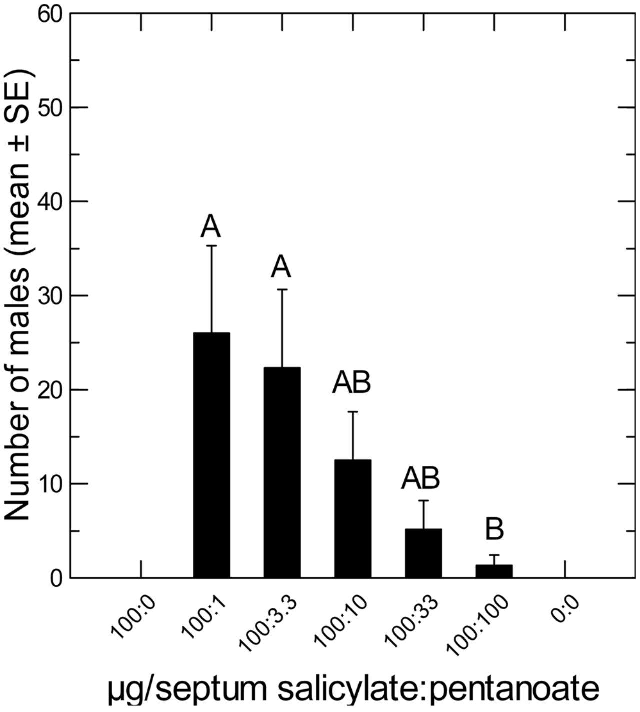


Fig. 4 Responses of *Polyergus lucidus* sensu stricto males to different ratios of its two pheromone components in Missouri. The Friedman analysis was highly significant, showing that the number of males landing on each of the different ratio lures was not the same (Friedman test statistic = 25.6, *N* = 6 blocks, $P < 0.001$). Bars with the same letters above them are not different from each other

virgin queens of the *P. lucidus* species group consists of two components, methyl 6-methylsalicylate and methyl 3-ethyl-4-methylpentanoate. Neither of the two compounds alone attracted males, indicating a strong synergism. In a blend ratio trial, we also determined that the optimal ratio of methyl 6-methylsalicylate and methyl 3-ethyl-4-methylpentanoate was 100:1 (Fig. 4), rather than the 100:17 ratio that we had been using while trying to determine which compounds were necessary and sufficient to obtain good levels of attraction. Methyl 6-methylsalicylate is also the major component in the corresponding pheromones of *P. topoffi* (Greenberg et al. 2004, 2007), *P. mexicanus* (see Results above) and the European *P. rufescens* (Castracani et al. 2008), all within the *rufescens-breviceps* group. In the latter three species, 3-ethyl-4-methylpentan-1-ol is the second crucial component of the pheromone blend, rather than methyl 3-ethyl-4-methylpentanoate component for the *P. lucidus* group queens. However, small amounts of 3-ethyl-4-methylpentan-1-ol were indeed present in the volatiles from squashed heads of *P. lucidus* group queens. In a previous study (Greenberg et al. 2004) with *P. topoffi* (reported as *P. breviceps*), analyses of concentrated CH_2Cl_2 extracts of dissected mandibular glands of four freshly collected queens determined that methyl 6-methylsalicylate (1608 ± 310 ng/queen) and 3-ethyl-4-methylpentanol (184 ± 39 ng/queen) composed more than 99% of the extractable volatile material in the glands. It is therefore likely that the same glands are producing the sex pheromones in the species analyzed here.

Both 3-ethyl-4-methylpentan-1-ol and methyl 3-ethyl-4-methylpentanoate have been identified from the volatiles of two European red wood ant species, *Formica rufa* L. and *Formica polyctena* Foerster (Buehring et al. 1976; Francke et al. 1985). In the latter species, methyl 3-ethyl-4-methylpentanoate reduced aggression between heterospecifics in laboratory bioassays (Francke et al. 1980) but, to our knowledge, no further bioassays have been carried out with any *Formica* species to determine whether one or both of these compounds may have additional roles as behavior-modifying chemicals.

We also tested the effects of mixing the major component, methyl 6-methylsalicylate, with the minor components of the two species groups. Field trials demonstrated that *P. lucidus* group males from Missouri and New York responded best to the *P. lucidus* type lure, with decreased responses to the mixed lure, indicating some degree of inhibition caused by the presence of 3-ethyl-4-methylpentan-1-ol, despite the fact that the volatiles from the *P. lucidus* queens contained small amounts of 3-ethyl-4-methylpentan-1-ol, in addition to methyl 3-ethyl-4-methylpentanoate. Thus, what may be crucial in determining the responses of males of this species is the relative ratio of the alcohol to the ester, as well as the ratio of one or both of these compounds to the major component. In contrast, *P. mexicanus* males appeared oblivious to the presence of methyl 3-ethyl-4-methylpentanoate, responding equally well to the mixed lure as to their own lure blend (Table 4).

We now have information on more than one species and/or location for each of the two North American *Polyergus* species groups. Specifically, we have identified the same pheromone components from both *P. sanwaldi* and *P. lucidus* from New York, and *P. lucidus* from Missouri. Among the *P. breviceps* group, besides *P. mexicanus* in Missouri and *P. topoffi* in Arizona (Greenberg et al. 2004, 2007), informal field tests with lures containing the blend of methyl 6-methylsalicylate and ethyl-4-methylpentan-1-ol also attracted *P. mexicanus* males from Rustler's Park in the Chiricahua Mountains of Arizona and *P. vinosus* Trager from the San Bernardino Mountains near Crestline, California (L.G., pers. obs.). Thus, it is possible that the pheromone difference between the *lucidus* and *breviceps* groups applies across all the species in those groups.

As mentioned above, the sex pheromone blends of queens of the North American *P. breviceps* group appear to be essentially identical to that of the European *P. rufescens*, based on analyses of volatiles from species from both continents and the results of field bioassays with reconstructed blends of the pheromone components. The sex pheromones of at least two species in the eastern North American *P. lucidus* group (*P. sanwaldi* and *P. lucidus)* also appear to be similar. On the other hand, their blend is distinct from both the western North American and the European *Polyergus*. Although the phylogeography of *Polyergus* is not yet known, our results suggest that in North America the sex pheromone blend has diverged at least once, which may reduce hybridization where populations were or are sympatric. This requirement would be particularly critical in areas of sympatry such as the North American Great Plains, to minimize cross-attraction of heterospecifics. To date, we have no information about the queen pheromones of the *P. samurai* species group, which consists of two species in East Asia and Japan (Trager 2013) that could shed some light on the evolution of *Polyergus* sex pheromones. It may also be useful to compare the pheromones of species within the *P. breviceps* and *P. lucidus* groups to determine whether there are discernible differences within species in each group, as well as between the groups.

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