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Structural complexity of chemical recognition cues affects the perception of group membership in the ants *Linephithema humile* and *Aphaenogaster cockerelli*

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Summary

Hydrocarbon profiles on the cuticle of social insects act as multi-component recognition cues used to identify membership in a species, a colony or, within colonies, cues about its reproductive status or task group. To examine the role of structural complexity in ant hydrocarbon recognition cues, we studied the species recognition response of two ant species, *Linepithema humile* and *Aphaenogaster cockerelli*, and the recognition of conspecifics by *L. humile*. The cuticular hydrocarbons of ants are composed of molecules of varying chain lengths from three structural classes, *n*-alkanes, methyl-branched alkanes and *n*-alkenes. We employed species recognition bioassays that measured the aggressive response of both species of ants to mixtures of hydrocarbon classes, single structural classes of hydrocarbons (*n*-alkanes, methyl-branched alkanes and *n*-alkenes), and controls. The results showed that a combination of at least two hydrocarbon structural classes was necessary to elicit an aggressive species recognition response. Moreover, no single class of hydrocarbons was more important than the others in eliciting a response. Similarly, in the recognition of conspecifics, *Linepithema humile* did not respond to a mixture of *n*-alkane cuticular hydrocarbons presented alone, but supplementation of nestmate hydrocarbon profiles with the *n*-alkanes did elicit high levels of aggression. Thus both *L. humile* and *A. cockerelli* required mixtures of hydrocarbons of different structural classes to recognize species and colony membership. It appears that information on species and colony membership is not in isolated components of the profile, but instead in the mixture of structural classes found in cuticular hydrocarbon profiles.

Key words: cuticular hydrocarbons, social recognition, colony recognition, species recognition.

Introduction

In social insects, an individual's hydrocarbon profile can provide cues about its membership in a species, a colony, or, within colonies, cues about its reproductive status or task group (Howard and Blomquist, 2005). Cuticular hydrocarbon profiles, detected by ants through antennal contact, are thought to be compared to a template that defines group membership (LeMoli et al., 1983; Vander Meer and Morel, 1998). Differences from the template can lead to a behavioural recognition response such as aggression. Template-label matching may involve neural mechanisms in the brain and has been shown to occur in sensilla peripheral to the central nervous system (Obin and Vander Meer, 1989; Ozaki et al., 2005; Vander Meer and Morel, 1998).

Cuticular hydrocarbon-based cues mediate the species recognition response in termites and ants of the genus *Pachycondyla* (Bagnères et al., 1991; Howard and Blomquist, 1982; Lucas et al., 2005; Vauchot et al., 1996). Nestmate recognition, identifying which conspecifics belong to the same colony, is mediated by hydrocarbons in paper wasps *Polistes dominulus* (Dani et al., 2001), the European hornet *Vespa crabro* (Ruther et al., 2002), honey bees *Apis mellifera* (Breed, 1998), and several species of ants (Lahav et al., 1999; Liang et al., 2001; Thomas et al., 1999; Wagner et al., 2000). Within colonies, harvester ant (*Pogonomyrmex barbatus*) workers recognize task-specific hydrocarbon profiles, which provide information used in task allocation (Greene and Gordon, 2003). Workers of the primitive ant *Myrmecia gulosa* discriminate among queens, fertile workers and non-fertile workers using variation in cuticular hydrocarbons (Dietemann et al., 2003).

Hydrocarbons are the most abundant class of chemicals coating the cuticle of social insects (Nelson and Blomquist, 1995). Their primary functions are to prevent water loss across and abrasion to the cuticle, and to protect against infection (Lockey, 1988). Cuticular hydrocarbons generally range in size from about 21 to over 40 carbons in chain-length, with three

known structural classes: *n*-alkanes, *n*-alkenes and methyl-branched alkanes (Nelson and Blomquist, 1995). Cuticular hydrocarbon profiles can differ among groups in the presence or absence of compounds and in the relative abundance of shared compounds (Bagnères et al., 1991; Haverty et al., 2000; Singer, 1998; Vauchot et al., 1996). Within a species, individual colonies generally share the same hydrocarbon components, but the relative abundance of each compound varies (Bonavita-Courgourdan et al., 1987; Howard, 1993; Singer, 1998; Vander Meer and Morel, 1998). The hydrocarbon profile of a colony can change over time and season (Haverty et al., 1996; Liu et al., 2001; Nielsen et al., 1999; Vander Meer et al., 1989). Within a colony, workers performing different tasks can vary in the relative abundance of hydrocarbon compounds (Haverty et al., 1996; Kaib et al., 2000; Wagner et al., 1998). Patriline differences in hydrocarbon profiles have been shown in the ant *Formica truncorum* (Boomsma et al., 2003).

Although considerable evidence shows that cuticular hydrocarbons are used as recognition cues, we know little about what information in these multi-component cues denotes nestmate, species, or task group status and how social insects perceive such information. Are certain components of the cuticular hydrocarbon profile more important than others? Is the relevant information present in the entire hydrocarbon profile, parts of the profile, or only in specific molecules? The recognition response may depend upon complex cues that vary subtly among species (Bagnères et al., 1991). It has been suggested that certain hydrocarbon components, such as *n*-alkenes and methyl-alkanes, have evolved signal function while other compounds, such as *n*-alkanes, serve little role in communication (Bonavita-Courgourdan et al., 1987; Dani et al., 2001; Dani et al., 2005; Espelie et al., 1994; Lucas et al., 2005).

In this study, we examined the role of structural complexity of hydrocarbon cues in the recognition of species and conspecifics. We considered two ant species, the Argentine ant (*Linepithema humile*) and the ant *Aphaenogaster cockerelli*. We investigated species recognition in both, and the recognition of conspecifics in *L. humile*. *L. humile* (subfamily Dolichoderinae) is a worldwide invader of Mediterranean-type habitats (Human and Gordon, 1999; Sanders et al., 2003). The ants are polygynous and polydomous, forming diffuse colonies composed of connected nests that reproduce by budding (Markin, 1968; Giraud et al., 2002). The ants exhibit little intraspecific aggression over large geographic areas (Heller et al., 2006). *L. humile* can acquire hydrocarbons from prey that elicit aggressive responses from conspecifics (Liang et al., 2001), and it interacts aggressively with other species including the ant *Formica moki* (Human and Gordon, 1996). *A. cockerelli* (subfamily Myrmicinae) is a monogynous, desert-dwelling ant native to the Southwestern USA. This ant competes for seed resources with other ants including the red harvester ant *Pogonomyrmex barbatus* (Barton et al., 2004).

In this study, recognition responses were elicited by presenting cuticular hydrocarbons in bioassays that measured aggression. We tested whether the ants respond with aggression to any mixture of hydrocarbons that differs from their own, or if their response depends on the structural complexity of the hydrocarbon cue.

Materials and methods

Extraction and purification of cuticular hydrocarbons

Hydrocarbon donor ants were collected in the field and killed by freezing. Surface lipids were extracted by soaking thawed ants in 2 ml 100% pentane for 10 min (Nelson and Blomquist, 1995). Hydrocarbons were separated from polar surface lipids by running samples through a solid phase of 2 cm of silica gel in a Pasteur pipette and eluting hydrocarbons with 2 ml 100% pentane (Nelson and Blomquist, 1995). Samples were stored at –20°C.

For experiment 2, heterospecific hydrocarbons were taken from the ant *Formica moki*, a species with which the *L. humile* were observed to fight (M.J.G., personal observation) (Human and Gordon, 1997; Human and Gordon, 1999). For experiment 3, heterospecific hydrocarbons were taken from the red harvester ant (*Pogonomyrmex barbatus*), a species with which *A. cockerelli* competes for seed resources (Barton et al., 2002).

Separation of cuticular hydrocarbon structural classes

Saturated and unsaturated hydrocarbons were separated by adding purified hydrocarbons to a silica gel column impregnated with 20% silver nitrate with 2 cm of solid phase (Nelson and Blomquist, 1995). Saturated hydrocarbons were eluted with 2.5 ml of 100% pentane. Unsaturated hydrocarbons were eluted into a separate tube with 2.5 ml of 10% ethyl ether in pentane. Straight chain *n*-alkane hydrocarbons were absorbed by 5 Å molecular sieves (Sigma Chemical Co., St Louis, MO USA) in iso-octane at 75°C for 12 h (Nelson and Blomquist, 1995). The molecular sieves were prepared by baking at 250°C for 12 h prior to adding the samples. As the *n*-alkanes were lost in the pockets of the molecular sieves, 4 μg of an *n*-alkane mixture (C_{21}–C_{31}, C_{33}: *n*-tricosane, *n*-tetracosane, *n*-pentacosane, *n*-hexaconsane, *n*-heptacosane, *n*-octacosane, *n*-nonacosane, *n*-triacontane, and *n*-tritriacontane; Sigma Aldrich) was used as the *n*-alkane structural class.

Experiment 1: the role of hydrocarbon structural complexity in the recognition of conspecifics by L. humile

This experiment tested whether (1) differences in relative abundance in conspecific hydrocarbons elicit a recognition response and (2) if a single structural class of hydrocarbons, *n*-alkanes, can elicit aggression from the ants. A mixture of synthetic saturated *n*-alkane hydrocarbons was added to *L. humile* lipid extracts. The surface lipid extracts supplemented with *n*-alkane standards were tested in a bioassay that measured aggressive behaviour from *L. humile*.

Linepithema humile (Mayr) were collected from focal ant trails the day before the experiment and killed by freezing. As a simple method to normalize the amount of surface lipid extracted, a 1 ml volume of thawed *L. humile* was extracted for

each stimulus. Extraction of 1 ml (average of 212 ants, *N*=3 samples) of *L. humile* yields on average 900 μg purified hydrocarbon. Thus, one ant-equivalent of *L. humile* hydrocarbon is equivalent to approximately 4.2 μg purified hydrocarbon.

The surface lipid extract was then divided into two aliquots, each of the same volume and therefore containing equal amounts of lipids. The first aliquot was left untreated and used as a control. The second aliquot was supplemented with 100 μg of a synthetic *n*-alkane mixture. The *n*-alkane mixture was composed of *n*-alkane hydrocarbons ranging from C23–C30 and C33 (*n*-tricosane, *n*-tetracosane, *n*-pentacosane, *n*-hexaconsane, *n*-heptacosane, *n*-octacosane, *n*-nonacosane, *n*-triacontane, and *n*-tritriacontane; Sigma Aldrich). Another stimulus was created by coating cotton with 100 micrograms of the synthetic *n*-alkane mixture alone. The blank control was created by soaking cotton in 100% pentane for 10 min and allowing the solvent to evaporate for at least 3 h until dry.

Both aliquots were then added to approximately 1 cm^3 of pentane washed cotton; the amount of cotton used for each sample was equal to the volume of ants that were extracted. The cotton was loosely packed into a screw-top tube to the same level as the ants in the extraction screw-top tube. The sample in pentane was then added so that cotton was submerged and the cotton was soaked through. The solvent was allowed to evaporate for at least 3 h so that the cotton was completely dry. Thus, each piece of cotton was treated with one ant-equivalent of hydrocarbon per volume unit area of cotton. This method ensured that all pieces of cotton used in the bioassays were treated approximately the same amount of extracted material.

All of the hydrocarbons in the *n*-alkane standard mixture are present on the cuticle of *L. humile* (Brophy et al., 1983; Cavill and Houghton, 1973; Liang et al., 2001), although only *n*-pentacosane (C25), *n*-octacosane (C28) and *n*-nonacosane (C29) have been reported to have average relative abundances of greater than 1.0% (Liang et al., 2001). Thus, the constituents of the *n*-alkane mixture differed in relative abundance, i.e. quantitatively but not qualitatively, from *L. humile* hydrocarbon profiles. Confirmation of samples using gas chromatography was performed using a DB-1 fused silica capillary column (30 m, 0.25 i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). During injection the oven temperature was held at 170°C for 5 min. Oven temperature was then raised to 220°C at 25°C min^{-1} and then to 310°C at a rate of 3°C min^{-1} with a 5 min hold. Peak areas of each chromatogram were measured and the relative abundance of each peak was calculated (peak area divided by total area of all peaks). Peaks were identified by comparing elution patterns to those reported elsewhere (Liang et al., 2001). The relative abundance of *n*-alkanes changed from 0.214 of total hydrocarbon abundance without the addition of hydrocarbon standard mixture to a relative abundance of 0.876 when the standard mixture was added to cuticular hydrocarbons. Thus, the supplemented levels of *n*-alkanes were not 'physiological doses', being much greater than levels of *n*-alkane abundance normally found on the cuticle of *L. humile* (Liang et al., 2001). However, the goal of this experiment was not to mimic natural conditions, but to determine if the ants responded differently to the *n*-alkane mixture when presented alone than to a mixture of cuticular hydrocarbons and *n*-alkane standards.

We used a behavioural bioassay that measured the ants' aggression towards stimuli presented on pieces of cotton. Treatments were: (1) whole surface lipid extract, including cuticular hydrocarbons, (2) surface lipid extract supplemented with synthetic *n*-alkane mixture, (3) synthetic *n*-alkane mixture alone, and (4) a blank control (solvent only). In all tests, surface lipid samples were collected from the nest being tested to avoid nestmate recognition responses. A response was considered aggressive if the ants bit or pulled the cotton fibres. Biting and pulling is displayed during encounters between *L. humile* and other ants (M.J.G., personal observation) (Roulston et al., 2003). In each trial, we placed treated pieces of cotton approximately 2 cm away from trails of foraging *L. humile*. The pieces of cotton used in the bioassay were pulled from the larger piece of cotton onto which surface lipids were added. These smaller pieces were approximately 3 mm×3 mm and were meant to approximate the size and amount of hydrocarbon of a live ant. Replicates within each experiment were conducted along trails leading to different nest entrances that were separated by at least 10 m. Stimuli were tested in a random order by an observer blind to the treatment order. We conducted the study at eight colonies at three sites in northern California, USA, all within 20 km of Stanford University: (1) on the campus of Stanford University, (2) in a suburban area of Redwood City, California, and (3) at Stanford University's Jasper Ridge Biological Preserve in Palo Alto, California. The number of ants biting or pulling the cotton and the number of ants in contact with the cotton but not aggressive towards it were counted every minute for a total of 10 min.

Experiment 2: the role of hydrocarbon structural complexity in the L. humile species recognition response

Bioassay 1

This experiment tested whether species recognition in *L. humile* is a response to entire hydrocarbon profiles or only to specific parts of the profile. Surface hydrocarbons from 20 *Formica moki* were separated into three structural classes: *n*-alkanes, methyl-alkanes and alkenes, according to the methods outlined above. Samples in pentane were added to 1 cm^2 pieces of cotton so that each piece of cotton was treated with a total of two-ant-equivalents of hydrocarbon extract or hydrocarbon class. For example, one half the amount, as determined by volume, of methyl-alkenes was added to the methyl-alkane and *n*-alkene mixture as compared to the methyl-alkane class alone. The solvent was allowed to completely dry, usually overnight, until used in the bioassay.

In each trial, the following stimuli were tested in a sequential, random order along eight replicate *L. humile* foraging trails using the bioassay described above for experiment 1: (1) solvent-treated cotton (blank control), (2) *n*-alkane fraction alone, (3) methyl-branched alkane fraction

alone, (4) *n*-alkene fraction alone, (5) a mixture of *n*-alkanes and methyl-branched alkanes, (6) a mixture of *n*-alkanes and *n*-alkenes, (7) a mixture of methyl-branched alkanes and *n*-alkenes, (8) a mixture of *n*-alkanes, methyl-branched alkanes, and *n*-alkenes and (9) hydrocarbon extract from *F. moki*.

We were not able to identify *F. moki* cuticular hydrocarbons, so as a surrogate we confirmed the separation methods by running *P. barbatus* hydrocarbons, which had been structurally identified and reported in the literature (Wagner et al., 1998), through the same protocols and analysing the results using gas chromatography (Varian 3900 gas chromatograph; DB-1 fused silica column, 30 m, 0.25 ID, 0.25 μm film thickness; J&W Scientific) using the temperature program described above for experiment 1. Peak areas of each chromatogram were measured and the relative abundance of each peak was calculated (peak area divided by total area of all peaks). Peaks were identified by comparing elution patterns to those of Wagner et al. (Wagner et al., 1998). The *n*-alkane structural class, created from synthetic standards, was composed of 100% *n*-alkanes. Analysis showed clear separation of structural classes; the methyl-alkane structural class was composed of a relative abundance of 1.0 for methyl-alkane hydrocarbons and the *n*-alkene structural class was composed of a relative abundance of 1.0 for *n*-alkenes.

In the bioassay, which was performed as described for experiment 1, we presented approximately 3 mm×3 mm treated pieces of cotton to ants about 2 cm away from active *L. humile* foraging trails. There were 10 min breaks between the presentation of stimuli during each trial, the stimuli were presented in a random order and the observer was blind to stimulus order. We conducted the study at eight colonies on the campus of Stanford University that were separated by at least 10 m. We measured the number of ants in contact with the stimuli and the number of ants pulling or biting the stimuli every minute for 10 min.

Bioassay 2

This experiment tested if the number of hydrocarbon compounds in each class contributed to a species-recognition response. This experiment replicated bioassay 1 but, since we were not able to identify structurally the constituents of *F. moki* cuticular hydrocarbon profiles, we used neat hydrocarbon standards to confirm the results.

We followed the methods outlined for bioassay 1, but synthesized hydrocarbon standards, rather than *F. moki* hydrocarbons, were used to make hydrocarbon structural classes. Two hydrocarbon compounds were used in each structural class: (1) *n*-tricosane (C_{23}) and *n*-pentacosane (C_{25}) for *n*-alkanes, (2) 5-methylpentacosane and 11-methylpentacosane for methyl-alkanes (Sigma Chemical Co.) and (3) 1-hexadecene and 1-octadecene for *n*-alkenes (Ultra Scientific, North Kingstown, RI, USA). 1 mg of each compound was added to stock solutions. Pieces of cotton (1 cm^2) were treated with stock solutions so that the cotton was soaked through. After treatment, the pieces of cotton were allowed to completely dry, and then treated with a total of 1 mg of hydrocarbon so that all stimuli had the same total amount of hydrocarbon on them. For example, the *n*-alkane class received 1 mg of the *n*-alkane standard while the mixture of *n*-alkanes and methyl-alkanes received 0.5 mg of *n*-alkane stock solution and 0.5 mg of methyl-alkane stock solution.

At eight active *L. humile* trails on the Stanford University campus, we presented approximately 3 mm×3 mm treated pieces of cotton to ants about 2 cm away from foraging trails. There were 10 min breaks between the presentation of stimuli during each trial and the stimuli were presented in a random order. The observer was blind to stimulus order. As in the other experiments, we measured the number of ants in contact with the stimuli and the number of ants pulling or biting the stimuli every minute for 10 min. Data were normalized by dividing the number of ants displaying agonistic behaviour by the total number of ants in contact with the cotton during 10 min observation periods.

Experiment 3: the role of hydrocarbon structural complexity in the A. cockerelli species recognition response

In another ant species from a different subfamily, *Aphaenogaster cockerelli* (André) we tested whether species recognition depends on the structural complexity of cuticular hydrocarbon cues. The following stimuli were tested in each trial, in a sequential, random order, at 11 *A. cockerelli* nests: (1) solvent-treated blank control, (2) nestmate hydrocarbons from the focal colony, (3) *n*-alkane fraction alone, (4) methyl-branched alkane fraction alone, (5) *n*-alkene fraction alone, (6) a mixture of *n*-alkanes and methyl-branched alkanes, (7) a mixture of *n*-alkanes and *n*-alkenes, (8) a mixture of methyl-branched alkanes and *n*-alkenes, (9) a mixture of *n*-alkanes, methyl-branched alkanes, and *n*-alkenes, (10) hydrocarbon extract from *P. barbatus*. The observer was blind to the order of stimuli presentation.

To perform the bioassay, treated 5 mm diameter glass beads (Fisher Scientific, Pittsburgh, PA, USA) were placed on *A. cockerelli* nest mounds 5–10 cm from the nest entrance. We measured the number of ants displaying aggressive behaviour and the total number of ants in contact with the cotton every 30 s for 5 min, with 5 min intervals between tests. Ants were considered aggressive if they flared their mandibles or bit the glass bead. These behaviours were observed during interactions with *Pogonomyrmex barbatus* in the field (M.J.G., personal observation). These trials were conducted at a field site near Rodeo, NM, USA (Barton et al., 2004; Sanders and Gordon, 2004). Structural class samples were created using hydrocarbons extracted from 20 *P. barbatus* foragers. Glass beads were added to each extract and hydrocarbon fraction in pentane in screw-top vials. The pentane was allowed to evaporate completely, coating the beads with hydrocarbons. Each bead was coated with a total of one-ant-equivalent of hydrocarbon extract or hydrocarbon structural class. One-ant-equivalent of *P. barbatus* hydrocarbon was estimated to be equivalent to a mass of 9 μg hydrocarbon.

Confirmation of hydrocarbon structural class separations was performed by analyzing structural class samples created for this experiment using *P. barbatus* worker hydrocarbons

using a Varian 3900 gas chromatograph (Varian, Inc.) with a DB-1 fused silica capillary column (30 m, 0.25 ID, 0.25 μm film thickness; J&W Scientific) and using the same temperature program as detailed above. Peak areas of each chromatogram were measured and the relative abundance of each peak was calculated (peak area divided by total area of all peaks). Peaks were identified by comparing elution patterns to those of Wagner et al. (Wagner et al., 1998). The *n*-alkane structural class, created from synthetic standards, was composed of 100% *n*-alkanes. Analysis showed that the methyl-alkane structural class was composed of 100% methyl-alkane hydrocarbons and that the *n*-alkene structural class was composed of 100% *n*-alkenes.

Data analysis

For all experiments, we calculated for each trial the sum of the number of ants aggressive towards the stimuli and the sum of the number of ants in contact with the stimuli without displaying aggression. To normalize for colony differences in the number of ants, data were converted to proportions, calculated as the total number of ants that were aggressive toward a stimulus in a given trial divided by the total number of ants in contact with a stimulus. To help meet the assumptions of normality for analysis of variance (ANOVA), the data were transformed using an angular transformation (arcsine of the square root of the proportion). ANOVA was used to test for statistical differences among treatments.

Results

Experiment 1: the role of hydrocarbon structural complexity in the recognition of conspecifics by L. humile

L. humile responded with a significantly greater proportion of aggressive ants towards the *n*-alkane standard + *L. humile* extract treatment than to the blank control, the *n*-alkane standard alone, or to *L. humile* extract alone ($F_{3,32}$=19.41, P<0.0001; Fig. 1). *Post-hoc* analysis revealed no significant differences between responses to the blank, *n*-alkane, and *L. humile* extract stimuli (LSD, P>0.05).

Experiment 2: the role of hydrocarbon structural complexity in the Argentine ant species recognition response

Bioassay 1

A higher proportion of *L. humile* were aggressive towards mixtures of hydrocarbon classes than to single classes or controls ($F_{8,63}$=17.87, P<0.0001; Fig. 2A). A higher proportion of *L. humile* responded aggressively to mixtures of the hydrocarbon classes than to individual classes of hydrocarbons presented alone or to the blank control (LSD, P<0.0001 for all comparisons). The proportion of ants that displayed aggression toward the mixtures of hydrocarbons was not significantly different from the proportion of ants that were aggressive to the *F. moki* hydrocarbon profile (LSD, P>0.05). *L. humile* responded to individual classes of hydrocarbons with little aggression; the mean proportion of ants aggressive toward *n*-alkanes alone, methyl-branched (*me*-) alkanes alone, or *n*-alkenes alone did not significantly differ from the proportion of ants aggressive toward the blank control (LSD, P>0.05 for all comparisons).

Bioassay 2

As in bioassay 1, a higher proportion of *L. humile* responded aggressively to mixtures of structural classes composed of synthetic hydrocarbons than to the blank control and the single classes of hydrocarbons ($F_{8,63}$=8.67, P<0.0001; Fig. 2B). There were no statistically significant differences between the proportion of *L. humile* that were aggressive to the *F. moki* hydrocarbon profile and any of the mixtures of synthetic hydrocarbon classes (LSD, P>0.05). There were no significant differences between the proportion of ants that were aggressive toward the blank control and the single hydrocarbon class stimuli (LSD, P>0.05).

Experiment 3: the role of hydrocarbon structural complexity in the A. cockerelli species recognition response

The response of *A. cockerelli* to the surface hydrocarbons of *P. barbatus* also depended upon having a mixture of structural classes in the stimulus ($F_{9,100}$=9.38, P<0.0001; Fig. 3). Mixtures of hydrocarbon structural classes elicited a significantly higher proportion of aggressive ants than did the single structural classes, nestmate hydrocarbons, or the blank control (LSD, P<0.05 for all comparison). The proportion of *A. cockerelli* that showed aggression toward the blank control did not differ from the response to the single structural classes of hydrocarbons or nestmate hydrocarbons (LSD, P>0.05).

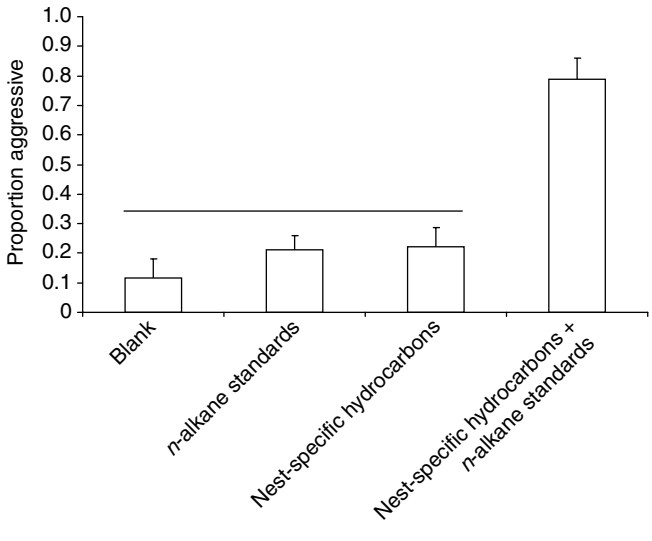

Fig. 1. The aggressive response of *L. humile* to conspecific surface lipids supplemented with *n*-alkane hydrocarbon standards. *L. humile* responded aggressively to changes in the relative abundance of *n*-alkane hydrocarbons in their cuticular hydrocarbon profile. The ants did not respond more aggressively to *n*-alkanes when presented alone than to the blank control or nestmate surface lipids. Values are means ± s.e.m. (N=8). The line above the bars indicates that LSD *post-hoc* analysis showed no statistical difference among the treatments.

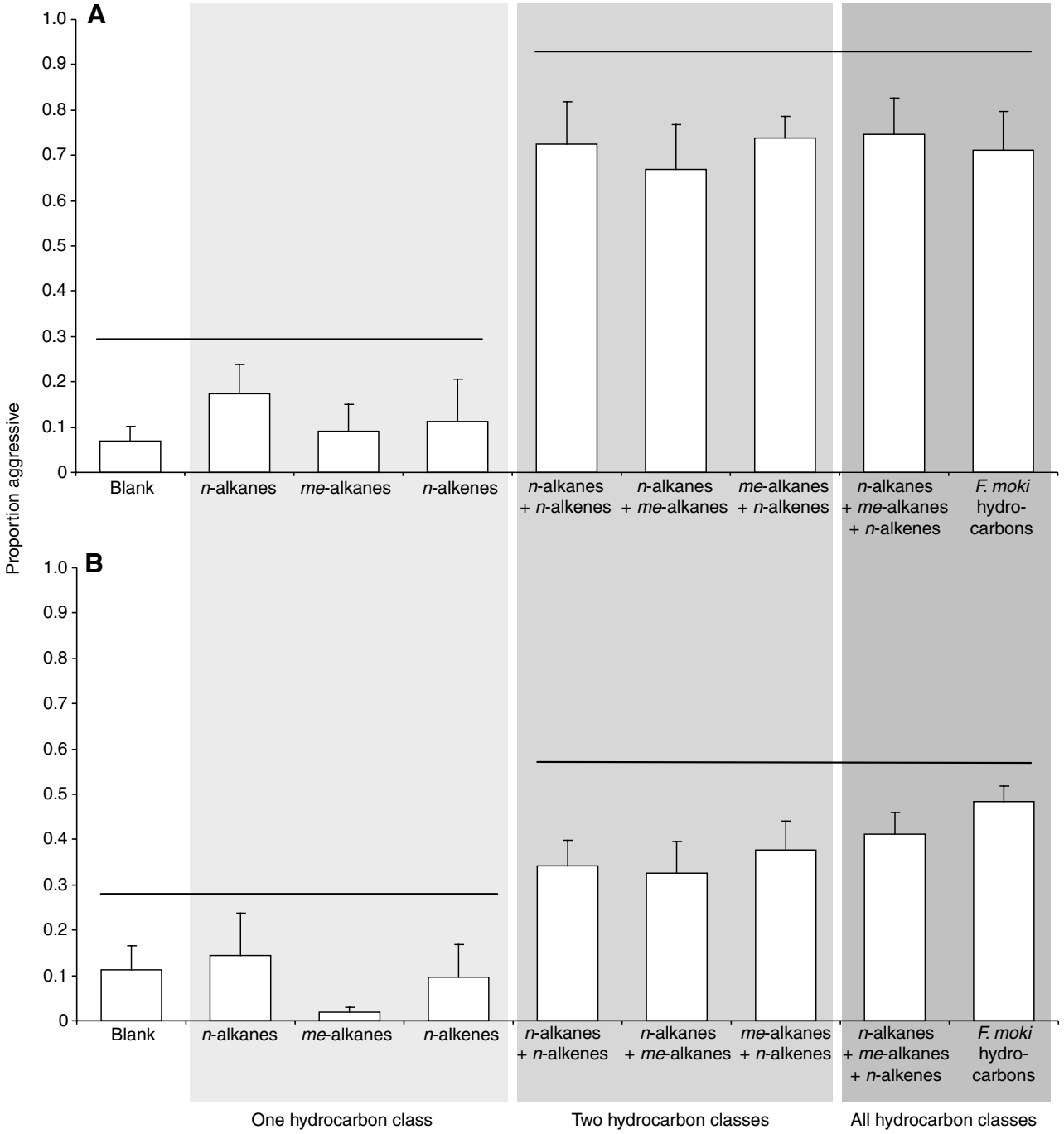

Fig. 2. The aggressive response of *L. humile* towards heterospecific hydrocarbons, structural classes and controls. (A) Argentine ant response to *Formica moki* hydrocarbons, individual hydrocarbon structural classes, mixtures of hydrocarbon classes, hydrocarbons extracted from *F. moki*, and controls. (B) Argentine ant response to mixtures of synthetic hydrocarbon standards and controls. Values are means ± s.e.m. (N=8). The line above the bars indicates that LSD *post-hoc* analysis showed no statistical difference among the treatments.

Discussion

Our data show that structural complexity of cuticular hydrocarbon profiles provides information about species and colony membership in the ants *Linepithema humile* and *Aphaenogaster cockerelli*. A mixture of different cuticular hydrocarbon structural classes was necessary to elicit species recognition responses, in both ant species and in the recognition of conspecifics in *L. humile*. Hydrocarbons of a single class, when presented alone, were not sufficient to elicit an aggressive recognition response in any of the experiments conducted. These results show that the recognition cues present in cuticular hydrocarbons are not based on any single component of the

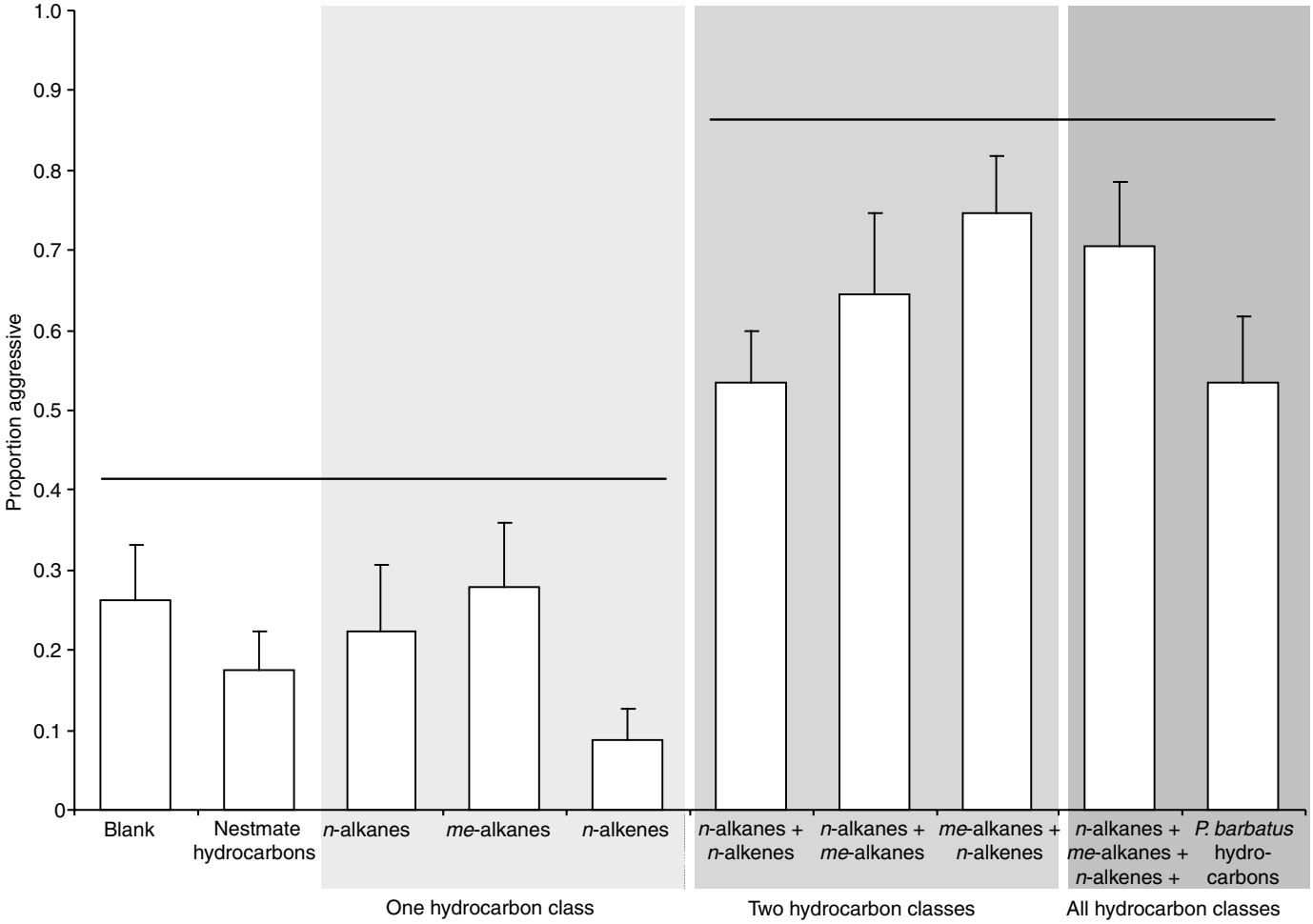

Fig. 3. The aggressive response of *Aphaenogaster cockerelli* towards heterospecific hydrocarbons from *Pogonmyrmex barbatus*, individual hydrocarbon structural classes, and mixtures of hydrocarbon classes. Values are means ± s.e.m. (N=10). The line above the bars indicates that LSD *post-hoc* analysis showed no statistical difference among the treatments.

profile alone. Instead, the perception of group membership depends on the detection of structural mixtures of cuticular hydrocarbons in the species studied.

A high proportion of *L. humile* responded aggressively toward conspecific hydrocarbons that had been supplemented with *n*-alkane standards. A low proportion of ants displayed aggression toward the *n*-alkane standards when presented alone in the bioassay, despite the fact that the hydrocarbons in this mixture differed in relative abundance from those found on the cuticle of the ants. Thus a recognition response can be elicited by changes to the relative abundances of *n*-alkane compounds in the colony hydrocarbon profile, but only as part of a structurally complex mixture of cuticular hydrocarbons. Previous work (Liang et al., 2001) showed that changes to *L. humile* cuticular hydrocarbons in the relative abundance of long chain methyl-branched hydrocarbons over 35 carbons in length elicited aggression towards conspecifics (Liang et al., 2001). The *n*-alkane standard mixture used in our study contained molecules of shorter chain-length, ranging in size from 21 carbons to 31 carbons in length, but also elicited aggression. Thus, changes in the relative abundances of both *n*-alkanes and long chain methyl-alkanes play a role in aggression towards conspecifics in this species.

Species recognition in *L. humile* and *A. cockerelli* occurs through the detection of recognition cues present in heterospecific hydrocarbon profiles. The relevant cues are in the mixture of structural classes within hydrocarbon profiles rather in particular components of the profile. Despite many differences in their social structure and responses to other species of ants (Human and Gordon, 1996; Giraud et al., 2002; Sanders et al., 2003; Markin, 1968), both *L. humile* and *A. cockerelli* exhibited very similar responses to the stimuli. For both species, fewer ants responded with aggression toward the blank control and pure structural classes than to any of the hydrocarbon class mixtures. The proportion of ants aggressive toward mixtures of structural classes was similar to those elicited by heterospecific hydrocarbons. No single hydrocarbon structural class elicited

more aggression than the others. Hydrocarbon molecules of all three structural classes appear to provide information about species membership.

The number of compounds in a hydrocarbon profile does not appear to provide additional information to *L. humile* in its species recognition response. There were no significant differences between the proportion of aggressive ants toward the *F. moki* hydrocarbon extract and toward the combination of two or three synthetic hydrocarbon classes. At least two hydrocarbon structural classes, containing only four compounds, were sufficient to elicit aggression from a high proportion of *L. humile*. This response is similar to the proportion of ants displaying aggression towards *F. moki* cuticular hydrocarbons, which contain more than a dozen compounds (M.J.G., personal observation). Thus, even a simple hydrocarbon profile, as long as it contains a mixture of structural classes, can elicit a recognition response from *L. humile*.

In the species recognition responses of *L. humile* and *A. cockerelli*, all structural classes were equally effective in eliciting an aggressive response. Other work indicates that some hydrocarbon structures may be more important than others in eliciting recognition responses (Boomsma et al., 2003; Breed, 1998; Dani et al., 2001; Espelie et al., 1994; Lucas et al., 2005; Singer, 1998). For example, ants of the genus *Pachycondyla* displayed greater levels of aggression toward branched methyl-alkanes than toward *n*-alkanes and alkenes; however, mixtures of hydrocarbon classes were not tested in this study (Lucas et al., 2005). In paper wasps (*Polistes dominulus*), methyl-alkanes elicited significantly more aggression from nestmates than *n*-alkanes and *n*-alkenes (Dani et al., 2001). Methyl-alkanes, *n*-alkanes, and *n*-alkenes have been implicated in honey bee (*Apis mellifera*) nestmate recognition, along with various fatty acids and esters in chemical derived mostly from comb wax (Breed, 1998; Dani et al., 2005). Also, the addition of synthetic (Z)-9-tricosene to the cuticle of the ant *Campanotus vagus,* was perceived by nestmates (Meskali et al., 1995). European hornets responded aggressively toward nestmates treated with a single *n*-alkane, heneicosane, or with a mixture of heneicosane, tricosane and (Z)-9-tricosene (Ruther et al., 2002) and honey bees recognize changes in the relative abundance of hexadecane and octadecane on nestmates (Breed, 1998). Our data support the suggestion (Breed, 1998) that any compound on the surface of a social insect could potentially play a role in the recognition cue.

Since cuticular hydrocarbons serve a primary function in prevention of water across the cuticle, variation in the relative abundance of compounds in order to facilitate communicative functions may be constrained. Structural complexity may provide the correct chemical context necessary to allow ants to discriminate group membership accurately. Recognition appears to be based upon subtle differences in many components of hydrocarbon profiles, not on larger differences in only a few hydrocarbon constituents. Mistakes in the recognition of group membership could lead to aggressive interactions among colony members, disrupting the social structure of the colony.

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