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Research article

Chemical communication in mating behaviour of the slave-making ant *Polyergus rufescens* (Hymenoptera, Formicidae): 3-ethyl-4-methylpentanol as a critical component of the queen sex pheromone

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Abstract. The aim of the research reported here was to determine whether 3-ethyl-4-methylpentanol, a minor but crucial component of the sex pheromone of the North American slave-making ant species *Polyergus breviceps*, was also a component of the sex pheromone of the European congener *Polyergus rufescens*. Thus, the contents of mandibular glands of *P. rufescens* virgin queen were extracted and analysed. The main component of the extracts was methyl 6-methylsalicylate and 3-ethyl-4-methylpentanol was identified as one of several minor components. Further analyses showed that the insects produce mainly the (*R*)-enantiomer of the alcohol. Males' responses to various blends of methyl 6-methylsalicylate with the racemate or the pure enantiomers of 3-ethyl-4-methylpentanol were tested in field behavioural bioassays. The data showed that blends of methyl 6-methylsalicylate and 3-ethyl-4-methylpentanol were strongly synergistic, with the most active ratios being biased toward the first component. The addition of other minor components to the binary blend neither increased nor decreased responses by males. Only the (*R*)-enantiomer of the alcohol was biologically active; its antipode did not inhibit attraction. The results are discussed in terms of the evolution of signals, and are compared with the results previously obtained for the allopatric species *Polyergus breviceps*.

Keywords: Sex pheromone, 3-ethyl-4-methylpentanol, methyl 6-methylsalicylate, enantiomer, *Polyergus rufescens*.

Introduction

The genus *Polyergus* consists of five ant species (*P. rufescens*, *P. breviceps*, *P. lucidus*, *P. nigerrimus,* and *P. samurai*) that share the same basic biology: they are all social parasites that are entirely dependent on the workers of the species that they enslave to carry out all nest maintenance duties. Periodically, they raid host colonies and capture host brood that is reared to adulthood and then integrated into the work force of the raiding species. Because of these habits, they are described as slave-making ants (Wilson, 1975; Hölldobler and Wilson, 1990).

Polyergus rufescens Latreille 1798 is a European member of the genus. Populations of this species are widespread along the northern and central part of the Italian peninsula. Although the main features of its parasitic lifestyle are the raiding behaviour and the dependent colony founding through usurpation (Mori et al., 2001), several other aspects of its morphology, biology, ecology, and behaviour can be related to its nature as a social parasite (Hölldobler and Wilson, 1990; Le Moli et al., 1994; Grasso et al., 1997, 2005; Billen et al., 2001). For instance, its mating behaviour is an example of "Female-calling Syndrome" (Hölldobler and Bartz, 1985), a strategy in which females typically call for males, do not disperse widely, and remain near a conspecific colony after mating (Mori et al., 1994). Because *P. rufescens* queens establish new colonies through usurpation, and host colonies generally have a patchy distribution, this mating strategy is advantageous because limited dispersal of queens reduces the risk of

leaving a habitat appropriate for host colony usurpation (Bourke and Franks, 1995).

Mate location is a crucial step in *P. rufescens* mating behaviour, in which chemical communication plays a very important role. In this species, virgin queens attract males with sex pheromones, and the mandibular gland has been shown to be the source of these signals (Grasso et al., 2003). Similar results have been demonstrated for the American species *P. breviceps* (Topoff and Greenberg, 1988), and the role of methyl 6-methylsalicylate as a component of the female sex pheromone has been demonstrated in both species (Greenberg et al., 2004, 2007; Castracani et al., 2005). In particular, *P. breviceps* males are strongly attracted by a highly synergistic, 7 : 1 blend of methyl 6-methylsalicylate with 3-ethyl-4-methylpentanol (Greenberg et al., 2004). More recent studies showed that *P. breviceps* queens produce exclusively the (*R*)-enantiomer of the alcohol, and only this enantiomer was attractive to male ants; the (*S*)-enantiomer neither increased nor antagonized responses to blends containing the (*R*)-enantiomer (Greenberg et al., 2007).

In order to compare the semiochemistry of these two congeneric but allopatric species, the aim of this research was to investigate the possible role of 3-ethyl-4-methylpentanol as a component of the *P. rufescens* sex pheromone. Thus, the contents of virgin queens' mandibular glands were extracted and analysed by coupled gas chromatography-mass spectrometry. Field behavioural bioassays then were performed to test responses of *P. rufescens* males to reconstructed blends of the components identified from the mandibular gland extracts, to determine the optimally attractive subset of compounds.

Materials and methods

Chemical analysis

Ants were collected at the University Campus in Parma (Italy) during summer 2006. *Polyergus rufescens* virgin queens were collected by aspiration at the entrance of the parasite nests during the period of nuptial flights between the end of June and the first half of August, in the early afternoon. A total of 11 glands were utilized to prepare 6 solvent extracts. Prior to dissection (under distilled water), the ants were placed in a freezer for 5 min, and then each gland was dissected from the head capsule and other mandibular structures using a binocular microscope (Zeiss©, Stemi 2000-C, 0.65x/5x). Each extract consisted of 20 µl of dichloromethane (CH_2Cl_2) as solvent and a variable number of mandibular glands: 1 gland in samples 2, 4, and 5; 2 glands in samples 3 and 6; 4 glands in sample 1. Glands were extracted for 24 hr before analysis.

Coupled gas chromatography-mass spectrometry (GC-MS) analysis was performed with an Agilent Technologies 6890N gas chromatograph coupled to a 5973N mass selective detector (Agilent Technologies, Santa Clara, CA, USA), using the following instrumental conditions: DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, Folsom, CA) with the oven temperature programmed from 40 °C for 1 min, then increased to 270 °C at 20 °C/min. Head pressure was 6.93 psi; injector temperature: 250 °C; injection mode: 1 min pulsed splitless at 20 psi, volume injected 2 µl; transfer line temperature: 280 °C; carrier gas: helium. MS conditions: ion source temperature 230 °C; electron impact ionisation: 70 eV; acquisition mode: scan (*m/z* 30–320). Compound structures were confirmed with authentic standards. In sample 2, analytes were quantified by adding 2.0 ng of tetradecane dissolved in methylene chloride as internal standard. In the remaining samples (1, 3, 4, 5, 6), analytes were quantified by comparison of peak areas in the total ion chromatogram (TIC) with those obtained for sample 2, working under the same experimental conditions.

The absolute configuration of the insect-produced 3-ethyl-4-methylpentanol was determined using a 25 m × 0.25 mm i.d. Chirasil-DEX CB GC column (Chrompack, Middleburg, The Netherlands), using the following instrumental conditions: oven temperature programmed from 50 °C for 1 min, then increased to 180 °C at 4 °C/min. Head pressure was 6.93 psi; injector temperature: 250 °C; injection mode: split with 20 : 1 split ratio, volume injected 2 µl; transfer line temperature 250 °C; carrier gas: helium. MS conditions: ion source temperature 230 °C; electron impact 70 eV; acquisition mode selected ion monitoring (*m/z* 69, 84, 134). Compound structures were confirmed with authentic (*R*)- and (*S*)-3-ethyl-4-methylpentanol standards (see below).

Field behavioural bioassays

Field bioassays were conducted at the University Campus in Parma (Italy) during the nuptial flight period in summer 2006 and 2007. Sticky traps consisting of a sheet of stiff paper with a sticky surface (21 cm × 21 cm) with a 11 mm grey rubber septum lure placed in the centre of the trap were used to catch *P. rufescens* males. Synthetic chemicals were loaded onto the rubber septa as hexane solutions. Each trap was placed on the ground, about 5 m apart, and at least 15 m from the nearest nest. Traps were left on the ground for 2 hr between 12 : 30 and 15 : 30, when both males and virgin queens were most active outside the nest (Mori et al., 2001). Traps were collected in late afternoon, and the numbers of trapped *P. rufescens* males were recorded.

In summer 2006, two series of bioassays were performed using binary blends of methyl 6-methylsalicylate and racemic 3-ethyl-4-methylpentanol. The two compounds were synthesized as previously described (Greenberg et al., 2004). In the first series, 7 blends with different methyl 6-methylsalicylate/3-ethyl-4-methylpentanol ratios were compared (salicylate/alcohol ratio: 10 : 0, 9 : 1, 3 : 1, 1 : 1, 1 : 3, 1 : 9, 0 : 10). Lures were loaded with test compounds as hexane solutions, with total doses of 100 micrograms of synthetic compounds per lure. The 7 treatments were randomized within a replicate, and only one replicate was performed daily for a total of 10 trials. In the second series, only 4 blends were tested (salicylate/alcohol ratio: 9 : 1, 7 : 1, 5 : 1, 3 : 1) and a total of 10 replicates were performed. The second series was designed to determine the optimum ratio of the two components as an attractant for the males of *P. rufescens*, with the blend ratios bracketing the best blend found in the first series. In summer 2007 an additional series of bioassays with binary blends was performed. Treatments were: pure hexane (control) and salicylate/alcohol ratios of 1000 : 1, 100 : 1, 50 : 1, 20 : 1 and 9 : 1. Differences among blends were statistically analyzed with One-Way ANOVA followed by Tukey's HSD Post Hoc tests for separation of means, both run on the logarithmically transformed data ($\alpha = 0.05$). Statistical analyses were performed using SPSS© 14.0 for Windows package (©SPSS Inc., Chicago, USA).

In summer 2007, two further series of bioassays were carried out. In the first series, the binary blend of methyl 6-methylsalicylate and racemic 3-ethyl-4-methylpentanol was compared with the entire reconstructed blend of the 10 chemicals identified from the mandibular glands by GC-MS analyses. According to the numbering of Table 1, chemicals **1** and **4** were synthesized as previously described (Greenberg et al., 2004), whereas chemicals **2**, **3**, **7**, **9**, and **10** were obtained from Aldrich Chemical Co. A sample of decyl butyrate **6** was synthesised by heating overnight at 60°C a mixture of decanol (1.6 g), butyric acid (1.4 g) (both from Aldrich Chemical), and 0.2 g of phosphosulphuric acid (Carlo Erba). The reaction mixture was diluted with methanol (10 ml), neutralized with excess solid potassium carbonate, further diluted to 30 ml with methylene chloride and hexane (1/1, v/v), mixed thoroughly, and filtered. The filtrate was concentrated under vacuum at 50°C and the oily residue (2.3 g) was dissolved in hexane (10 ml),

filtered through a short silica gel column (2 × 10 cm) and again concentrated. The residue (1.9 g) corresponded to decyl butyrate (GC purity: 92%, mass and NMR spectra as expected). Dodecyl butyrate **8** (90% purity by GC) was synthesised in the same way, starting from dodecanol (Aldrich Chemical) and butyric acid. Ethyl 6-methylsalicylate was synthesized from 2-hydroxy-6-methylbenzoic acid (Greenberg et al., 2004) by refluxing the acid (0.31 g, 2 mmol) in a mixture of 3 ml EtOH and 0.5 ml conc. H_2SO_4 for 12 hr. The mixture was then poured into cold water and extracted 3 times with ether. The combined ether extracts were washed thoroughly with saturated aqueous $NaHCO_3$ and brine, then dried over anhydrous Na_2SO_4. Concentration under vacuum gave the desired product as a crystalline solid, >95% pure by GC, which was used without further purification. Spectral data agreed with those previously reported (Snider et al., 1988).

Table 1. Compounds identified in solvent extracts of the mandibular glands of *P. rufescens* virgin queens. For each compound, the retention time (R.t.), and the mean amount calculated from 6 samples (ng/gland ± SE, and %) are shown. Sample 2 was analysed three times with standard deviation < 5% for major peaks. Samples 1, 3, 4, 5, 6 were analyzed twice.

Chemical Compound	R.t. (min.)	Mean (ng/gland)	Mean (%)
1) 3-Ethyl-4-methylpentanol	6.4	6.6 ± 1.7	1.7%
2) Nonanal	7.1	2.0 ± 0.9	0.5%
3) Methylsalicylate	7.9	1.9 ± 0.5	0.5%
4) Methyl 6-methylsalicylate	8.9	359.7 ± 95.3	92.1%
5) Ethyl 6-methylsalicylate	9.4	3.2 ± 0.8	0.8%
6) Decyl butyrate	10.5	3.4 ± 3.0	0.9%
7) Tetradecanoic acid	11.4	1.2 ± 0.7	0.3%
8) Dodecyl butyrate	11.5	0.8 ± 0.6	0.2%
9) Hexadecanoic acid	12.5	4.8 ± 1.8	1.2%
10) Oleic acid	13.5	7.2 ± 2.2	1.8%
Total		390.7	100%

Ten replicates were performed and four treatments were tested: 1) a binary blend with a 9:1 ratio (salicylate/alcohol), 2) a binary blend with a 50:1 ratio, 3) a full blend with a 9:1 ratio (salicylate/alcohol) and corresponding amounts of the other components based on the ratio versus the salicylate (see Table 1), and 4) full blend with the 50:1 ratio with corresponding amounts of the other components. The 9:1 and 50:1 ratios were selected because both were highly attractive in the previous binary blends bioassays, with 9:1 being the most effective ratio and 50:1 being the closest ratio to that found by chemical analysis of the gland extracts. Comparisons between the binary and the full blends were carried out to determine whether the other minor chemicals might increase attraction to the blends. Differences among blends were statistically analyzed with One-Way ANOVA run on the logarithmically transformed data ($\alpha = 0.05$). Statistical analyses were performed using SPSS© 14.0 for Windows package (©SPSS Inc., Chicago, USA).

In the second series of bioassays, the biological activities of pure enantiomers of 3-ethyl-4-methylpentanol were tested using binary blends of methyl 6-methylsalicylate and (*R*)- or (*S*)-3-ethyl-4-methylpentanol. The two enantiomers were synthesized as previously described (Greenberg et al., 2007). Seven treatments (replicated 11 times) were tested: salicylate:alcohol 9:0.5(*R*), 9:0.5(*S*), 9:0.4(*R*):0.1(*S*), 50:0.5(*R*), 50:0.5(*S*), 50:0.4(*R*):0.1(*S*), and solvent control (a blend of hexane and dichloromethane). Lures were loaded with total doses of 100 micrograms of synthetic compounds per lure. Differences among blends were statistically analyzed with One-Way ANOVA followed by Tukey's HSD Post Hoc tests for separation of means, both run on the logarithmically transformed data ($\alpha = 0.05$). Statistical analyses were performed using SPSS© 14.0 for Windows package (©SPSS Inc., Chicago, USA).

Results

Chemical analysis

A total of 10 compounds were identified by GC-MS analyses of extracts of the mandibular glands of virgin queens (Table 1). Methyl 6-methylsalicylate was the main component ($\overline{X} \pm$ S.E. = 360 ± 95 ng) corresponding to 92.1% of the blend (Fig. 1). Minor compounds included oleic acid (7.2 ± 2.2 ng; 1.8%), 3-ethyl-4-methylpentanol (6.6 ± 1.7 ng; 1.7%), and hexadecanoic acid (4.8 ± 1.8 ng; 1.2%). The other components each corresponded to less than 1% of the blend. Enantioselective gas chromatographic analyses of the extracts using a chiral stationary phase revealed that the extracts contained a 5:1 mixture of the (*R*)- and (*S*)-enantiomers of 3-ethyl-4-methylpentanol (Fig. 2).

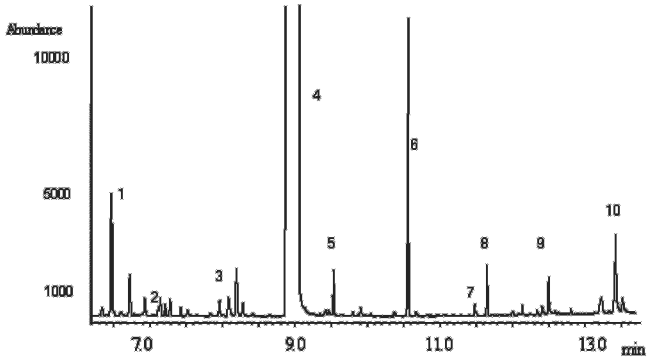

Figure 1. GC-MS total ion chromatogram profile of a methylene chloride extract of 4 mandibular glands of *P. rufescens* virgin queens (sample 1, see Methods). Numbers 1–10 refer to compounds listed in Table 1.

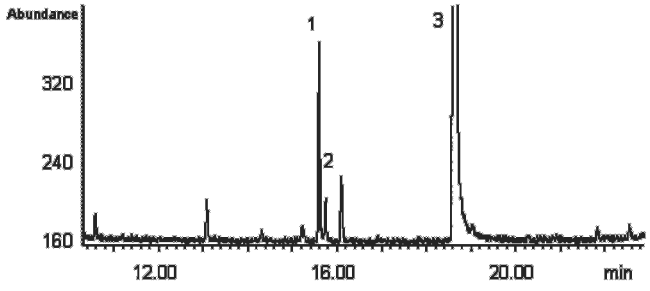

Figure 2. GC-MS selected ion monitoring chromatogram of a methylene chloride extract of mandibular glands of *P. rufescens* virgin queen (sample 1, see Methods) analysed with a Chirasil-DEX CB capillary column for the determination of the enantiomeric composition of 3-ethyl-4-methylpentanol. Peak 1: (*R*)-3-ethyl-4-methylpentanol; peak 2: (*S*)-3-ethyl-4-methylpentanol; peak 3: methyl 6-methylsalicylate.

Field behavioural bioassays

Bioassays with binary blends of methyl 6-methylsalicylate and racemic 3-ethyl-4-methylpentanol showed substantial differences in the attraction of male ants to the various blends. In the first series of bioassays, the 7 blends attracted significantly different numbers of *P. rufescens* males (One-Way ANOVA: $F_{(6,63)} = 75.5$; $p < 0.001$). From ANOVA and subsequent Tukey's HSD Post Hoc tests, in increasing order of attraction, the blends can be classified as follows: $10:0 = 0:10 < 1:9 < 1:3 = 1:1 < 3:1 = 9:1$ (salicylate/alcohol ratio) (Fig. 3A). In particular, traps baited with only a single component of the blend were unattractive to males: no males were found in traps baited with 3-ethyl-4-methylpentanol, and only 3 males were captured in traps baited with methyl-6-methylsalicylate. Traps baited with the other blends all caught males, and lures containing a high percentage of methyl 6-methylsalicylate were most attractive.

In the second series of bioassays, there were no significant differences among the four blends tested (salicylate/alcohol ratio: 9:1, 7:1, 5:1, 3:1; One-Way ANOVA: $F_{(3,36)} = 2.1$; $p = 0.12$; Fig. 3B). In the third series of bioassays, no ants were attracted to the solvent controls, whereas the 5 blends attracted significantly different numbers of *P. rufescens* males (One-Way ANOVA: $F_{(5,54)} = 92.5$; $p < 0.001$). From ANOVA and subsequent Tukey's HSD Post Hoc tests, in increasing order of attraction, the blends were ranked as follows: solvent control $< 1000:1 < 100:1 < 50:1 = 20:1 = 9:1$ (salicylate/alcohol ratio) (Fig. 3C).

Although the best 2-component blends were highly attractive, a further set of bioassays was conducted to determine whether the remaining minor components identified in the mandibular gland extracts might further increase the attraction of males. However, in bioassays comparing the attraction of males to binary blends versus more completely reconstructed blends, the four treatments were equivalent in activity (One-Way ANOVA test: $F_{(3,36)} = 1.5$; $p = 0.23$; Fig. 4). Thus, the full effect of the pheromone can be demonstrated by the combination of the 2-component blend of methyl 6-methylsalicylate and 3-ethyl-4-methylpentanol.

The results of bioassays with the enantiomers of 3-ethyl-4-methylpentanol showed that only the (*R*)-enantiomer was biologically active as part of the sex pheromone blend (One-Way ANOVA test: $F_{(6,70)} = 72.8$; $p < 0.001$). From ANOVA followed by Tukey's HSD Post Hoc tests, the blends broke into two groups (Fig. 5). Solvent controls and binary blends with methyl 6-methylsalicylate and (*S*)-3-ethyl-4-methylpentanol (ratios: 50:0.5 and 9:0.5) were minimally attractive. In contrast, binary blends with methyl 6-methylsalicylate and (*R*)-3-ethyl-4-methylpentanol (ratios: 50:0.5 and 9:0.5) and blends with the salicylate and mixtures of the two enantiomers (ratios: 50:0.4(*R*):0.1(*S*) and 9:0.4(*R*):0.1(*S*)) were highly attractive to *P. rufescens* males.

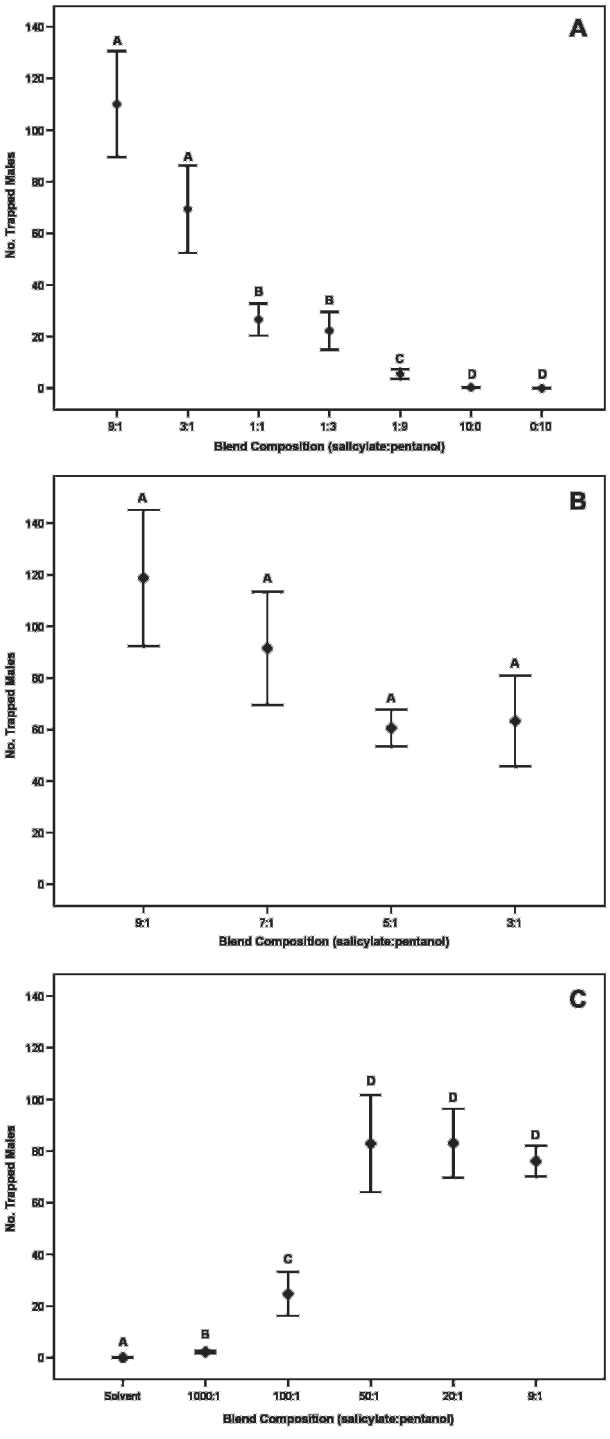

Figure 3. Attractiveness of blends of methyl 6-methylsalicylate and racemic 3-ethyl-4-methylpentanol to *P. rufescens* males. For each blend the mean number of trapped males and the standard error are shown. A: first series of bioassays (N = 10 replicates); means with the same letter are not significantly different (One-Way ANOVA followed by Tukey's HSD Post Hoc tests on the logarithmically transformed data). B: second series of bioassays (N = 10 replicates); means with the same letter are not significantly different (One-Way ANOVA on the logarithmically transformed data). C: third series of bioassays (N = 10 replicates); means with the same letter are not significantly different (One-Way ANOVA followed by Tukey's HSD Post Hoc tests on the logarithmically transformed data).

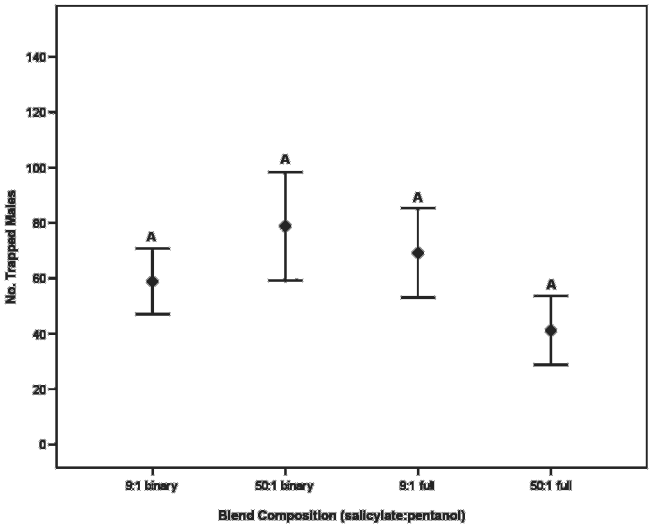

Figure 4. Comparison between attractiveness of binary blends and fully reconstructed blends to *P. rufescens* males (N = 10 replicates). For each blend, the mean number of trapped males and the standard error are shown. Means with the same letter are not significantly different (One-Way ANOVA on the logarithmically transformed data).

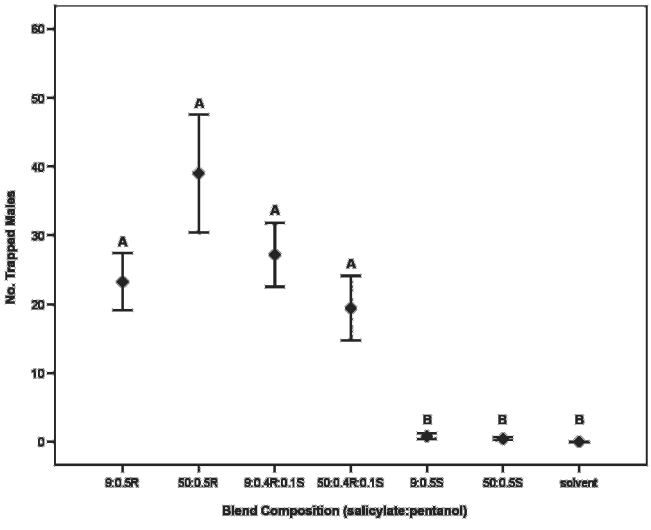

Figure 5. Attractiveness of blends of methyl 6-methylsalicylate and the enantiomers of 3-ethyl-4-methylpentanol to *P. rufescens* males (N = 11 replicates). For each blend the mean number of trapped males and the standard error are shown. Means with the same letter are not significantly different (One-Way ANOVA followed by Tukey's HSD Post Hoc tests on the logarithmically transformed data).

Discussion

Ten components were conclusively identified in GC-MS analyses of solvent extracts of mandibular glands of *P. rufescens* virgin queens. Methyl 6-methylsalicylate was the most abundant component, as was found previously using SPME extraction (Castracani et al., 2003). However, analyses of solvent extracts allowed the identification of several minor components that had not been found in the previous analyses. Four of these (nonanal, tetradecanoic acid, hexadecanoic acid, and oleic acid) were common lipid components and seemed unlikely to be involved in chemical communication. Five of the remaining chemicals were deemed to be possible components of the *P. rufescens* sex pheromone, and among them 3-ethyl-4-methylpentanol seemed to be the most probable because it was the most abundant of the five, and its biological role had been demonstrated in the congeneric American species *P. breviceps* (Greenberg et al., 2004, 2007). Thus, comparisons between binary blends of methyl 6-methylsalicylate/3-ethyl-4-methylpentanol and the full reconstructed blend were tested in a series of field bioassays. The results showed that there were no differences in the attraction of male ants to binary blends or the more fully reconstructed blends, and confirmed that methyl 6-methylsalicylate and 3-ethyl-4-methylpentanol are the two key components of the sex pheromone.

Decyl butyrate was identified as one of the minor components of the extracts by GC-MS analysis. Previous research had demonstrated that this ester was the most abundant volatile component in the Dufour's gland of *P. rufescens* queens, and that it acts to appease host workers during nest usurpation (D'Ettorre et al., 2000; Mori et al., 2000; Visicchio et al., 2000). The presence of this compound in the mandibular glands of queens suggests that it may have an additional role in intraspecific interactions. Thus, future research on *P. rufescens* could focus on this compound, and on its possible role in sexual communication or other communicative contexts.

Field bioassays showed that blends containing both 3-ethyl-4-methylpentanol and methyl 6-methylsalicylate were required to elicit strong attraction of *P. rufescens* males. Thus, the pheromone emitted by calling females must consist of at least these two components, which contribute to increase the complexity of the message. Two main evolutionary advantages are suggested. First, an increase in complexity is an adaptive strategy to ensure the promotion of privacy by discouraging illicit senders/receivers, such as predators, parasites, or competitors, from interfering during communication (Hölldobler and Wilson, 1990). Furthermore, a more complex message can also increase the amount of transmitted information. Although field bioassays demonstrated that both compounds were essential for attracting males, previous laboratory research had shown that methyl 6-methylsalicylate was a key stimulus for orientation during mate location by *P. rufescens* males (Castracani et al., 2005). Taken together, these results suggest that the two semiochemicals might communicate different information, or that they might act over different ranges. From the perspective of males locating females in open fields, one component could allow orientation over longer ranges, whereas the second one might be used to pinpoint the signaller over shorter ranges. Further bioassays are needed to clarify the possible different roles of each pheromone component.

Our results also demonstrated that the optimal ratio between the two compounds was biased towards methyl

6-methylsalicylate, both in terms of the gland contents and in terms of the ratios of synthetic chemicals required to obtain optimal attraction of males. In particular, the most attractive blends were characterized by a salicylate/pentanol ratio ranging from 50 : 1 to 3 : 1. This range included the ~50 : 1 ratio found in analyses of the mandibular gland extracts. Methyl 6-methylsalicylate, the most abundant substance, has been previously reported to be involved in several communicative contexts in ant semiochemistry (Duffield and Blum, 1975; Longhurst et al., 1980; Morgan and Ollett, 1987; Kohl et al., 2000; Ayasse et al., 2001). In contrast, 3-ethyl-4-methylpentanol has been reported from only a few ant species (Francke et al., 1985; do Nascimento et al., 1993; Greenberg et al., 2004, 2007). However, because of the small quantities in which it is present and its high volatility, this chemical was more difficult to find in extracts than methyl 6-methylsalicylate (e.g., Castracani et al., 2003; Greenberg et al., 2004). Thus, more careful analyses may reveal that it is also relatively common in other ant species.

Our results showing that binary blends consisting of 75–98% methyl 6-methylsalicylate with 3-ethyl-4-methylpentanol were the most effective attractants for *P. rufescens* males, were in accord with those reported for *P. breviceps* (Greenberg et al., 2004, 2007), although the ratio of components may be more heavily biased towards methyl 6-methylsalicylate in *P. rufescens*. Similarities between the two congeneric species were also confirmed through investigations of the absolute configuration of 3-ethyl-4-methylpentanol produced by the two species. In both species, only the (*R*)-enantiomer was attractive to male ants whereas the (*S*)-enantiomer neither increased nor antagonized responses. According to Mori's classification (1998a,b), the relationship between the two enantiomers falls in category n.1, in which only a single enantiomer is bioactive and its opposite enantiomer does not inhibit the action of the pheromone. Other examples in this group include the alarm pheromone of the leaf-cutting ant *Atta texana*, the trail pheromone of the pharaoh's ant *Monomorium pharaonis* (cf. Mori 1998a,b and references cited therein). The fact that ants are able to discern the absolute configuration of 3-ethyl-4-methylpentanol suggests that they possess highly specific pheromone binding proteins and/or pheromone receptors. From an evolutionary point of view, chemical communication is advantageous for these insects because semiochemicals are low cost signals both in terms of their production and transmission, and chirality enhances the specificity of the message and the communication system (Hölldobler and Wilson, 1990; Mori, 1998a).

In conclusion, the composition of the queen-produced sex pheromone is remarkably similar between *P. rufescens* and *P. breviceps*. Because these congeners presumably have lived in allopatry for millions of years, the similarity in their sex pheromone blends appears to represent a very conservative character inherited from their common ancestor. The fact that there is essentially no difference in the sex pheromone blends of these two geographically isolated species may also indicate that there has not been strong selection pressure on the pheromone blend. Thus, in their respective home ranges, there may be few other species that use these compounds or blends for communication. Alternatively, species that might use one or both of these compounds as semiochemicals may do so during periods of the day or periods of the year when *Polyergus* males are not active. Nevertheless, the close resemblance between the sex pheromone blends of these two geographically isolated species suggests that the blend compositions may be chemotaxonomic characters of use in delineating phylogenetic relationships among species within the genus *Polyergus*. Thus, we anticipate an extension of this research to other *Polyergus* species.

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