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RESEARCH PAPER

Individual variation of (*S*)-4-methyl-3-heptanone in heads of braconid wasp, *Leiophron uniformis*, and *Pogonomyrmex* ants indicates costs of semiochemical production

John A. Byers · Anat Levi-Zada

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Abstract (*S*)-4-Methyl-3-heptanone is an alarm pheromone released from the mandibular glands in heads of harvester ants (*Pogonomyrmex* spp.). We used gas chromatography–mass spectrometry (GC–MS) to study the variation in amounts of this ketone among individual ants of a colony. *P. barbatus* contained about 2,000 ng per head, while only about half of this amount was found in heads of *P. rugosus* and *P. californicus*. Individuals of *P. barbatus* from three different nests contained rather uniform amounts of the alarm pheromone within each colony (16–30% coefficient of variation CV; normal distributions skewed left), but one nest under food stress had a significantly lower mean amount. In contrast, both sexes of a small braconid wasp *Leiophron uniformis*, a parasitoid of *Lygus* plant bugs, contained up to 10 ng of the same volatile enantiomer in their heads; and groups of either sex of the wasp exhibited normal distributions of quantities (64% CV, skewed right). The differences in the distributions between parasitoids and ants suggest that related members within a social ant colony may attempt to maintain a uniform level of ketone compared to independent variation in unrelated, solitary wasp individuals. When the wasp's leg was grasped with forceps, it tried to escape and bite the forceps as it ejected (*S*)-4-methyl-3-heptanone (detected by solid phase microextraction, SPME, and GC–MS). Since adult wasps are nonsocial and feed only on nectar, their sharp piercing mandibles in combination with this escape/biting behavior indicate the ketone is used for defense rather than for an alarm function as in harvester ants. Costs of producing the semiochemical in wasp *L. uniformis* and ants *P. barbatus* and *P. californicus* are suggested since populations exhibited a significant linear increase in the amount of (*S*)-4-methyl-3-heptanone with an increase in body weight of individuals.

Keywords Braconidae · Formicidae · Hymenoptera · Parasitoid · Alarm pheromone · Defensive secretion

J. A. Byers (✉)
USDA-ARS, Arid-Land Agricultural Research Center,
21881 North Cardon Lane, Maricopa, Arizona 85138, USA
e-mail: john.byers@ars.usda.gov

A. Levi-Zada
Department of Entomology-Chemistry,
Institute of Plant Protection,
Agricultural Research Organization,
POB 6, Bet Dagan 50250, Israel

Introduction

Pogonomyrmex barbatus Smith and *P. rugosus* Emery are large harvester ants (8–9 mm long) common in southern Arizona that have been rated among the ant species with the most powerful stings (Schmidt and Blum 1978; Schmidt 2003). There are about 60 species of *Pogonomyrmex* ants in North, Central, and South America (MacMahon et al. 2000), another being the smaller (about 7.5 mm long) and more slender *P. californicus* (Buckley). Wilson (1958) showed that heads of another species of the same genus, *P. badius* (Latreille), when crushed near workers caused them to become "alarmed" and attempted to engage any enemies invading the colony. This alarm pheromone was identified from 700 g of *P. barbatus* by GC–MS and NMR as (*S*)-4-methyl-3-heptanone and it was further located in the ant's mandibular glands (McGurk et al. 1966). The compound was also found (by GC analysis only) in several other harvester ant species, including *P. badius*, *P. rugosus* and *P. californicus*, and only the *S*-enantiomer was bioactive causing general excitement

and aggressive behavior (McGurk et al. 1966; Vick et al. 1969). Phenotypic variation in semiochemicals is key to understand sexual and natural selection and thus vital to population genetics, evolution, and ecology (Roughgarden 1979; Smith 1986). However, no study has quantified the variation in the amounts of alarm pheromone among individuals of a *Pogonomyrmex* species.

Using GC–MS we discovered the same compound, 4-methyl-3-heptanone, in both sexes of a small wasp *Leiophron uniformis* (Gahan) (Hymenoptera: Braconidae) that along with other species of the genus are important parasitoids of plant-feeding bugs (Loan 1974; Goulet and Mason 2006). *L. uniformis* specializes in early instar nymphs of plant bugs (Hemiptera: Miridae) such as *Lygus hesperus* and *L. lineolaris* and garden fleahoppers (*Halticus bractatus*) (Debolt 1981, 1989; Graham et al. 1986). The *Lygus* plant bugs are among the most important crop pests of Europe and North America (Wheeler 2001; Blackmer et al. 2004; Blackmer and Byers 2009). For example, *L. hesperus* feeds on over 100 species of plants and it can be a serious pest of cotton, alfalfa, beans, strawberries, peaches, and various seed crops (Scott 1977; Fye 1982; Leigh et al. 1988; Blackmer et al. 2004). Volatile compounds produced by *L. uniformis* have not been reported, nor have any other braconid species been reported to contain this compound.

Several hemipteran bugs including *Lygus* species release defensive esters and ketones when their legs are grasped by forceps or when attacked by ant predators such as *P. rugosus* (Byers 2006a, Byers unpublished). Thus, our first objective was to investigate whether grasping a leg of the small parasitoid wasp (body length 2.5 mm) with fine forceps to simulate predator attack would elicit defensive behaviors such as biting and release of 4-methyl-3-heptanone that might act as defensive venom. We also wanted to locate the compound within the wasp and determine the compound's enantiomeric composition by comparison to synthetic standards of the two enantiomers and the *S*-enantiomer of harvester ant alarm pheromone on a chiral column with GC–MS. In addition, variation in 4-methyl-3-heptanone quantities in male and female wasps would be related to fresh weights of individuals. Our hypothesis was that the populations would exhibit an increase in ketone amounts with an increase in body weight, which would indicate a cost for producing the semiochemical (Birgersson et al. 1988; Byers 2005, 2006a). In comparison, the harvester ants, *P. barbatus, P. rugosus* and *P. californicus*, were similarly studied during the year to determine relationships between amounts of (*S*)-4-methyl-3-heptanone in individuals' heads and body weights that may suggest production costs. We conducted various behavioral tests (ants fighting other ants or attacking moth caterpillars) to better describe some of the circumstances that elicit release of alarm pheromone in harvester ants, and whether there could be an additional function that was defensive/aggressive in nature. The normality and degree of variation of ketone amounts among individuals of male and female solitary wasps were compared to that in *P. barbatus* ants from three nests for significant differences that may depend on their different ecology and relatedness.

Materials and methods

Insect rearing and collection

Leiophron uniformis were reared in the laboratory on host plant bugs, *Lygus hesperus* (Debolt 1981). The plant bugs (originally from Maricopa, Arizona, USA) were reared on green beans and synthetic diet within 5 × 10 × 0.5 cm Parafilm® packets (Debolt 1982) placed inside paper cartons (15 cm diam. × 15 cm high) with nylon mesh tops (at 26°C, 65% R.H., 14L:10D photoperiod). Males and females of *L. uniformis* emerged from cocoons and they were held together in transparent plastic chambers (5 cm diam. × 9 cm high) containing tissue paper under the same conditions as mentioned above. The parasitoid adults were allowed to mate freely (females laid eggs in *L. hesperus* nymphs twice weekly) and were used in experiments at 5–10 days of age. The adults were fed commercial honey droplets placed on nylon mesh over the top of the chambers with 2 ml glass vials of drinking water and sponge protruding through a hole cut in the chamber wall.

The harvester ants *P. barbatus*, *P. rugosus*, and *P. californicus* were collected in the field from several nests during 2005–2008 near Maricopa, AZ, USA (N33,5,15; W111,58,19; 358 m) and they were taken within 30 min to the laboratory for extraction or use in experiments. Some *P. rugosus* ants also were collected in Utah (4 May 2007; N37,38,11; W110,48,5; 1,373 m) and extracted on site while a cohort of *P. californicus* were collected from a nest near Needles, CA, USA (24 April 2008; N34,52,16; W114,39,32; 250 m) and taken to the laboratory within 24 h. All ants were collected from 10 to 60 cm from the nest entrance. Species determinations of the ants were confirmed by Dr. Robert Johnson, Arizona State University (Tempe, AZ, USA).

Collecting volatile emissions from insects by SPME and analysis by GC–MS

To elicit release of potentially defensive volatiles as in other insects (Byers 2006a, unpublished), a 2 ml glass vial containing a single male or female *L. uniformis* wasp was opened and the insect's leg or antenna was grabbed by a fine metal forceps for 5–10 s (termed *molested*), the insect was released inside the vial that was quickly recapped, and

volatiles from the headspace was immediately collected for 2 min by solid phase microextraction (SPME). The vial cap had a Teflon liner with a 0.5 mm hole, allowing insertion of a needle with a 65 μm Carbowax-divinylbenzene SPME fiber (Supelco, Bellefonte, PA, USA) that was desorbed in the GC injection port at 250°C for 5 min. Background air of resting wasps that had not yet been molested in vials were collected by SPME for 20 min as controls. The same methods were used for ants with 5 ml or 25 ml vials. GC–MS analysis of volatiles collected on SPME fibers was carried out with a Varian 3800 GC with a 30 m × 0.25 mm ID column coated with 0.25 μm CP-Sil 8 CB (Varian CP5860, Walnut Creek, CA, USA) coupled to a Varian Saturn 2000 MS (ion trap, 70 eV EI). Helium carrier gas was programmed for a constant flow (1.2 ml/min). The injection at 250°C was splitless for 5 min (then split 20:1), and the oven temperature program (#1) was held at 40°C for 5 min and then increased 5°C/min to 180°C, then at 20°C/min to 250°C and held for 5 min. The NIST05 (National Institute of Standards, USA) and Wiley7 (John Wiley & Sons, Inc., Hoboken, NJ, USA) MS spectral libraries were used to identify 4-methyl-3-heptanone as well as by comparison to (*S*)-4-methyl-3-heptanone from the three harvester ant species. A chiral column (Cyclodex-B, J&W Scientific, now Agilent, Folsom, CA, USA) of 60 m × 0.25 mm ID coated with 0.25 μm permethylated *β*-cyclodextrin was used to compare harvester ants having the (*S*)-enantiomer to synthetic enantiomers of (*S*)- and (*R*)-configuration (Zada et al. 2004) and to the compound in the parasitoid wasps. Separation of enantiomers occurred with a program (#2) of 40°C for 2 min, then 30°/min to 55° and hold for 45 min, then 30°/min to 230°C and hold 10 min.

Harvester ants of *P. barbatus* and *P. californicus* collected from several nests in the field were kept in groups from their own colony and then individuals were taken and placed in a 5 ml or 25 ml vial depending on the experiment. After some minutes, usually the background volatiles were collected by SPME for 2 min as a control. After this, ants were molested by grabbing their legs or antenna with a fine forceps for about 10 s in the 5 ml vial, the cap replaced, and after 1 min SPME was done for another 2 min. In other cases *P. barbatus* were allowed to interact with individuals of other colonies or with *P. californicus* and after a bout of fighting, SPME was done in 25 ml vials to collect odors as above. In other experiments, *P. barbatus* individuals in 25 ml vials were allowed to attack second and third instar beet armyworms (*Spodoptera exidua*, Lepidoptera: Noctuidae) or second and third instar caterpillars of cabbage looper moth (*Trichoplusia ni*, Lepidoptera: Noctuidae) that had been reared in the laboratory (Ignoffo 1963). After an ant attacked a caterpillar, SPME was done similarly and GC–MS analysis was performed as described above.

Solvent extractions of parasitoid wasps and harvester ants and analysis by GC–MS

To locate the source of 4-methyl-3-heptanone in ants and parasitoids, individuals were weighed inside 300 or 700 μl plastic vials on a Mettler Toledo UMT2 micro-balance (Columbus, OH, USA) with μg resolution, after which they were cooled to 1°C and their head, thorax, and abdomen separated with a razor blade against a glass surface. Each body part was quickly deposited in a 100 μl conical glass vial containing 50 or 100 μl hexane with an internal standard of (+)-carvone and/or ethyl heptanoate (each 1 ng/μl, Sigma–Aldrich, St. Louis, MO, USA). However, ethyl heptanoate was used to quantify results, since no significant differences were found in quantities even when analyses used carvone. The parts (or whole wasp) in solvent were crushed with a blunt, nickel-plated tapestry needle (Prym-Dritz Corp., Spartanburg, SC, USA) and then the extract was immediately sucked off the mashed insect and placed in a new vial stored at −20°C, until analysis by GC–MS within a few days to prevent degradation of compounds (Byers 2006a).

A Varian CP-8400 autosampler was used for 1 μl sample injections at 250°C. The GC temperature program for extracts on the non-polar column was the same as for SPME, but it was held 15 min at 250°C and the injection was split-less until 0.75 min when it was split 60:1 and then 20:1 at 5 min and thereafter. The same injection methods that were used with the non-polar column in regard to SPME and solvent extracts were used for the chiral column. However, for extracts analyzed on the chiral column, the temperature program (#3) was 40° for 2 min, 10°/min to 60°C and hold 10 min, then 3°/min to 150°, then 20°/min to 230°C and hold 10 min; and for SPME, either the same program without 10 min hold at 230° or a shorter program (#4) was used at 40° for 2 min, 15°/min to 120°C hold 5 min, then 20°/min to 230°C and hold 10 min.

Statistical analysis

The mean amounts of (*S*)-4-methyl-3-heptanone were compared between groups by two-tailed *t* tests. Distributions of amounts were analyzed for differences from a normal distribution by Chi-square and by kurtosis and skewness (Sokal and Rohlf 1995). To determine if there was a linear relationship between body weight and chemical amount, we used linear regression and tested whether the slope was significantly different from zero (Sokal and Rohlf 1995). Frequency histograms of individuals categorized within amount ranges were compared by Kolmogorov–Smirnov two-sample tests (Sokal and Rohlf 1995). To compare the histogram shapes between samples differing in amount ranges, the sample with the lower range was scaled equivalent to the higher range. This was

done by multiplying amounts in the lower range by a constant (largest bin value of larger range divided by largest bin value of smaller range).

Results

Collecting volatile emissions from insects by SPME and analysis by GC–MS

Male and female *L. uniformis* released undetectable or trace amounts of 4-methyl-3-heptanone when resting (not molested) in a 2 ml vial. The SPME fiber collected trace amounts of 4-methyl-3-heptanone from resting males (mean ± SE 0.006 ± 0.005 ng per 2 min, $N = 6$) and resting females (0.004 ± 0.002 ng, $N = 6$), while within 10 s after molestation with the forceps the collection of 4-methyl-3-heptanone increased about 350-fold to 1.51 ± 0.36 ng for males ($N = 4$) and 2.27 ± 1.09 ng for females ($N = 5$) over 2 min ($P < 0.01$, t tests, for both sexes). 4-Methyl-3-heptanone was the only volatile detected by GC–MS. The SPME fiber collected 4.63 ± 1.26 ng 4-methyl-3-heptanone from males and females over 2 min that were squished briefly in the vial ($N = 4$). These amounts are certainly less than the absolute amounts released since SPME only collects a fraction of the vapor but does reflect relative differences in amounts reliably. The ketone was most likely ejected in liquid, but due to its high volatility, it would soon vaporize and encounter the SPME fiber. The initial discharge percentage (IDP) is the proportion of the insect gland's defensive volatiles that are released in response to an attack by a predator (Byers 2006a). The IDP for *L. uniformis* can be estimated as 41% by comparing the amount collected by SPME from squished insects divided by the amount collected from molested insects. In all cases, individuals of either sex that had their antenna or leg grasped by a fine forceps beat their wings vigorously, while biting the forceps repeatedly and released 4-methyl-3-heptanone (collected by SPME over 2 min). Both sexes of *L. uniformis* have sharp piercing mandibles (see Fig. 1) that could open wounds in adversaries into which the ketone as venom could be spit.

Pogonomyrmex barbatus ants held individually in vials as controls released low amounts of alarm pheromone (0.11 ± 0.06 ng, $N = 36$, collected on SPME), although some ants were apparently still alarmed after being taken from the colony (>1 ng was collected from 2 of 36 ants). Grasping the leg of ants with a forceps usually elicited some alarm pheromone release (3.51 ± 1.41 ng collected, $N = 24$, $P = 0.005$, t test), but a significant proportion (9 of 24) appeared to release little or no alarm pheromone (<0.5 ng collected) during the molestation. Individual *P. barbatus* placed with individuals of the same species from other

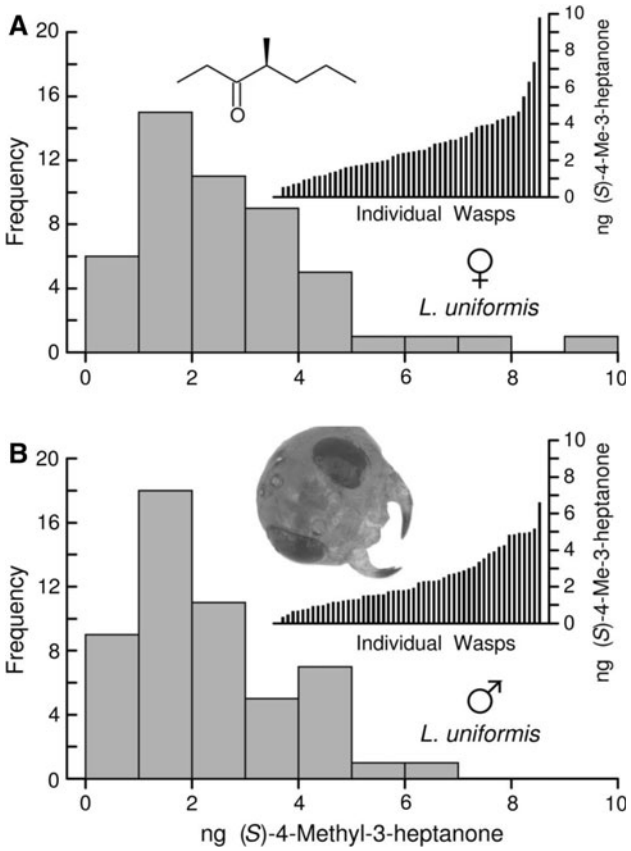

Fig. 1 Ordering of amounts of (*S*)-4-methyl-3-heptanone extracted from heads of female (**a**) and male (**b**) *Leiophron uniformis* parasitoid wasps (*inset graphs*) and the frequency distribution of individuals within ranges of these amounts (*larger graphs*). The image in (**b**) shows the front of a *L. uniformis* head (0.5 mm wide) and its needle-like piercing mandibles that may defend against enemies more effectively by secretion of the ketone

colonies ($N = 4$ pairs), or with *P. californicus* ($N = 17$ pairs), usually began to fight within a few minutes, and after this bout alarm pheromone was usually collected (4.98 ± 1.69 ng, $N = 21$, $P < 0.001$; with >0.5 ng collected from 16 of 21 antagonistic pairs). It is not possible to determine which species, or if both species, released alarm pheromone except when only *P. barbatus* were together (5.63 ± 3.05 ng, $N = 4$). *P. barbatus* attacked beet armyworm and cabbage looper caterpillars by biting and stinging and released alarm pheromone (6.20 ± 2.48, $N = 21$, $P = 0.002$; with >0.5 ng collected from 12 of 21 individuals). No ketone was detected from beet armyworm or cabbage looper caterpillars held alone in vials by SPME/GC–MS.

Solvent extractions of parasitoid wasps and harvester ants and analysis by GC–MS

Solvent extraction of parasitoids (22 August 2003) showed male heads ($N = 9$) contained a mean of 3.54 ± 1.21 ng of 4-methyl-3-heptanone but none was detected in their

thoraxes or abdomens when each was extracted after ablation. Similarly, female *L. uniformis* heads ($N = 5$) contained 2.67 ± 0.84 ng and none was detected in their thoraxes or abdomens. Temperature program (#2) and the chiral column achieved complete separation of the two synthetic enantiomers of 4-methyl-3-heptanone [(*R*) at 39.1 min and (*S*) at 39.64 min]. Using the chiral column to compare the synthetic enantiomers with those from heads of male and female *L. uniformis* as well as worker ants of *P. barbatus*, *P. californicus*, and *P. rugosus*, only (*S*)-4-methyl-3-heptanone was found. Histograms of amounts of (*S*)-4-methyl-3-heptanone in both *L. uniformis* sexes (extracted in 2006–2007) indicate that many individuals had smaller amounts with a few having larger amounts (Fig. 1). These females contained a mean amount of 2.76 ± 0.25 ng per individual ($N = 50$) and the males ($N = 52$) had 2.36 ± 0.21 ng (sexes not significantly different, $P > 0.1$, *t* test). The distribution of female wasp amounts was not different from a normal distribution ($\chi^2 = 9.87$, $df = 8$, $P > 0.05$) and had a leptokurtic kurtosis of 4.04 ($P < 0.001$) and skewness of 1.64 that tailed right ($P < 0.001$), while male amounts also were not different from a normal distribution ($\chi^2 = 9.21$, $df = 8$, $P > 0.05$) and had a kurtosis of 0.018 and skewness of 0.87 that tailed right ($P < 0.05$). The coefficient of variation (CV) is a measure of the relative variability (standard deviation/mean × 100) of amounts among individuals in the population and was 64.9% for females and 63.1% for males. No significant difference was detected between the male and female frequency histograms of numbers of wasps with various ketone amounts (Fig. 1, $P > 0.1$, Kolmogorov–Smirnov).

Extraction of *P. barbatus*, *P. californicus*, and *P. rugosus* heads revealed much larger amounts of the compound (Table 1) at up to about 2,000 ng or from 400 to 1,000 times more than *L. uniformis*. The lighter-weight species (*P. californicus*) and a black-form of *P. rugosus* contained significantly less alarm pheromone than *P. barbatus* (Table 1). Inter-colony variation was observed in *P. barbatus*, with individuals from nest #1 weighing slightly but significantly more than individuals from the other two nests (November 15–19, 2007) while nest #2 individuals had significantly less alarm pheromone than those from the other two nests (Table 1). In *P. barbatus*, nest #2 exhibited a truncated frequency distribution of individual's with amounts indicating many ants with relatively uniform amounts of alarm pheromone (Fig. 2a). The uniformity also was indicated by a CV of 29.7%, although this distribution was not different from a normal distribution ($\chi^2 = 9.90$, $df = 5$, $P > 0.05$) and had a kurtosis of 1.10 and skewness of −1.21 that tailed left ($P < 0.05$). The same distribution is seen in the pooled results of nests #1 and #3, but a considerable number had higher amounts than in nest #2 (Fig. 2b). This distribution also was not different from a normal distribution ($\chi^2 = 4.87$, $df = 6$, $P > 0.05$) and had a kurtosis of 1.23 and skewness of −0.98 that tailed left ($P < 0.05$). Compared to the CV of the wasps (about 64%), both ant nests #1 and #3 also had less

Table 1 Mean (±SE) amounts of (*S*)-4-methyl-3-heptanone (ng) extracted per head and mean fresh body weights (mg) of *Pogonomyrmex* spp. harvester ants collected at various locations and dates

Ant species	Collection		(*S*)-4-methyl-3-heptanone	Body weight
	Date	Location[a]		
P. barbatus	5 October 2006	Maricopa, AZ	1,660 ± 333 ($N = 9$)	16.38 ± 0.60 ($N = 6$)
P. rugosus (black)[b]	5 October 2006	Maricopa, AZ	597 ± 72 ($N = 8$)[c]	11.41 ± 1.09 ($N = 5$)[c]
P. californicus	5 October 2006	Maricopa, AZ	820 ± 122 ($N = 6$)	6.52 ± 0.22 ($N = 3$)
P. rugosus	4 May 2007	Utah	1,021 ± 97 ($N = 4$)	N.a.[d]
P. barbatus nest #1	15–19 November 2007	Maricopa, AZ	1,971 ± 81 ($N = 16$)[e]	21.12 ± 0.72 ($N = 16$)[e]
P. barbatus nest #2	15–19 November 2007	Maricopa, AZ	1,228 ± 74 ($N = 24$)[f]	18.89 ± 0.29 ($N = 24$)
P. barbatus nest #3	15–19 November 2007	Maricopa, AZ	1,520 ± 105 ($N = 16$)	18.28 ± 0.50 ($N = 16$)
P. barbatus	5 December 2007	Maricopa, AZ	1,621 ± 177 ($N = 8$)	N.a.
P. californicus	24 April 2008	Needles, CA	1,338 ± 146 ($N = 12$)	8.99 ± 0.24 ($N = 12$)

[a] Refers to materials and methods for latitude/longitude

[b] Black form, other *P. rugosus* were red colored

[c] Black *P. rugosus* contained significantly less alarm pheromone ($P < 0.01$) and weighed significantly less ($P < 0.01$) than *P. barbatus* on October 5, 2006 (*t* tests)

[d] Weights not taken

[e] Ants from nest #1 had significantly more alarm pheromone and weighed significantly more than ants from nest #2 or nest #3 ($P < 0.01$) on November 15–19, 2007 (*t* tests)

[f] Ants from nest #2 had significantly less alarm pheromone than ants from nest #3 ($P < 0.01$) on November 15–19, 2007 (*t* test)

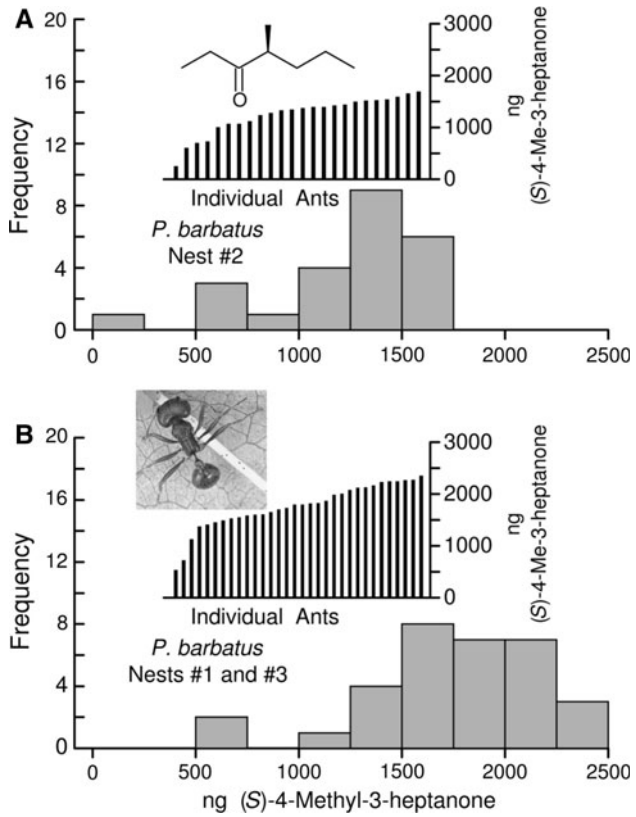

Fig. 2 Ordering of amounts of (*S*)-4-methyl-3-heptanone extracted from heads of red harvester ants (*Pogonomyrmex barbatus*) from nest #2 (**a**) and nests #1 and #3 (**b**) during 15–19 November 2007 (*inset graphs*) and the frequency distribution of individuals within ranges of these amounts (*larger graphs*). The image in (**b**) shows a dorsal view of a *P. barbatus* worker on a cotton leaf

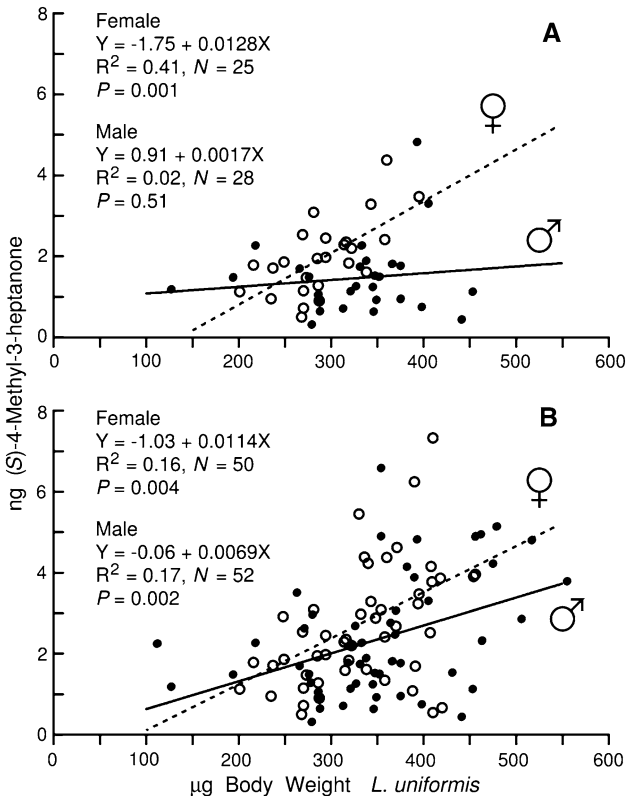

Fig. 3 Relationships between body weight of *Leiophron uniformis* and amounts of (*S*)-4-methyl-3-heptanone in heads of females (*circles*, $N = 25$) and males (*dots*, $N = 28$) in a cohort extracted February.14–15, 2007 (**a**) and this cohort plus two others (25 March 2005 and 4 October 2006) (**b**)

variation (more uniformity) with CV of 16.4 and 27.6%, respectively. The distributions of nest #2 compared to nests #1 and #3 were different (Kolmogorov–Smirnov test, $P = 0.001$) because of the lack of ants in nest #2 with higher amounts. In order to compare the histogram shapes, the amounts in nest #2 were scaled (multiplied by 2,500/1,850) to correspond to the range of nests #1 and #3, and then no significant difference resulted ($P = 0.11$). However, there was a significant difference in the histogram shapes of the male and female parasitoid wasps (Fig. 1) compared to the pooled ant nest data ($N = 56$) when the parasitoid amounts were scaled (multiplied by 2,500/10) to correspond to the ant range ($P < 0.001$ for both wasp–ant comparisons).

A significant linear relationship was found between the fresh weights of female *L. uniformis* (291 ± 9 µg) and the amounts of (*S*)-4-methyl-3-heptanone in their heads (1.97 ± 0.18 ng) for those extracted 14–15 February 2007 ($R^2 = 0.42$, $N = 25$, $P = 0.001$, Fig. 3a). Male body weights (326 ± 13 µg) and amounts (1.46 ± 0.17 ng) appeared not linearly related ($R^2 = 0.02$, $N = 28$, $P = 0.51$, Fig. 3a). However, a larger data set of females (334 ± 9 µg, 2.76 ± 0.25 ng, $N = 50$) and males (350 ± 12 µg, 2.36 ± 0.21, $N = 52$) showed a linear relationship for both sexes (Fig. 3b), although in both data sets the female slope was higher. Unhealthy outliers might bias the relationship, thus the smaller data set ($N = 25$) had five of the lightest weight females removed (20%), but still there was a significant positive relationship ($P = 0.01$), while in the larger data sets (about $N = 50$) removal of the five lightest weight individuals and the five smallest amount individuals of each sex (about 20%) still gave significant positive relationships (both sexes $P \leq 0.01$).

A significant linear relationship between body weight and amount of alarm pheromone in their heads was also found in harvester ants, *P. barbatus* (Fig. 4a) and *P. californicus* (Fig. 4b). Removing the five lightest weight individuals and the five smallest amount individuals of *P. barbatus* (16%) still gave a significant positive relationship ($P = 0.013$, $N = 47$). However, after removal of the two lightest *P. californicus* ants (13%) there was no significant relationship ($P = 0.13$) due in part to the low sample number.

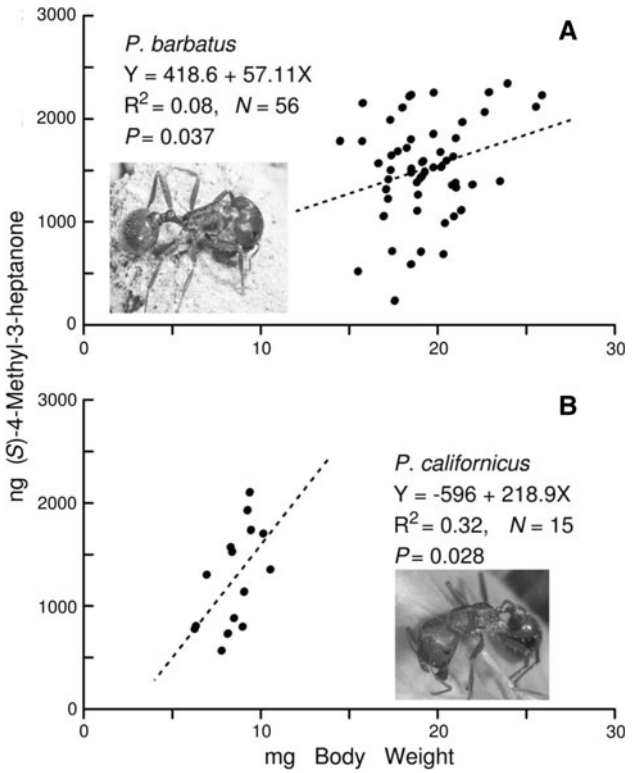

Fig. 4 Relationship between body weight of *Pogonomyrmex barbatus* from three nests (15–30 November 2007, Maricopa, Arizona) and amounts of (*S*)-4-methyl-3-heptanone in their heads (**a**) and the relationship between weight of *P. californicus* (24 April 2008, Needles, California) (**b**). *Image inserts* show these two species

Discussion

To our knowledge, this is the first report of a volatile chemical found in the heads of braconid wasps, one of the largest families of Hymenoptera with over 40,000 species (Whitfield et al. 2004). The behavior of both males and female parasitoids was identical, when their legs were grasped by fine forceps: violent beating of the wings, repeated biting of the forceps, and emission of (*S*)-4-methyl-3-heptanone which volatilized into the air. Since the wasps are solitary and unlikely to be near relatives when releasing the compound in nature, it is unlikely that the ketone serves as an alarm signal (Byers 2005). Rather, a defensive nature for the compound is suggested since it is found in both sexes. The sharp piercing mandibles of both sexes (Fig. 1) would be suited to biting a potential predator (e.g., lacewing nymphs, small spiders) and spitting 4-methyl-3-heptanone as an irritant to enhance defensive efforts.

The wasps appear to release a significant portion of the volatile contents of their head (about 40%) during molestation, as estimated from the relative amounts collected on SPME fibers from crushed insects and from those molested by the forceps. SPME only collects a portion of that emission but serves well in determining a ratio of volatile quantities. The IDP (initial discharge percentage) of the defensive compound amounts from glands upon attack by a predator was proposed by Byers (2006a) as a species-specific characteristic. The adult plant bug, *L. hesperus*, has defensive aldehyde-ketones (4-oxo-2-hexenal) and esters (hexyl butyrate, hexenyl butyrate) that are secreted from the metathoracic gland in response to harvester ant attacks and its IDP was estimated at about 60% (Byers 2006a). Insects that are occasionally attacked by predators might release a significant portion of their defensive secretion compared to other insects that are frequently attacked or must be alarmed for longer periods of time such as red harvester ants (Wilson 1958).

The distribution of amounts of (*S*)-4-methyl-3-heptanone in *L. uniformis* males and females indicates a normal variation with several containing larger than average amounts (>4 ng) so the distribution is skewed to the right (Fig. 1). The volatile was found in the heads of both wasp sexes, and the source could be in the salivary glands, mandibular glands, or regurgitant (alimentary canal). In contrast, the harvester ants had roughly 300× larger amounts in their heads' mandibular glands and the frequency distributions skews to the left in the opposite direction to that of the wasp, meaning that relatively few ants had lower amounts compared to most with more uniformly higher amounts (Fig. 2). These patterns suggest an uncoordinated normal variation in compound amounts among unrelated wasp individuals (CV of about 64% in both sexes) compared to a coordinated and more uniform variation among red harvester ants of the same colony (nests #1–#3 of *P. barbatus* had lower CV of 16.4–29.5%). The lower variation in alarm pheromone amounts among individuals in ant colonies may result because of genetic relatedness and social feeding that would maintain uniform vigor among colony members. For example, nest #2 had no individuals with alarm pheromone >1,850 ng, perhaps because the weeds in their foraging area were recently burned, eliminating seed collection and depleting the grain reserves of the colony causing incipient starvation. In fact, this nest became deserted about 2 month later while the other two nests remained active with foraging on nearby weeds. In addition, it is probable that only foraging ants near the colony nest entrance were sampled and thus would be a more uniform age group (temporal polyethism, Oettler and Johnson 2009) which might have contributed to the uniformity in alarm pheromone amounts. In any case, it would be advantageous for ants to maintain uniform levels of alarm pheromone among guarding-foraging individuals if possible, since any individual at the surface with inadequate amounts that encountered an adversary would fail to warn the colony, while an individual with extra amounts

might waste colony resources (or too frequently alarm members).

An important question in chemical ecology concerns what factors during evolution determine the level of pheromone content and emission in a species. For example, are there costs involved that hinder smaller individuals from producing amounts of pheromone allowing higher fitness? Such a cost of producing nanogram amounts of alarm pheromone (*E*)-β-farnesene was suggested in cotton aphids (*Aphis gossypii*) because as weight of first-instar nymphs (5 μg) increased over a 60-fold range to adults (300 μg), the amounts of alarm pheromone increased from 0.1 ng to 1.2 ng (variation in body weight explained 62% of the variation in alarm pheromone, Byers 2005). It is expected that adult aphids with the largest pheromone amounts have not produced more than the optimal fitness levels (as down-regulation of pheromone production should be effortless). Birgersson et al. (1988) looked for costs in producing pheromone in male bark beetles, *Ips typographus* L., attacking Norway spruce and found that an increase in the dry weight of males was weakly correlated with an increase in one component of its aggregation pheromone, 2-methyl-3-buten-2-ol (MB). However, variation in male weight explained only 3.2% of the variation in MB, while a second pheromone component, *cis*-verbenol was not correlated with size. In the butterfly *Colias eurytheme*, the variation in size of male forewings was not significantly correlated with amounts of three saturated hydrocarbons (heptacosane, 13-methylheptacosane, and nonacosane) of the male courtship pheromone in 6 of 7 samples, and in all samples, the variation in forewing size could explain only about 2.4% of the variation in pheromone content (Sappington and Taylor 1990). The lack of significant relationships between size and sex pheromone content in some studies may be explained by that (a) adult insects vary much less in size compared to juveniles + adults, (b) the tiny amounts of pheromone in adults may not have a measureable cost, or (c) there is substantial random variation due to environmental factors.

On the other hand, the significant linear relationship between fresh body weight and amounts of (*S*)-4-methyl-3-heptanone in adult *L. uniformis* does suggest a cost of producing the volatile, especially for females (Fig. 3). If the ketone is used as a venom, as suggested by its release when biting, then production of even higher levels than found here would probably enhance fitness, but may increase costs beyond the capacity of the individual. Because lighter, unhealthy individuals might have less ketone and thereby bias the slope in a positive direction, a conservative test was done where about 20% of the lightest-weight and lowest-amount individuals were removed from the analysis, but a significant positive slope still resulted. Female wasps were producing eggs at the time of extractions and may have needed to divert resources to reproduction rather than for defense, while males might more easily afford biosynthesis of the compound. Similarly, in plant bug *L. hesperus*, large amounts of defensive volatiles increased slightly with body weight of females (explaining about 18% of volatile variation), suggesting smaller females may divert a larger proportion of resources to eggs rather than to defensive volatiles (about 10,000 ng hexyl butyrate per female), while males did not show any relationship suggesting resources were not as limited for producing defensive volatiles (Byers 2006a). Linear regression of body weights of *P. barbatus* ants from the three nests with significant amounts of (*S*)-4-methyl-3-heptanone (about 1,500 ng/ant) also indicated there is a cost of producing the alarm pheromone in adults (Fig. 4). As above, a conservative removal of about 10% of the lightest weight and 10% of the lowest amount individuals from the analysis did not alter the relationship significantly. For the smaller *P. californicus*, the linear relationship between body weight and alarm pheromone amounts also indicated a cost, but the large variation and lower numbers make for a tentative conclusion.

If *L. uniformis* releases (*S*)-4-methyl-3-heptanone during bites with its piercing mandibles to defend against arthropod predators, then by inference it can be supposed that red harvester ants, having considerably larger amounts, could also use the volatile in a defensive manner (in addition to the alarm function) against insects. However, we found little conclusive evidence for this hypothesis. Most harvester ants did release some alarm pheromone when molested by forceps but this might be a call to nestmates for help in repelling the enemy. The ants usually released their alarm pheromone when fighting other ants, possibly as a defensive measure, but this is also justified as an alarm function. Finally, about half the ants released alarm pheromone when attacking caterpillars, possibly to help subdue the prey (but all ants stung the larvae) or to recruit other ants. In all cases, the possible defensive function of the ketone is confounded by the alarm pheromone function. The defensive function hypothesis is further weakened because the mandibles of harvester ants are broadened (Fig. 4b) for crushing seeds (Oettler and Johnson 2009) and thus not as well suited to introduce the ketone as venom compared to the braconid wasp. Perhaps because harvester ants have such potent stingers, there are less benefits to evolve the ketone as a venom.

Alarm pheromones, such as (*E*)-β-farnesene used by many aphid species, evolved in communal living with related individuals (Byers 2005). If an aphid is captured by a predator, such as a lacewing larva, the aphid emits the volatile as it is being eaten which causes not only clonally-related aphids but also unrelated aphids of the same and different species to leave the vicinity of the predator. In

Pogonomyrmex ants, colony members are also related, as sisters or half-sisters, and the alarm pheromone in all species consists of (*S*)-4-methyl-3-heptanone (McGurk et al. 1966; Vick et al. 1969, and data here) probably due to a common evolutionary origin in the genus. Similar to aphids, the alarm signal is understood between colonies and between *Pogonomyrmex* species perhaps as a means of maintaining territories (in the case of ants). If a harvester ant queen did mutate to produce the (*R*)-enantiomer and mated with the prevailing (*S*)-males, then any hybrid workers might not understand the alarm signal as well, and the colony would be at a disadvantage. In addition to harvester ants, several species in other genera (*Atta*, *Manica*, and *Trachymyrmex*) in the sub-family Myrmicinae contain 4-methyl-3-heptanone that functions as an alarm pheromone (Moser et al. 1968; Fales et al. 1972; Crewe and Blum 1972; Riley et al. 1974; Do Nascimento et al. 1997; Hernández et al. 1999; Hughes et al. 2001). Queens and males of leaf-cutting ants (*A. sexdens rubropilosa* and *A. laevigata*) also contain 4-methyl-3-heptanone that alarms/excites workers for enhanced protection of sexuals during their emergence from the nest on the nuptial flight (Do Nascimento et al. 1997; Hernández et al. 1999). While it is well known that sex and aggregation pheromones are species-specific, there may also be a communication advantage to conformity in alarm pheromones and territorial pheromones between species (Byers 1989, 2006b).

A mutant *L. uniformis* producing the *R*-enantiomer (theoretically equally effective in defense from a chemical perspective) that mated with the usual (*S*)-individual might have progeny where the biosynthetic enzymes with chirally-active sites were compromised. The predominating use of the (*S*)-enantiomer in braconid parasitoids (*L. uniformis*), harvester ants, and some other ants (Moser et al. 1968; Riley et al. 1974; Do Nascimento et al. 1997; Hughes et al. 2001) may simply be a coincidence of early evolution and the advantages of conformity, once one or the other enantiomer became widely used by a species. However, not all ants use the *S*-enantiomer since *Aphaenogaster albisetosus* uses an 80:20 ratio of (*S*):(*R*) enantiomers of 4-methyl-3-heptanone in its trail pheromone secreted by the poison gland in their abdomen (Hölldobler et al. 1995). The inference from the compound's putative defensive function in *L. uniformis* is that the trail pheromone in this ant may also serve as a poison.

Many insects contain quite small amounts of defensive volatiles or alarm pheromones relative to their body weight. For example, first-instar cotton aphids store alarm pheromone at about 0.002% of their body weight, while the percentage declines to only 0.0004% in adult aphids (Byers 2005). Interestingly, the smallest aphids produced the largest relative concentrations of pheromone. This was also true of *P. barbatus* where smaller individuals contained relatively more (*S*)-4-methyl-3-heptanone at 0.0085% of body weight compared to larger ants with 0.0074%. The relative increase in semiochemical concentrations in smaller individuals was not found in *L. uniformis* wasps (ranging from 0.0006 to 0.0009% of body weight in small to large female adults, respectively). Similarly, adult female tarnished plant bugs at 10 days of age produced their defensive volatiles, hexyl and hexenyl butyrate, in smaller relative amounts in smaller adults at 0.039% of body weight compared to larger adults at 0.076% (Byers 2006a). Despite that several insects produce minute amounts of semiochemicals compared to their body weights, there appears to be a cost of producing these volatiles since smaller individuals, in all species above, did not produce the presumably higher-fitness amounts that larger adults did. The costs are likely metabolic involving biosynthesis of semiochemicals and regulation of their autotoxicity and storage. The semiochemical costs and benefits are likely balanced against costs and benefits for growth and reproduction.

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