JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12162

The evolution of queen pheromones in the ant genus *Lasius*

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Keywords:

cuticular hydrocarbons;
fertility signal;
phylogenetic analysis;
worker sterility.

Abstract

Queen pheromones are among the most important chemical messages regulating insect societies yet they remain largely undiscovered, hindering research into interesting proximate and ultimate questions. Identifying queen pheromones in multiple species would give new insight into the selective pressures and evolutionary constraints acting on these ubiquitous signals. Here, we present experimental and comparative evidence that 3-methylalkanes, hydrocarbons present on the queen's cuticle, are a queen pheromone throughout the ant genus *Lasius*. Interspecific variation in the chemical profile is consistent with 3-methylalkanes evolving more slowly than other types of hydrocarbons, perhaps due to differential selection or evolutionary constraints. We argue that the sensory ecology of the worker response imposes strong stabilizing selection on queen pheromones relative to other hydrocarbons. 3-Methylalkanes are also strongly physiologically and genetically coupled with fecundity in at least one *Lasius* species, which may translate into evolutionary constraints. Our results highlight how honest signalling could minimize evolutionary conflict over reproduction, promoting the evolution and maintenance of eusociality.

Introduction

Communication within social insect colonies is predominantly chemical. Queen pheromones are particularly important because they simultaneously regulate many processes including reproductive division of labour (Hoover *et al.*, 2003; Smith *et al.*, 2009, 2012; Holman *et al.*, 2010b; Matsuura *et al.*, 2010), worker behavioural development and learning (Beggs *et al.*, 2007; Vergoz *et al.*, 2007) and colony productivity (Yamamoto & Matsuura, 2011; Holman *et al.*, 2013a), yet until very recently they had been definitively identified in only a single species: the honeybee (Le Conte & Hefetz, 2008). Now, the first queen pheromones affecting reproductive division of labour have been experimentally isolated in ants (physiological effect: Holman *et al.*, 2010b; behavioural effect: Smith *et al.*, 2009, 2012) and termites (Matsuura *et al.*, 2010). This has allowed researchers to document their effects on workers and queens (Smith *et al.*, 2009, 2012; Holman *et al.*, 2010b, 2013a; Yamamoto & Matsuura, 2011) and to begin to address ultimate questions about their origins and function (Holman *et al.*, 2010b, 2013b; Holman, 2012).

Reconstructing queen pheromone evolution could provide fresh insight into the selective pressures and evolutionary constraints acting upon these important signals. For example, queen pheromones have been proposed to be agents of control, used by queens to suppress reproduction in workers in spite of fitness costs to the latter. This situation could lead to an arms race between queens and workers, potentially leading to the rapid diversification of queen pheromones (Keller & Nonacs, 1993; Heinze & d'Ettorre, 2009). Conversely, queen pheromones have been suggested to be honest signals that are closely linked to the queen's fecundity or overall quality, to which workers respond because doing so increases their inclusive fitness (Keller & Nonacs, 1993; Heinze & d'Ettorre, 2009). If workers impose stabilizing selection on queen pheromones (rather than diversifying selection as in the case of an arms race), and/or if these pheromones were physiologically coupled with fecundity (implying strong evolutionary

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constraints), queen pheromones would evolve slowly and may be conserved among distantly related species.

Here, we investigate the evolutionary history of queen pheromones in the ecologically diverse and speciose ant genus *Lasius*. A queen pheromone, 3-methylhentriacontane (hereafter 3-MeC_{31}), was recently isolated in *Lasius niger* (Holman *et al.*, 2010b), and we aimed to evaluate whether this compound is also a queen pheromone throughout the genus. To this end, we tested whether 3-MeC_{31} signals queen fecundity and inhibits worker reproduction in *Lasius flavus*, an early-branching species that is genetically and ecologically divergent from *L. niger*. We then measured the chemical profiles of queens and workers in 11 *Lasius* species and used phylogenetic comparative analyses to assess patterns of evolutionary change.

Materials and methods

Maturation of the cuticular hydrocarbon profile of *Lasius flavus* queens

Fifty-eight mated *L. flavus* queens were collected during a mating flight in Copenhagen, Denmark, in July 2009 and housed immediately in individual plastic cylinders (26 × 38 mm) with a ball of moist cotton wool. Subsets of these queens were frozen at −20 °C on days 0 ($n = 10$), 14 ($n = 14$), 32 ($n = 17$) and 67 ($n = 17$) post-mating, in order to assess changes in the cuticular hydrocarbon (CHC) profile as queens began to reproductively mature (Holman *et al.*, 2010a). Their hydrocarbons were later analysed in a random order as described below, in order to identify changes in the CHC profile as queens activated their ovaries and began to oviposit. We also analysed the CHCs of three highly fecund queens that were visibly swollen with eggs, which were excavated from mature colonies containing many thousands of workers in Tvärminne, Finland, in June 2010. These queens laid 50–100 eggs each in the 24 h prior to being frozen (i.e. were an order of magnitude more fertile than the recently mated queens) and were likely a number of years old.

Effect of 3-MeC_{31} on worker fertility in *Lasius flavus*

Groups of intranidal *L. flavus* workers were collected from six wild colonies in Copenhagen in July 2010. The six colonies were split into three groups of 50 workers (giving 18 subcolonies), each of which was housed in an 8 × 6 × 5 cm plastic box with a moist plaster base and *ad libitum* water, honey and mealworms. Each group of workers was provided with a glass model 'queen', made from 1 cm of the tip of a Pasteur pipette. The three groups from each colony were randomly allocated to one of three treatments: a pentane-only control, hentriacontane (n-C_{31}) in pentane (0.01 $\mu g\ \mu L^{-1}$; Sigma-Aldrich) and 3-MeC_{31} in pentane (0.01 $\mu g\ \mu L^{-1}$, synthesized as described in Holman *et al.*, 2010b). Highly pure, HPLC-grade pentane was used throughout. The linear alkane n-C_{31} is a strong control because it is also a major component of the *L. flavus* queen profile (*c.* 21%; Supplementary Data S1), has the same carbon chain length as 3-MeC_{31} and also increased with reproductive maturity (see Results). Every 24 ± 0.5 h for 30 days, 10 μL of hydrocarbon solution was applied to the glass model queens. The workers were then frozen for subsequent ovary dissection ($n = 30$ per subcolony, total $n = 540$). The ovaries were removed, and the number of developing eggs present was counted at 40× magnification. All experimental procedures and statistical analyses were performed blind to treatment.

Characterization of queen and worker cuticular hydrocarbons in 11 *Lasius* species

Lasius ants were collected from a range of sources around Europe (Table S1) and were identified based on morphological characters and (where applicable) nest architecture and habitat type (Czechowski *et al.*, 2002). Our sample included species from diverse habitats with a variety of distinguishing traits that might affect hydrocarbon evolution; for example, *Lasius umbratus* and *Lasius fuliginosus* engage in social parasitism, *L. flavus* is largely subterranean, *Lasius neglectus* is supercolonial, and *Lasius psammophilus* nests in sand (Czechowski *et al.*, 2002). The difficulty of obtaining queens meant that the sample size was limited to 1–3 queens per species (mean 2.0 ± 0.26), with the exception of *L. niger* for which 47 queens were available (Table S1). The number of workers per species was 2–5 (mean 4.44 ± 0.34), except for *L. niger* ($n = 85$).

Hydrocarbons were extracted by immersing ants in pentane (150 μL for queens, 20 μL for workers) for 10 min. Queen extracts were evaporated in a flow cabinet and rediluted in 40 μL pentane before being injected into an Agilent Technologies 6890N gas chromatograph (capillary column: Agilent HP-5MS, 30 m × 25 μm × 0.25 μm; split–splitless injector; carrying helium gas at 1 mL min^{-1}) using an Agilent 7683B autosampler, whereas worker extracts were immediately manually injected using a Hamilton syringe (2-μL injection volume for both castes). Different extraction methods were used because the castes differ greatly in size; these methods should have no effect on the relative abundance of extracted hydrocarbons. Hydrocarbons were identified using an Agilent 5975 mass selective detector coupled with the gas chromatograph (70 eV electron impact ionization) based on their retention times, diagnostic ions and comparisons with known standards and published data (e.g. Holman *et al.*, 2010a). We were able to ascertain the positions of methyl branches and the number of double bonds, but not the position of double bonds or

stereochemistry. We then calculated the percentage of each peak in the total profile following integration with MSD Chemstation software (Agilent, Santa Clara, CA, USA). For the phylogenetic analyses, we averaged peaks across individuals for each combination of caste and species.

Statistical analyses

We first performed bootstrap analyses to test whether our interspecific hydrocarbon data were reliable despite the small sample size of some species–caste combinations (Table S1). Assuming within-species chemical variability is similar in *L. niger* and other species, the large *L. niger* data set allows us to estimate our sampling error. We first calculated the mean percentage of each hydrocarbon in the total profile of *L. niger* queens and workers using the full sample (n = 47 and 85, respectively) and assumed these values were negligibly different from the population mean. We then (i) sampled n individuals from the available ants with replacement, (ii) found the sample mean percentage of each hydrocarbon, (iii) calculated the absolute difference between these peak means and the 'true' peak means and (iv) took the mean of these differences. This procedure was repeated 10^4 times for different values of n, giving their respective distributions of expected sampling errors per hydrocarbon peak.

For some analyses, we ignored carbon chain length and classified individual compounds into one of the following ten functional groups: *n*-alkanes, *n*-alkenes, 3-methylalkanes, 5-methylalkanes, other monomethylalkanes, 3,y-dimethylalkanes, 5,y-dimethylalkanes, other dimethylalkanes, trimethylalkanes and dienes (which included linear alkadienes and methylated alkadienes) (Supplementary Data S1). This classification scheme reflects what is known about hydrocarbon biosynthesis: common enzymes and precursor substances are used to make hydrocarbons that have the same methyl branch or double-bond position/number, but different chain lengths (Blomquist & Bagnères, 2010). Moreover, ants are known to similarly perceive (or at least respond to) hydrocarbons of the same functional group but different chain lengths in at least two species (van Wilgenburg *et al.*, 2010; Bos *et al.*, 2012). Additionally, some hydrocarbon groups have very similar retention times (e.g. 13- and 11-methylalkanes) and thus cannot be reliably separated using standard gas chromatography.

We estimated the topology of the phylogenetic tree using methods outlined in Lanfear & Bromham (2011). We downloaded all available sequences for five genes (COI, COII, tRNA-leucine, 16S and defensin) from GenBank for each species in our data set, then aligned these sequences by eye in Geneious v5.4 (Drummond *et al.*, 2011). Sequence data were not available for *Lasius piliferus* and *Lasius grandis*, so we constrained these species to be sisters of *L. psammophilus* and *Lasius emarginatus*, respectively, in the light of morphological data from Seifert (1992) and Janda *et al.* (2004). We estimated a partitioning scheme and models of molecular evolution for the data set using the Bayesian Information Criterion in PartitionFinder v0.9.2 (Lanfear *et al.*, 2012), using the 'greedy' search algorithm to find an appropriate partitioning scheme, and the set of models of molecular evolution implemented in MrBayes v3.1.2 ('models = mrbayes'). We then estimated a phylogenetic tree in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003), using the partitioning scheme and models of molecular evolution estimated in PartitionFinder. The Bayesian MCMC was run for 11 million generations with two independent runs of two chains each, and trees were sampled from the chain every 1000 generations. The first 1 million generations of each run were discarded as burn-in, and all results were checked for convergence and sufficient sampling (ESS > 200) in Tracer v1.5 (Rambaut & Drummond, 2007). A 50% majority-rule consensus topology was then estimated in MrBayes v3.1.2 using the 'sumt' command. Trees were rooted at *L. umbratus*, following Maruyama *et al.* (2008).

We employed two complementary statistical tests for phylogenetic signal, that is, the tendency of related species to resemble one another more than randomly selected species (Münkemüller *et al.*, 2012), in queen and worker hydrocarbon profiles. Significant phylogenetic signal implies that the evolutionary rate of the focal trait is comparable to the rate of change in the characters used to build the phylogeny (here, gene sequences).

Firstly, we calculated the genetic distances between all pairs of species from the nine species for which sequence data were available, using the branch lengths from the 50% majority-rule tree estimated above, and the chemical distances among the same species (measured by calculating Aitchison distances on the mean proportions; Aitchison, 1986). We then tested whether the genetic and chemical distance matrices were correlated using Mantel tests (as in van Wilgenburg *et al.*, 2011). Secondly, we reduced the hydrocarbon data set for the nine species with genetic data using a phylogenetically controlled principal component analysis (using the phyl.pca function in the phytools package for R; Revell, 2012), then estimated Blomberg's K on the first principal component (Blomberg *et al.*, 2003), using the 50% majority-rule nine-species tree. Blomberg's K was chosen over other commonly used measures of phylogenetic signal (e.g. Pagel's λ) because it is reliable for small data sets (Blomberg *et al.*, 2003). A value of $K = 1$ means that the distribution of traits corresponds exactly to the predicted distribution assuming a Brownian motion model of evolutionary change, whereas $K = 0$ means no correspondence.

We also performed ancestral state reconstruction (ASR) in order to identify which hydrocarbon families

were consistently associated with caste throughout the evolutionary history of the genus. The ASR was performed in Mesquite v2.75 (Maddison & Maddison, 2011) using maximum parsimony. We used parsimony because our tree of 11 species does not have meaningful branch lengths for the two species without DNA sequence data, and parsimony does not require information on branch lengths when calculating ancestral states.

For the ASR, we used the 50% majority-rule tree of all 11 species. To quantify whether a hydrocarbon functional group was more strongly expressed in queens or workers within each species, we simply scored whether the mean proportion of the profile composed of that group was higher in workers or queens. Each hydrocarbon family was thereby placed into one of three categories for each species: queen-type, worker-type or absent (defined as composing < 1% of the total profile). Although this classification scheme provides no measure of how consistently or strongly caste-specific particular hydrocarbon families are, it allowed us to perform a robust ASR given the limitations of the data. Specifically, it was not feasible to ensure that queens and workers had equal levels of reproductive development across all species, and the queen-like portions of the hydrocarbon profile are known to increase with fecundity in ants (Monnin, 2006; Peeters & Liebig, 2009; Holman *et al.*, 2010a, b; the present study). Therefore, uncontrolled variation in age and fecundity probably explains some of the interspecific variation in the absolute magnitude of the queen specificity of each hydrocarbon type, meaning that the absolute magnitudes are not necessarily informative for assessing ancestral states. To be conservative, we therefore chose a measure of caste specificity (and an ASR method) that ignores the absolute magnitude of the queen–worker difference.

The raw hydrocarbon data and files relating to the phylogenetic analyses are archived at Dryad.

Results

3-MeC_{31} signals queen fertility and causes worker sterility in *Lasius flavus*

As *L. flavus* queens acquired fertility in the first 67 days after mating, three CHCs significantly increased in proportion, namely *n*-C_{31}, 3-MeC_{31} and *n*-C_{33} (binomial GLMs; all $p_{56} < 0.0001$) (Fig. 1a). Mature, highly fecund *L. flavus* queens possessed a very high proportion of 3-methylalkanes, particularly 3-MeC_{31} (Supplementary Data S1; Fig. 1a).

Queenless workers exposed to synthetic 3-MeC_{31} had significantly fewer eggs in their ovaries (typically zero; mean ± SE: 0.3 ± 0.05) than those treated with the control substances pentane (1.1 ± 0.1; contrast from a Poisson GLMM with colony as a random factor: $z = 8.6$, $P < 0.0001$, $n = 540$) or *n*-C_{31} (0.97 ± 0.09; $z = 7.2$, $P < 0.0001$) (Fig. 1b). There was no significant difference between the latter two groups ($z = 1.7$, $P = 0.08$).

Evolution of cuticular hydrocarbon profiles in *Lasius*

The results of the bootstrap analysis are shown in Fig. S1. Sampling only a single individual caused the percentage of each hydrocarbon to be estimated with a relatively small error of 0.67 ± 0.002% for queens or 0.48 ± 0.002% for workers (means ± SE). Sampling error declined rapidly with increased sampling effort, with the rate of decline slowing markedly around $n = 5$ (Fig. S1). Therefore, we expect that the limited

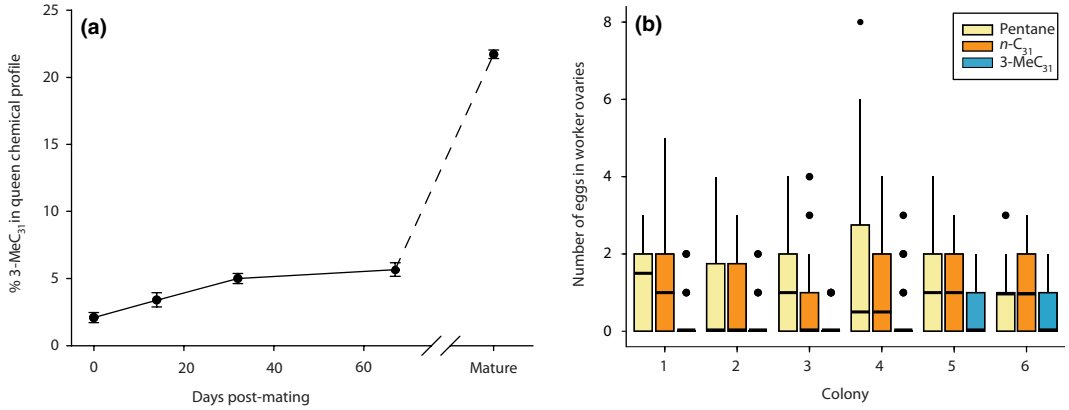

Fig. 1 Fertile *Lasius flavus* queens produce 3-MeC_{31}, which inhibits worker reproduction. Panel a shows the change in the percentage of the chemical profile composed of 3-MeC_{31} with days since mating; queens after the break were collected from mature colonies, were highly fecund and physogastric and were likely a number of years old ($n = 61$; points show mean + 1 SE). Panel b shows the effects of synthetic hydrocarbons on worker fertility (scored as the number of eggs present in the ovaries) relative to the pentane control ($n = 30$ per plot, $n = 540$ in total), replicated using workers from six colonies.

availability of some caste–species combinations did not compromise our ability to detect broad interspecies trends, because hydrocarbon proportions are substantially more similar within than between species.

Hydrocarbon profiles were widely divergent across the genus, and sister species were sometimes highly chemically distinct (Figs 2, 3 and S2; Supplementary Data S1). For example, alkadienes were only present in two species, the early-branching *L. umbratus* and the derived *L. neglectus*, and evolutionary gains and losses of linear alkanes, alkenes, dimethylalkanes and trimethylalkanes were detected (Fig. S2). The methylalkanes

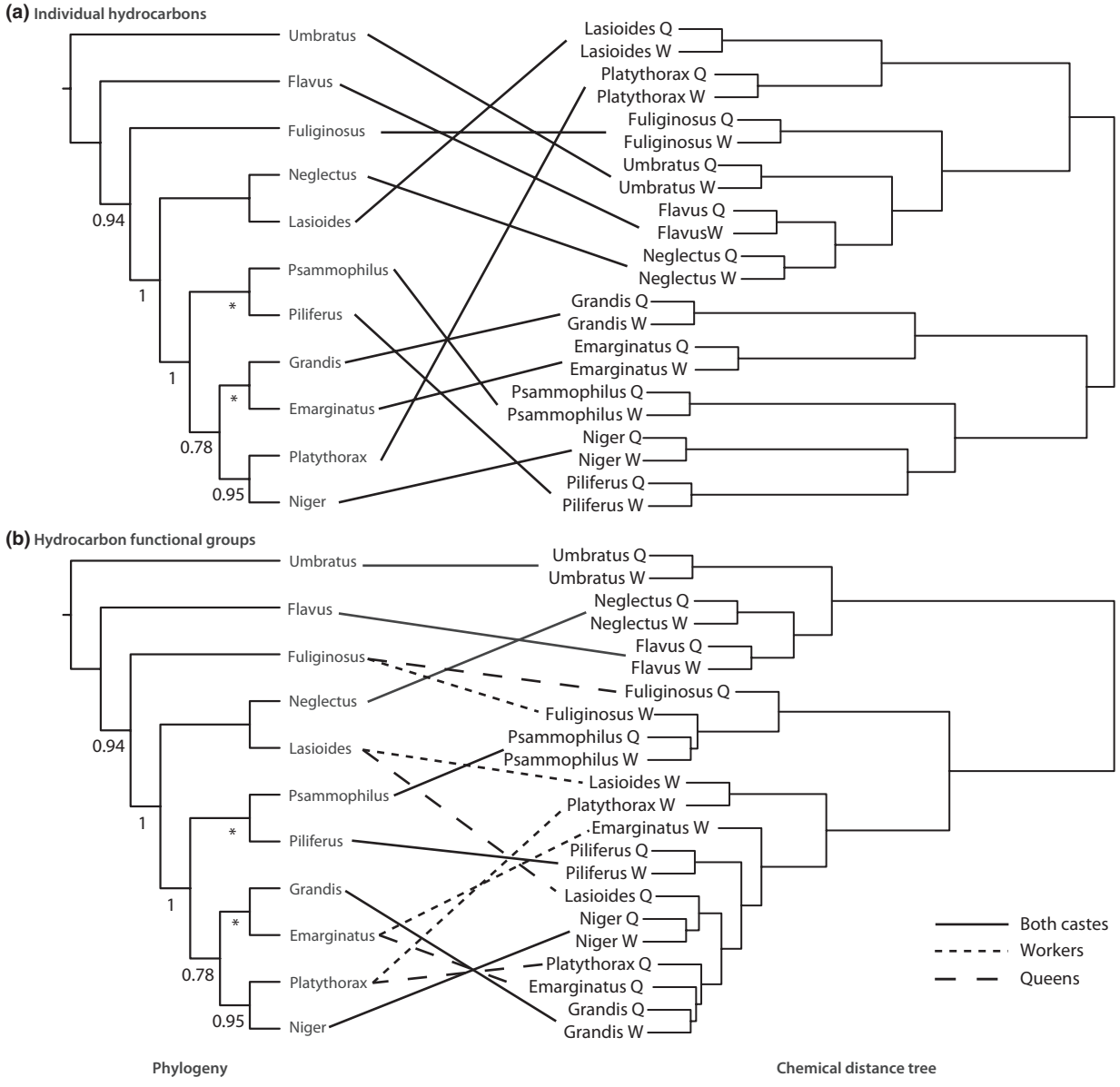

Fig. 2 Comparisons of the phylogeny (left; branch lengths are arbitrary) with two different chemical distance trees (right; branch lengths are proportional to chemical similarity); the chemical distance trees were calculated from a matrix of Aitchison distances by hierarchical clustering using Ward's method. Chemical distances were calculated using either the relative amounts of the 149 different hydrocarbon peaks detected in at least one species (Panel a; this measure incorporates information on chain length) or the relative amounts of each of the 10 hydrocarbon functional groups, that is, after ignoring hydrocarbon chain length (Panel b). Black lines connect species between the trees, whereas dashed and dotted lines connect queens and workers, respectively, for species in which the castes did not cluster together in the chemical tree. Numbers on the phylogeny show Bayesian posterior probabilities, and asterisks denote species whose position was assumed based on morphological data.

were the only functional group present in all 11 species.

Mantel tests (which used the nine-species tree that includes branch lengths; Fig. S3) found no significant concordance between the genetic and chemical distance matrices when examining the data for workers, regardless of whether chemical distances were calculated from proportion data for all 149 hydrocarbons detected in the study (Mantel test with 10^4 permutations; $r = 0.03$, $P = 0.47$) or from the relative abundances of the ten hydrocarbon functional groups (i.e. when disregarding variation in chain length; $r = 0.26$, $P = 0.15$). For the queen data, there was no significant relationship when hydrocarbons were treated individually (Mantel test: $r = 0.009$, $P = 0.49$). However, when the chemical distance matrix was instead calculated on hydrocarbon functional groups, there was a strong, significant correlation between genetic and chemical distances ($r = 0.59$, $P = 0.016$).

Analyses using Blomberg's K (also using the tree in Fig. S3) concurred with the Mantel tests. Blomberg's K for the first phylogenetically corrected principal component (% variance explained: 49.1) of the queen functional group hydrocarbon data set was 0.78 (significantly > 0: $P = 0.047$), suggesting significant phylogenetic signal in functional groups of queen hydrocarbons. As with the Mantel tests, calculating Blomberg's K on the queen (% variance: 49.1, $K = 0.12$, $P = 0.99$) and worker (% variance: 36.0, $K = 0.16$, $P = 0.93$) individual hydrocarbon data sets or the worker functional group data set (% variance: 42.6, $K = 0.24$, $P = 0.64$) yielded no evidence for significant phylogenetic signal.

Lastly, there was no significant phylogenetic signal in the mean chain length (calculated by multiplying each peak's relative abundance by its chain length and summing across peaks) of queen (Blomberg's $K = 0.21$, $P = 0.69$) or worker ($K = 0.48$, $P = 0.16$) hydrocarbon profiles.

Ancestral state reconstruction of cuticular hydrocarbon caste specificity

3-Methylalkanes were more abundant in queens than in workers in all 11 species (Fig. 3). The magnitude of this difference varied among species, but because we obtained ants from a range of sources (Table S1), some of this variation is probably due to uncontrolled differences in age and fecundity. Accordingly, ASR using parsimony suggested that 3-methylalkanes were characteristic of queens in the last common ancestor of these species and at every node in the phylogeny. We stress,

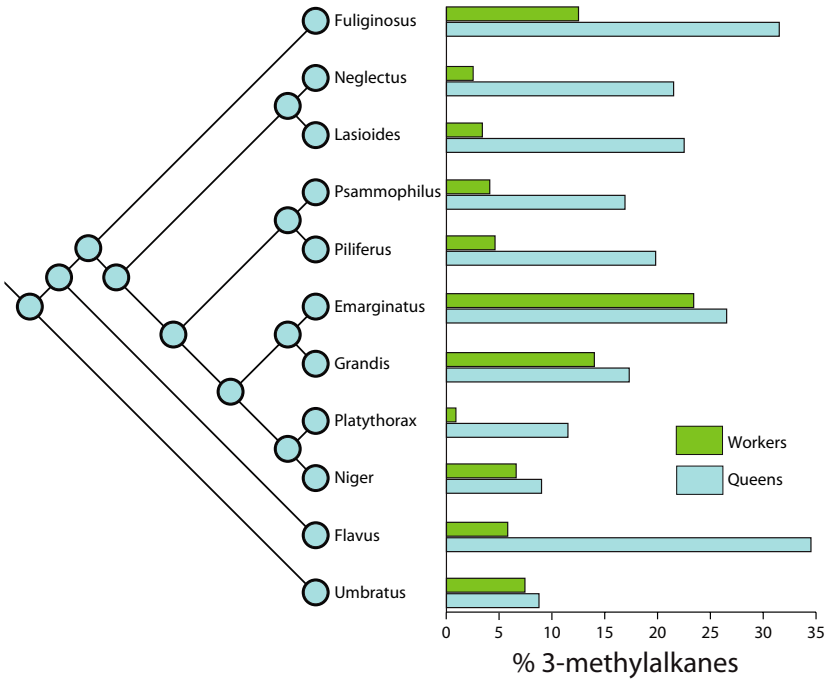

Fig. 3 3-Methylalkanes are more abundant in queens than in workers in all 11 species studied, likely reflecting the ancestral condition. The tree diagram shows that 3-methylalkanes are characteristic of queens rather than workers in all extant species and that the most parsimonious ancestral state at every node of the phylogeny is that 3-methylalkanes were more abundant in queens. The bar chart shows mean 3-methylalkane production by queens and workers of each species. Comparable figures for the other hydrocarbon functional groups are shown in Fig. S2.

however, that the chain length of these 3-methylalkanes varied. For example, relatively short 3-methylalkanes (3-MeC_{25}, 3-MeC_{27} and 3-MeC_{29}) dominated in *L. emarginatus* and *L. fuliginosus*, whereas longer ones (3-MeC_{33} and 3-MeC_{35}) were abundant in the chemical profile of *Lasius lasioides* queens (Supplementary Data S1). No other hydrocarbon family was consistently overexpressed in queens, and the ASR suggested that between two and five evolutionary shifts in caste specificity (or presence/absence) have occurred in the evolutionary history of every hydrocarbon family except 3-methylalkanes (Fig. S2).

Because some queens and workers had a very similar amount of specific chemicals in some cases, meaning that the queen- or worker-type labels might not be appropriate, we ran another ASR with a fourth state added, termed 'Equal'. The Equal state was assigned in cases in which queens and workers had the same amount to within 1%. Adjusting the ASR in this fashion made little difference to the results, and our main conclusion (that 3-methylalkanes alone are 'queen-type' in all species) was unaffected (Fig. S4).

Discussion

Our results provide evidence that 3-methylalkanes are an evolutionarily conserved queen pheromone in *Lasius*. We found that 3-methylalkanes are more abundant in the chemical profile of queens than in workers throughout the genus (uniquely among the hydrocarbon families we examined) and that this was the only hydrocarbon family that has not undergone any evolutionary transitions in caste specificity or presence/absence. Additionally, experiments on *L. flavus* showed that 3-MeC_{31} is a queen pheromone that inhibits worker reproduction and signals queens' reproductive development. *Lasius flavus* and *L. niger* are ecologically and genetically distinct and may be separated by millions of years of independent evolution, yet 3-MeC_{31} is a queen pheromone in both species. While 3-methylalkanes characterize queens in all species and are queen pheromones in the basal *L. flavus* and the derived *L. niger*, we cannot be certain they are queen pheromones in the other species.

The ratios of hydrocarbon functional groups making up the queen chemical profile showed significant phylogenetic signal, whereas those making up the worker profile did not. Given that interspecific variance in worker functional group composition is comparatively high, this result is consistent with queen hydrocarbons evolving more slowly than worker hydrocarbons. The detection of significant phylogenetic signal in queen hydrocarbon functional groups (ignoring information on hydrocarbon chain length), but not in individual queen hydrocarbons (incorporating chain length information), is consistent with chain length being more evolutionarily labile than the relative abundances of the different hydrocarbon functional groups.

One reason why queen pheromones might evolve at a different rate to other hydrocarbons is a difference in selection pressures arising from the role of queen hydrocarbons in signalling fertility (Smith *et al.*, 2009; Holman *et al.*, 2010b; Smith *et al.*, 2012; the present study). Available evidence implies that the worker response to queen pheromone is innate rather than learned in *Lasius* (because workers from all surveyed colonies display the same response; Holman *et al.*, 2010b; the present study), as with other pheromones (Wyatt, 2010). Therefore, evolutionary transitions to a new queen pheromone might be relatively rare because they require a corresponding novel worker response, perhaps mediated by a modified odorant receptor (Niehuis *et al.*, 2013). Because complementary mutations in the pheromone and odorant receptors are expected to be infrequent, selection from receivers should slow the evolution of queen pheromones relative to hydrocarbons that are not pheromones. Our results therefore contrast with the evolution of insect sex pheromones, which differ among closely related species despite the apparent 'fitness valley' imposed by pheromone–receptor codependence (Roelofs & Rooney, 2003; Niehuis *et al.*, 2013). Part of the difference may be due to a discrepancy in the strength of selection; incipient species are often under strong selection to evolve distinct sexual signals in order to avoid costly hybridization (e.g. Hoskin & Higgie, 2010), while there is presently little evidence for diversifying selection on queen pheromones (Heinze & d'Ettorre, 2009).

It is informative here to contrast queen pheromones with hydrocarbons that function in nestmate recognition (reviewed in van Zweden & d'Ettorre, 2010). Nestmate recognition cues are 'signature mixtures' (sensu Wyatt, 2010); in contrast to pheromones, the response to signature mixtures is learned rather than innate. Current evidence suggests that ants learn their colony's hydrocarbon profile from their social environment and are capable of updating their odour 'template' to include novel hydrocarbons (Guerrieri *et al.*, 2009; Stroeymeyt *et al.*, 2010; Bos *et al.*, 2011). Therefore, a mutant colony with a novel hydrocarbon affecting nestmate recognition would be simultaneously able to produce and respond to the new hydrocarbon. Signature mixtures should therefore be expected to evolve faster than pheromones, all else equal, because novel signature mixture components will generally elicit a receiver response whereas novel pheromones may not.

Differing rates of evolution in the chain length of queen hydrocarbons vs. the ratios of different functional groups may also be explained in the light of selection resulting from receiver sensory ecology. Two species of ants have been shown to similarly perceive (or respond to) hydrocarbons with the same methyl branch positions but different chain lengths (van

Wilgenburg *et al.*, 2010; Bos *et al.*, 2012), suggesting, for example, that queens with abundant 3-MeC_{27} might elicit similar worker responses to those with similar amounts of 3-MeC_{31}. If true, chain length would experience less stabilizing selection from receivers than functional group composition and might therefore evolve faster. The perceptual similarity of hydrocarbons has yet to be evaluated in the context of queen–worker communication, and our results highlight that this question deserves experimental study. Furthermore, the synthetic enzymes that determine hydrocarbon chain length are separate from those controlling the positions of methyl branches and double bonds (Blomquist & Bagnères, 2010), suggesting that chain length and the ratio of functional groups could evolve independently. Our data suggest that this has indeed been the case, corroborating a previous phylogenetic study (van Wilgenburg *et al.*, 2011). The evolutionary forces producing diversity in chain length are unclear, although dry environments are thought to select for longer hydrocarbons (Kwan & Rundle, 2010; van Wilgenburg *et al.*, 2011), which may offer superior protection from desiccation.

Another reason why queen pheromones might evolve more slowly is evolutionary constraints on their expression. In support of this interpretation, production of eggs and queen-like hydrocarbons (principally 3-MeC_{31}) are both affected by juvenile hormone treatment in *L. niger*, suggesting either causal links between fecundity and pheromone production or common endocrine regulation (Holman, 2012). Additionally, almost all of the hydrocarbons of *L. niger* were found to be positively genetically correlated with ovarian activity, with some evidence that the genetic correlations are especially strong for 3-methylalkanes, which again suggests constraints (Holman *et al.*, 2013b). These physiological and genetic relationships likely result from the dependence of oogenesis on hydrocarbons. Hydrocarbons are used to provision developing eggs, into which they are shuttled by plasma lipophorins that carry these hydrophobic molecules through the hemolymph (Fan *et al.*, 2002). Lipophorins also deliver hydrocarbons to the cuticle for external secretion (Blomquist & Bagnères, 2010), so the cuticle may provide a window into the hydrocarbon content of the hemolymph. Therefore, ants that are actively producing hydrocarbons for oogenesis may be physiologically constrained to possess a particular hydrocarbon profile (see also Smith *et al.*, 2009) whenever particular functional groups of hydrocarbon are preferentially utilized in oogenesis. The independent evolution of the hydrocarbon profile and fecundity may therefore be difficult, ensuring that these traits remain coupled over evolutionary time.

A remaining question is whether 3-methylalkanes function as queen pheromones across a broader taxonomic range than we have examined here. These compounds have been found to be more abundant in queens in several other ant and wasp species and even a termite (Holman, 2010; Bonckaert *et al.*, 2012), although to date a large-scale, systematic analysis is lacking. It is also unclear whether they are the only queen pheromones in *Lasius*. We urge a transition from descriptive studies that characterize chemical caste dimorphism to experiments aimed at identifying pheromones by bioassay, which will allow us to comprehensively map the evolution of queen pheromones over longer periods of evolutionary time.

In particular, it would be interesting to determine whether honest signals of fertility predate the evolution of eusociality. Such signals might facilitate the origin of eusociality, by allowing offspring to adopt a facultative helping strategy that depends on their mother's fecundity. For example, offspring perceiving their mother to be highly fertile (or alternatively, more fertile than themselves) may decide to stay and help at the natal nest rather than dispersing. This type of facultative helping strategy could be evolutionarily stable in parameter spaces in which an 'always stay and help' strategy was not. Theoretical models and further comparative analyses of fertility signalling in eusocial taxa and cooperative breeders are warranted.

Acknowledgments

We are very grateful to H. Helanterä, X. Esplander, A. Lenoir, A. Tartally and R.S. Larsen for providing ant samples and to C. Leroy for technical support. LH was funded by a Marie Curie fellowship, and PdE by a Marie Curie reintegration grant ('Identity Code').

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Results of the bootstrap analysis, showing estimated sampling error for sample sizes 1–10 (see text).

Figure S2 Bar charts showing the mean proportion of the chemical profile composed of each hydrocarbon functional group for queens and workers of the 11 *Lasius* species.

Figure S3 The nine-species phylogenetic tree used in the Mantel tests and calculations of Blomberg's K.

Figure S4 Bar charts showing the mean proportion of the chemical profile composed of each hydrocarbon functional group for queens and workers of the 11 *Lasius* species.

Table S1 Collection information and sample sizes for ant chemical data presented in Figs 2, 3 and S2.

Supplementary Data S1 Conserved queen pheromone in Lasius - all CHC means.

Data deposited at Dryad: doi: 10.5061/dryad.802t8

Received 28 December 2012; revised 21 February 2013; accepted 14 March 2013