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Blending of heritable recognition cues among ant nestmates creates distinct colony gestalt odours but prevents within-colony nepotism

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Abstract

The evolution of sociality is facilitated by the recognition of close kin, but if kin recognition is too accurate, nepotistic behaviour within societies can dissolve social cohesion. In social insects, cuticular hydrocarbons act as nestmate recognition cues and are usually mixed among colony members to create a *Gestalt* odour. Although earlier studies have established that hydrocarbon profiles are influenced by heritable factors, transfer among nestmates and additional environmental factors, no studies have quantified these relative contributions for separate compounds. Here, we use the ant *Formica rufibarbis* in a cross-fostering design to test the degree to which hydrocarbons are heritably synthesized by young workers and transferred by their foster workers. Bioassays show that nestmate recognition has a significant heritable component. Multivariate quantitative analyses based on 38 hydrocarbons reveal that a subset of branched alkanes are heritably synthesized, but that these are also extensively transferred among nestmates. In contrast, especially linear alkanes are less heritable and little transferred; these are therefore unlikely to act as cues that allow within-colony nepotistic discrimination or as nestmate recognition cues. These results indicate that heritable compounds are suitable for establishing a genetic *Gestalt* for efficient nestmate recognition, but that recognition cues within colonies are insufficiently distinct to allow nepotistic kin discrimination.

Introduction

Levels of selection theory predicts that evolutionary changes at the higher level are more likely to occur when potential conflicts at lower levels have been reduced or eliminated (Keller, 1999). For social insect colonies containing individuals with varying degrees of relatedness to each other (e.g. due to multiple mating of the queen – polyandry), nestmate recognition would be more effective if recognition cues were homogenized among genetic lineages. This would maximize differences among colonies, but would likely imply that differences between genetic lineages become so small that nepotistic discrimination is impossible (Boomsma & Franks, 2006). Indeed, unambiguous cases of nepotistic behaviour have never been found (e.g. Queller *et al.*, 1990; reviewed in Keller, 1997; but see Hannonen & Sundström, 2003; Holzer *et al.*, 2006).

Nestmate recognition in social insects is largely chemically mediated and based on cuticular hydrocarbons, particularly in ants, and these compounds are typically mixed throughout the colony by means of liquid food transfer and grooming (Soroker *et al.*, 1995; reviewed in van Zweden & d'Ettorre, 2010), thereby creating a *Gestalt* colony odour (Crozier & Dix, 1979). Young social insect workers start their adult life practically without any cues on their body, after which they synthesize and acquire

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hydrocarbons to blend in with the colony odour (e.g. Dahbi *et al.*, 1998; Breed *et al.*, 2004). Direct evidence for the use of hydrocarbons in nestmate recognition has been obtained in several social insect species, by testing the level of aggression to either nestmates supplemented with synthetic hydrocarbons (Lahav *et al.*, 1999; Dani *et al.*, 2001, 2005; Guerrieri *et al.*, 2009) or inert materials treated with the hydrocarbon profile of fellow workers or with synthetic mixtures of hydrocarbons (Wagner *et al.*, 2000; Akino *et al.*, 2004; Greene & Gordon, 2007; Martin *et al.*, 2008b). There is other evidence suggesting that cuticular hydrocarbon profiles can carry information about an individual's fertility (Monnin, 2006; Liebig, 2010), worker task specialization (Greene & Gordon, 2003) and genetic heterogeneity within the colony (Boomsma *et al.*, 2003). Hence, multiple messages can apparently be encoded within the same cuticular hydrocarbon profile, which implies that not all hydrocarbons within the profile may have equal importance for nestmate recognition.

Nestmate recognition is generally considered to be based on the similarity between a label (the multicomponent pattern of nestmate recognition cues, i.e. the *Gestalt*) and a template (the neural representation of these cues) (Lenoir *et al.*, 1999). The acquisition of the template is thought to occur early in the adult life of a social insect (Hölldobler & Michener, 1980; Gamboa *et al.*, 1986), but templates can be updated throughout adult life (Stuart, 1988; Errard *et al.*, 2006), so that templates are ultimately considered to be 'environmentally derived'. The location of templates in the social insect nervous systems remains unknown. Traditionally, it was believed that the configuration of the hydrocarbon pattern of nestmates is stored in the long-term memory (Lenoir *et al.*, 1999), but this has recently been challenged by the hypothesis that desensitization of the olfactory receptor neurons on the antennae is responsible for nestmate recognition (Ozaki *et al.*, 2005). Receptor adaptation or habituation (Guerrieri *et al.*, 2009) now appear to provide a more parsimonious explanation for template updating.

Nestmate recognition cues can have a significant genetic component (Vander Meer & Morel, 1998), as in the sweat bee *Lasioglossum zephyrum*, where relatedness predicts the acceptance of individuals in a nest (Greenberg, 1979). Evidence for a genetic component in nestmate recognition has also been obtained in the ants *Temnothorax* (*Leptothorax*) *ambiguus* and *T.* (*L.*) *longispinosus*, where workers that eclosed in isolation were better accepted in their nestmate colony than in non-nestmate colonies. Also, cross-fostering of workers in these species showed that individual odour has a heritable component and that these individual contributions are mixed within the colony (Stuart, 1988). The environment is another possible source of nestmate recognition cues, as food and nesting material have been shown to influence *Gestalt* odours in ants (Heinze *et al.*, 1996; Liang & Silverman, 2000) and honeybees (Kalmus & Ribbands, 1952; Downs & Ratnieks, 1999). However, genetic cues originating from nestmates may also affect the odour phenotype of a focal individual (i.e. indirect genetic effects; cf. Linksvayer, 2006), but to our knowledge no study has investigated how synthesis (own genotype) and acquisition (nestmates genotypes and abiotic environment) may differentially influence the phenotypic expression of cuticular hydrocarbon recognition cues.

In the present study, we use a cross-fostering design to investigate the pattern of nestmate recognition cue synthesis by young workers of the ant *Formica rufibarbis*. We test the degree to which nestmate recognition cues are heritable using bioassays and subsequently use methods from quantitative genetics and multivariate statistics to estimate the degree to which the hydrocarbons are heritably synthesized by newly eclosed workers and transferred by their foster workers. We use these results to elucidate whether any subsets of these compounds are suitable for: (i) efficient discrimination between nestmates and non-nestmates (*sensu* Hölldobler & Michener, 1980); (ii) nepotistic discrimination between individuals of different degree of kin within colonies (*sensu* Page & Breed, 1987); and (iii) the assessment of colony-level genetic heterogeneity, and hence relatedness asymmetry, as would for example be required to let workers bias the sex ratio towards the sex with the highest kin value (*sensu* Boomsma *et al.*, 2003).

Materials and methods

Study animals

Eight *F. rufibarbis* colonies were collected in a grassland field near Regensburg, Germany, and transported to Copenhagen, Denmark (henceforth, stock colonies). Colonies A and B were collected in April 2005 and colonies C, D, E, F, G and H were collected in May 2007. In the laboratory, each colony was kept in a nest consisting of two plastic boxes ($26.5 \times 17.5 \times 8.0$ cm) connected with a short plastic tube, and both were coated with Fluon® (De Monchy International BV, Rotterdam, The Netherlands). One half of boxes served as nest, was darkened with a lid, had a regularly moistened plaster floor and contained glass tubes filled with water and a cotton plug; the other half was uncovered and served as foraging area. The colonies were fed diluted honey and mealworms, *Tenebrio molitor*, three times a week and were kept at room temperature (20–25 °C).

Pilot field vs. laboratory experiment

When excavating colonies A and B in 2005, the queen and about 500 workers were collected whereas the other half of workers were left in the field in Regensburg. In June 2006, the remaining (queenless) workers of colonies A and B were collected from the field and

transported to the laboratory in Copenhagen. Hence, these workers had been separated from their kin for over a year. At this point, we conducted aggression bioassays between either kin individuals (field–field, $N = 40$; lab–lab, $N = 40$; field–lab, $N = 40$) or non-kin individuals (field–field, $N = 40$; lab–lab, $N = 40$; field–lab, $N = 40$) to test whether physical environment had an effect on nestmate recognition. Differences with the bioassays described below were that in this case both workers were alive, yet only the behaviour of one focal individual in each pair was recorded, and that data were not log-transformed before statistical analysis. Data were analysed using a Kruskal–Wallis test in STATISTICA 7.0 (StatSoft Inc., Tulsa, OK, USA).

Experimental setup

We created small experimental colonies: for each, 65 workers were picked randomly from one of the colonies (henceforth, foster workers), marked with a small dot of enamel paint on their abdomen and placed in a nest resembling the stock colony nests, but smaller ($8.0 \times 6.0 \times 5.0$ cm). They received the same diet as stock colonies. After 1 h of acclimatization, the foster workers were provided with 30 randomly picked cocoons (pupae) from either a non-nestmate or their own stock colony. Experimental colonies were labelled by a capital symbol (indicating the stock colony from which cocoons came) and a subscript (indicating the stock colony from which foster workers came): we created ten cross-fostered colonies (A_B, B_A, A_C, B_C, A_D, B_D, E_F, F_E, G_H, H_G) and six control colonies (A_A, B_B, E_E, F_F, G_G, H_H). Of the workers that eclosed from these cocoons (henceforth, adoptees), the first ten were allowed to stay in their experimental colony for 5 days, after which they were taken out for analysis of hydrocarbon profiles. The remaining adoptees were allowed to stay in the experimental colonies for 30 days, until the onset of aggression tests. By this time, each experimental colony contained on average 15 adoptees (range 9–18) and 65 foster workers.

Aggression bioassays

We tested whether foster workers' exposure to the hydrocarbon profiles of their adoptees reduced their aggression towards kin of their adoptees. Aggression bioassays were conducted between a live foster worker taken from one of the experimental colonies, and a freshly killed adult worker from one of the stock colonies. The latter was killed by freezing at −20 °C to prevent any behaviour from influencing the foster worker and subsequently left at room temperature for 5–10 min to warm up. Killing does not alter the properties of substances involved in nestmate recognition (Nowbahari *et al.*, 1990). In each aggression bioassay, the killed worker was placed inside an opaque plastic cylinder (Ø 2.2 cm, h 3.5 cm) coated with Fluon® in the middle of a Petri dish lined with a clean filter paper. The foster worker was subsequently placed just outside the smaller cylinder, but was surrounded by a larger transparent cylinder (Ø 5.2 cm, h 6.1 cm), also coated with Fluon®. The foster worker was allowed to move around freely for 3 min to acclimatize. The test began when the inner cylinder was removed, so that the foster worker could contact the killed worker for 3 min, during which we recorded the duration of four behaviours using Etholog 2.25 (© E.B. Ottoni 1999). Each behaviour was given a score indicating increasing aggression: 0 = investigate with antennae, 1 = open mandibles, 2 = bite, 3 = curl abdomen forward (to be ready to spray formic acid). Overall aggression indices for each test were calculated, using the formula by Errard & Hefetz (1997):

$$\mathrm{AI}_{\mathrm{overall}} = \frac{\sum_{i=0}^{3} \mathrm{AI}_i \times t_i}{T},$$

where $\mathrm{AI}_{\mathrm{overall}}$ is the overall aggression level, AI_i and t_i are the aggression index and duration of the i'th behaviour, and T is the sum of the durations of all interactions.

Foster workers (and not adoptees) of experimental colonies A_A, A_B, B_A, B_B, A_C, B_C, A_D and B_D were tested towards individuals of each of the involved stock colonies (i.e. A_B vs. A, A_B vs. B, A_B vs. C, A_B vs. D, B_B vs. A, etc.), replicating each combination ten times, resulting in a total of 320 encounters of five different types (Fig. 1a). Foster workers from cross-fostered colonies were tested against individuals of their own stock colony ('kin', X_Y vs. Y, $N = 60$), against individuals of the stock colony of their adoptees ('kin of adoptees', X_Y vs. X, $N = 60$), and against individuals of stock colonies that were neither kin to their adoptees nor to themselves ('non-kin', X_Y vs. Z, $N = 120$). Foster workers of control colonies were tested against individuals of their own stock colony ('kin', X_X vs. X, $N = 20$) and against individuals of non-kin stock colonies ('non-kin', X_X vs. Z, $N = 60$). After log-transformation, aggression indices were compared using a General Linear Mixed Model (GLMM) in SAS 9.1 (SAS Institute Inc., Cary, NC, USA), with 'encounter type' as fixed variable, and 'stock colony of foster worker' and 'stock colony of killed worker' as random variables.

Chemical analysis

Cuticular lipids were extracted by immersing workers individually in 200 μL HPLC-grade pentane (Sigma-Aldrich, Brøndby, Denmark) for 10 min, of which the first and last 15 s involved gentle vortexing. The solvent was then allowed to evaporate, after which the extract was re-suspended in 50 μL pentane, of which 2 μL was injected in an Agilent 6890N gas-chromatograph, equipped with a HP-5MS capillary column ($30 \text{ m} \times 250 \times 0.25$ μm), a *split-splitless* injector in splitless mode and a flame ionization detector. The carrier gas was helium at

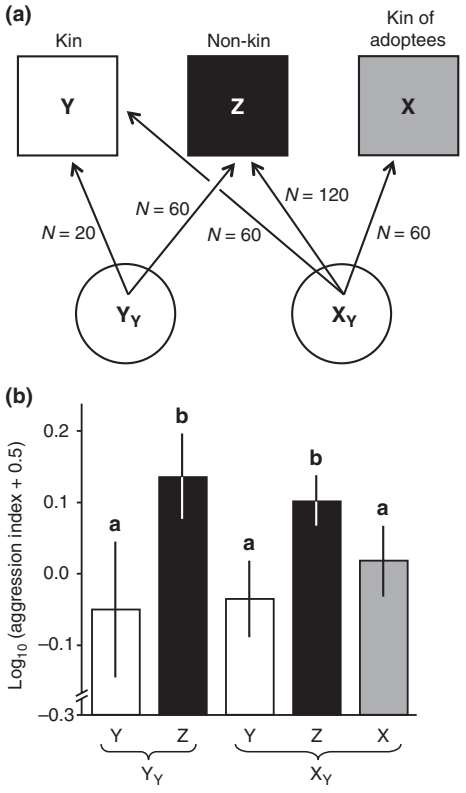

Fig. 1 Nestmate recognition cues are heritable compounds. (a) Scheme of aggression tests with sample sizes. Foster workers were exposed to either eclosing nestmate adoptees (Y_Y) or non-nestmate adoptees (X_Y) for 1 month in experimental colonies (circles), after which their aggression towards freshly killed workers of stock colonies (squares) was tested: their own kin (Y), non-kin (Z) or, in the case of cross-fostered colonies, kin of their adoptees (X). (b) The mean aggression levels observed (± 95% confidence limits). Lowercase letters indicate significant differences at $P < 0.01$ (GLMM with Tukey's post hoc tests, $F_{4,312} = 8.30$, $P < 0.0001$). Foster workers exposed to non-nestmate workers (X_Y) significantly reduced their aggression towards kin of their adoptees (X) in comparison with other non-kin workers (Z), indicating that there is a significant heritable component in the hydrocarbon profiles that mediates nestmate recognition.

1 mL min^{-1}. After an initial hold of 1 min at 70 °C, the temperature increased to 220 °C at a rate of 30 °C min^{-1} and then to 310 °C at 3 °C min^{-1} with a final hold time of 5 min. The compound identities (Fig. 2) were determined on the basis of their mass spectra produced by gas chromatography – mass spectrometry (GC-MS) performed using a Agilent 6890N GC coupled with an Agilent 5375 MS with electron ionization (70 eV).

Qualitative analysis of relative heritability of hydrocarbons

In these analyses, we used adoptees of each of experimental colonies A_A, B_B, A_B, B_A, E_E, F_F, E_F, F_E, G_G, H_H, G_H and H_G. To determine qualitatively whether hydrocarbon profiles are heritable and differences in heritability of direct and indirect effects among hydrocarbons, we used a recently developed multivariate pattern recognition method that allows comparison of entire chemical profiles (Christensen & Tomasi, 2007). The chromatographic data were divided into six test sets, altogether including all experimental colonies for each of the combinations A–B, E–F and G–H. Specifically, each test set consisted of one cross-fostered colony (e.g. A_B or B_A) and two control colonies (e.g. A_A and B_B). For each test set, we created a validation set consisting of worker ants from stock colonies (e.g. A and B). For each of these six data sets, a tiered approach was employed for the processing of the chromatographic data to remove variation that is unrelated to the chemical composition such as constant chromatographic baselines, retention time shifts and changes in detector sensitivity (see Supporting Information for an outline of the methods). We then performed principal component analyses (PCAs) on each of the mean-centred test sets, using the validation set as an independent. An explanatory scheme is shown in Fig. 3, and an example score plot and corresponding loadings of hydrocarbons on PC1 and PC2 are shown in Fig. 4 (see also Fig. S1).

Quantitative analysis of relative heritability of hydrocarbons

Peak areas of the hydrocarbons (or blends of a few hydrocarbons because of co-elution) that were found on the cuticles of all workers were quantified using the software Agilent ChemStation (v. D.02.00.237; Agilent Technologies, Waldbronn, Germany), after which the data were sum-normalized. Hydrocarbon profiles were described in terms of both relative concentration of separate hydrocarbons and separate principal components, extracted by a PCA including all stock and experimental colonies. We used the following model to describe the expected hydrocarbon profile of an adoptee:

$$\text{Profile} = \text{Synthesized} + \text{Transferred} + \text{Common environment},$$

where the synthesized profile components are produced by the adoptee, depending on their own genotype and pre-foster environmental factors (e.g. type of food consumed during the early larval stage). Transferred profile components are hydrocarbons that are transferred from foster workers to the adoptees; these components are influenced by the genotypes as well as the pre-foster environment of the foster workers (e.g. nutritional and nest environmental factors that influence the hydrocarbon profile of field-collected foster workers). Finally, the common environment components include those hydrocarbons that are influenced by shared post-foster synthesized and transferred factors.

Label	Compound(s)	Label	Compound(s)	Label	Compound(s)	Label	Compound(s)
1	n-C_{25}	11	4,12-, 4,10-, 4,8-diMeC_{28}	21	8-MeC_{30}	30	5,19-, 5,17-, 5,15-, 5,9-diMeC_{31}
2	5-MeC_{25}	12	n-C_{29}	22	4-MeC_{30}	31	3,y-diMeC_{31}
3	n-C_{27}	13	15-, 13-, 11-, 9-MeC_{29}		13,17-, 12,14-diMeC_{30}	32	16-,15-,14-,13-MeC_{32}
4	13-, 11-, 9-MeC_{27}	14	7-MeC_{29}	23	6,14-, 6,12-diMeC_{30}	33	8-MeC_{32}
5	7-MeC_{27}	15	5-MeC_{29}	24	4,14-, 4,12-, 4,10-diMeC_{30}	34	6,16-, 6,14-diMeC_{32}
6	5-MeC_{27}	16	13,17-, 9,15-, 9,19-diMeC_{29}	25	n-C_{31}	35	17-,15-,13-MeC_{33}
7	3-MeC_{27}	17	7,12-diMeC_{29}	26	15-, 13-, 11-, 9-MeC_{31}	36	5-MeC_{33}
8	5,17-, 5,15-diMeC_{27}		3-MeC_{29}	27	7-MeC_{31}		13,17-, 5,19-diMeC_{33}
9	n-C_{28}	18	5,15-, 5,17-, 5,19-diMeC_{29}	28	5-MeC_{31}	37	7,y-diMeC_{33}
10	3,15-, 3,9-, 3,7-diMeC_{27}	19	3,11-, 3,9-, 3,7-diMeC_{29}		13,17-diMeC_{31}		3-MeC_{33}
		20	15-, 14-, 13-, 12-, 11-, 10-MeC_{30}	29	7,17-, 7,15-diMeC_{31}	38	5,19-, 5,17-, 5,15-diMeC_{33}

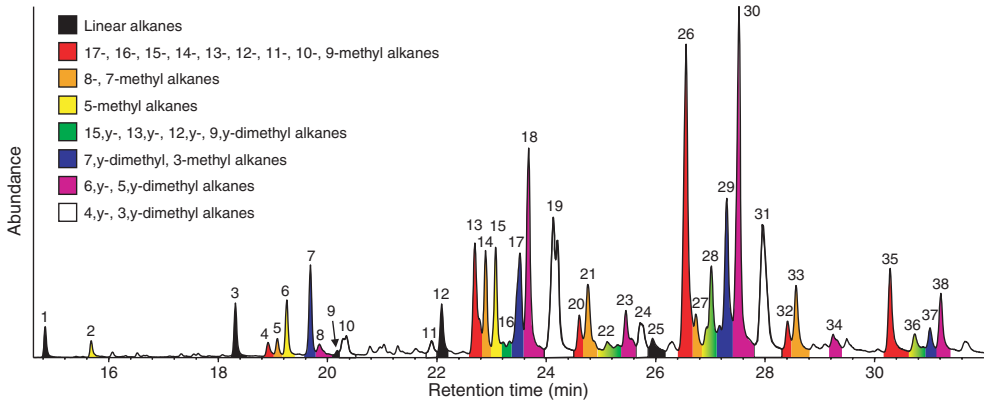

Fig. 2 Typical cuticular hydrocarbon profile of *Formica rufibarbis*. The 38 identified (numbered) peaks used in the analyses are colour-coded according to the different structural groups that they belong to.

The covariance in profiles between cross-fostered individuals from a colony X and control individuals from colony X is the variance between groups of adoptees derived from the same colony (e.g. A_A and A_B; Fig. 3) and is:

$$\mathrm{Cov}(X_Y, X_X) = rS_G + S_E,$$

where S_G is the genetic variance between colonies in synthesized hydrocarbon profile components because of similarities in the genotypes of adoptees from colony X; r is the relatedness between adoptees from colony X; and S_E is variance between colonies in the hydrocarbon profiles of focal individuals because of similarities in the pre-foster environment of individuals from colony X. Assuming that the pre-foster environment has negligible influence on hydrocarbon synthesis at the adult stage ($S_E \approx 0$), this covariance is proportional to the heritability of direct effects on the hydrocarbon profile (Fig. 3; cf. Linksvayer, 2006). Hence, the values presented in this article are not true heritabilities, but measures that are proportional to the relative heritabilities of direct and indirect effects of hydrocarbons.

The covariance between the hydrocarbon profiles of individuals from colony X reared by Y foster workers and individuals from colony Y reared by Y foster workers is the variance between groups of adoptees reared by foster workers derived from the same colony (e.g. A_A and B_A; Fig. 3) and is:

$$\mathrm{Cov}(X_Y, Y_Y) = rT_G + T_E,$$

where T_G is genetic variance between colonies in hydrocarbon components of focal individuals that are transferred from foster workers to adoptees. This genetic component is due to similarities in the genotypes of foster workers from colony X; r is the relatedness between foster workers from colony X; and T_E is the environmental variance between colonies that was transferred to adoptees, which is because of similarities in the pre-foster environment of foster workers from colony X. For example, workers were field-collected and any between-colony pre-foster environmental variation that affects transferable foster worker hydrocarbon profile components will contribute to $\mathrm{Cov}(X_Y, Y_Y)$. Assuming that the pre-foster environment has negligible influence on transferred hydrocarbons ($T_E \approx 0$), this covariance is proportional to the heritability of indirect effects on the hydrocarbon profile (Linksvayer, 2006).

The covariance between the hydrocarbon profiles of control individuals (colony X reared by X foster workers) is the total variance between colonies (Fig. 3d) and includes additional more complex terms:

$$\mathrm{Cov}(X_X, X_X) = rS_G + S_E + rT_G + T_E + 2r\mathrm{Cov}(S_G, T_G) + C,$$

where r is the relatedness between foster workers and focal adoptees from colony X; $\mathrm{Cov}(S_G, T_G)$ is the covariance between genetic effects on synthesized and transferred hydrocarbon components; and C is variance between colonies because of similarities in the post-foster environment of experimental colonies, as well as any covariances between pre- and post-foster environmental components.

We estimated the above covariances using standard sib analysis. Specifically, for $\mathrm{Cov}(X_X, X_X)$, we used ANOVA

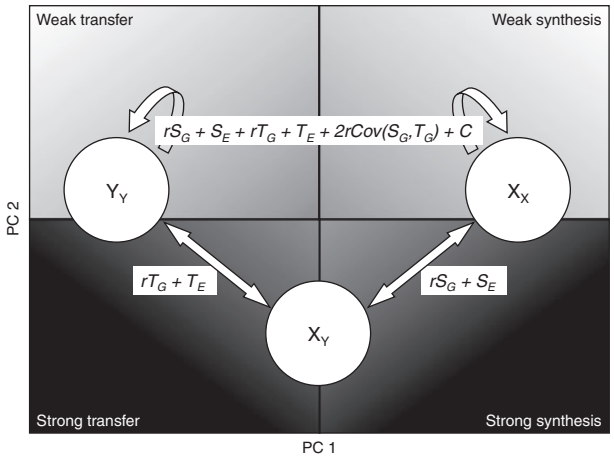

Fig. 3 Multivariate statistics and heritability estimates of hydrocarbon profiles. Explanatory scheme of relative influence of synthesis and transfer of hydrocarbons, corresponding to PCA patterns in Fig. 4a, b. Compounds with high relative abundance in the genetic control and in the cross-fostered individuals (high positive loading on PC1, high negative loading on PC2), show a strong synthesis variance component. Variance components estimated: $\mathrm{Cov}(X_X,X_X) = rS_G + S_E + rT_G + T_E + 2r\mathrm{Cov}(S_G, T_G) + C$ (total among-colony component), $\mathrm{Cov}(X_Y,Y_Y) = rT_G + T_E$ (hydrocarbon transfer component), $\mathrm{Cov}(X_Y,X_X) = rS_G + S_E$ (hydrocarbon synthesis component).

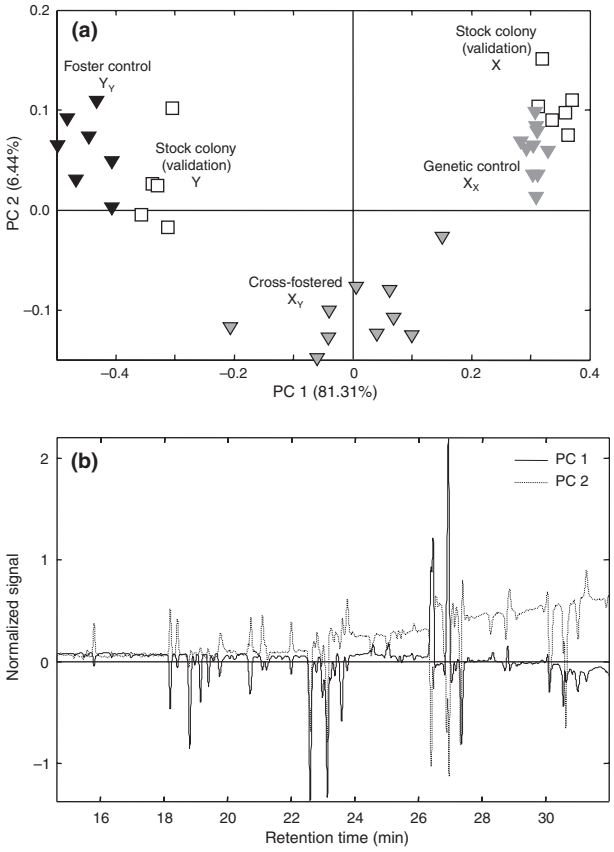

Fig. 4 General pattern observed in principal component analyse (PCAs) and associated PC loadings. (a) PCA example plot of the hydrocarbon profiles of cross-fostered individuals (X_Y), a genetic control (X_X), and a foster control (Y_Y), depicted by triangles. Hydrocarbon profiles of stock colony individuals (open squares) were used as validation set. (See Supporting Information for the full set of plots.) (b) Loadings of chromatographic peaks (hydrocarbons) on PC 1 and PC 2, corresponding to (a). The peaks at retention time 25.0 min (15-, 13-, 11-, 9-MeC_{29}), 25.5 min (13,17-, 9,15-, 9,19-diMeC_{29}), 28.3 min (15-, 13-, 11-, 9-MeC_{31}) and 29.4 min (5-MeC_{31}, 13,17-diMeC_{31}) are responsible for the separation between controls on PC 1. These same peaks and those at retention time 30.0 (5,19-, 5,17-, 5,15-, 5,9-diMeC_{31}), 32.6 min (17-,15-, 13-MeC_{33}) and 33.0 min (5-MeC_{33}, 13,17-, 5,19-diMeC_{33}) separate cross-fostered individuals from controls on PC 2.

with full-sib analysis to partition total phenotypic variance into between- and within-colony variance components (Lynch & Walsh, 1998; Linksvayer, 2006). For $\mathrm{Cov}(X_Y, X_X)$ and $\mathrm{Cov}(X_Y, Y_Y)$, we used a nested analysis of variance, equivalent to a half-sib design, where treatment (cross-fostered and control) was nested within colony of origin to partition total phenotypic variation into variance between colonies for synthesized/transferred components, variance between cross-fostered and control groups within colonies, and variance within groups (Lynch & Walsh, 1998). We present these covariance estimates as proportion of total phenotypic variance (i.e. intra-class correlations), not heritabilities, because we are interested in the relative heritability, when comparing synthesized and transferred components for the whole hydrocarbon profile, using PCA, and for individual hydrocarbons, using ANOVA. Furthermore, our modest sample sizes mean that we cannot reliably estimate heritabilities of direct and indirect effects for the whole population, also because our estimates will be upwardly biased by any pre-foster environmental effects.

We also estimated the diagnostic power (DP) of each compound (Christensen *et al.*, 2005), which is calculated as the standard deviation of the standardized peak area over all colonies divided by the pooled standard deviation within these colonies. Hence, compounds with the highest DP are most variable between colonies, but comparatively most consistent within colony, and are therefore the most likely to act as nestmate recognition cues. We used only individuals from stock colonies to calculate DP.

Results

Pilot field vs. laboratory experiment

Encounters between non-kin were always highly aggressive (non-kin field–field, aggression index (AI) = 2.05 ± 0.90 (mean ± SD); non-kin lab–lab, AI = 1.87 ± 0.97; non-kin field–lab, AI = 2.54 ± 0.56) and independent

of the origin of the ants (Kruskal–Wallis test with multiple comparisons, $H_{5,240} = 176.38$, $P < 0.0001$; non-kin field–field vs. non-kin lab–lab, $Z = 0.80$, $P > 0.95$; non-kin field–field vs. non-kin field–lab, $Z = 1.13$, $P > 0.95$; non-kin lab–lab vs. non-kin field–lab, $Z = 1.93$, $P = 0.80$), whereas encounters between kin were generally not aggressive (kin field–field, AI = 0.04 ± 0.08; kin lab–lab, AI = 0.07 ± 0.09). Even when kin had been separated for a year under very different conditions (kin field–lab, AI = 0.31 ± 0.51), encounters were mostly docile and only resulted in clear aggression (biting or curling the abdomen forward) in four of 40 encounters, so there was little influence of origin in kin encounters (kin field–field vs. kin lab–lab, $Z = 1.40$, $P > 0.95$; kin field–field vs. kin field–lab, $Z = 2.96$, $P = 0.05$; kin lab–lab vs. kin field–lab, $Z = 1.56$, $P > 0.95$). As a result, there was a clear difference in aggression between kin and non-kin encounters ($Z > 4.97$, $P < 0.0001$ for all nine comparisons).

Aggression bioassays

Aggression tests showed that there is a significant genetic component to nestmate recognition in *F. rufibarbis* (Fig. 1). Foster workers (Y individuals) from mixed colonies (X_Y) were significantly less aggressive to nestmates of their adoptees (X) than to unfamiliar non-nestmates (Z) (GLMM with Tukey's post hoc tests, $F_{4,309} = 7.04$, $P < 0.0001$; X_Y–X vs. X_Y–Z, $P < 0.05$), whereas they were equally nonaggressive to nestmates (Y) (X_Y–X vs. X_Y–Y, $P = 0.164$). Furthermore, foster workers from mixed (X_Y) and control colonies (Y_Y) were equally highly aggressive to unfamiliar non-nestmates (X_Y–Z vs. Y_Y–Z, $P = 0.374$) and equally nonaggressive towards nestmates (X_Y–Y vs. Y_Y–Y, $P = 0.796$). When entered as fixed variables, there was also a significant effect of 'stock colony of foster worker' ($F_{3,309} = 9.83$, $P < 0.0001$), but not of 'stock colony of killed worker' ($F_{3,309} = 1.00$, $P = 0.395$).

Chemical analysis

The typical chromatogram of the cuticular hydrocarbon profile of *F. rufibarbis* consists of about 40 peaks containing linear and methyl-branched alkanes (Fig. 2). We colour-coded the different structural groups of hydrocarbons to better visualize how different structural groups of compounds performed in the different analyses.

Qualitative analysis of relative heritability of hydrocarbons

The general pattern observed in the PCAs can be used to determine whether a hydrocarbon is synthesized to a high degree by the adoptees (Fig. 3). This is the case if the relative abundance of the hydrocarbon is consistently higher in the genetic control than in the foster control (high positive loading on PC1). If the hydrocarbon also has high relative abundance on the cross-fostered individuals (high negative loading on PC2), this must have been attributable to synthesis by the adoptees themselves. Figure 4a shows how hydrocarbon profiles of cross-fostered individuals fall in between the genetic and foster control on PC1, confirming that both synthesis and social transfer of hydrocarbons together create the observed pattern, with Fig. 4b showing the associated PC loadings of hydrocarbons on PC1 and PC2.

Quantitative analysis of relative heritability of hydrocarbons

The quantitative analysis (Fig. 3; Table 1) shows that only a limited set of methyl-branched hydrocarbons (especially 13,17-dimethyl alkanes) have a high synthesis component (> 0.7; Table 1), a high transfer component and high DP. On the other hand, all linear alkanes and a number of branched alkanes (especially some of the 7-methyl alkanes) show relatively low heritability of direct effects and are transferred to a much lower degree among individuals within the colony (low heritability of indirect effects). Furthermore, whenever a hydrocarbon has a strong synthesis component (high $rS_G + S_E$), it is also transferred extensively among individuals within the colony (high $rT_G + T_E$) (Fig. 5). Assuming that the pre-foster environment has negligible influence on hydrocarbon synthesis at the adult stage ($S_E \approx 0$), there is a clear correlation between direct and indirect genetic effects, where some compounds undergo a strong influence of both effects, whereas other compounds undergo very little influence of either.

Discussion

We have documented the relative heritability of direct and indirect effects of different hydrocarbons in the ant *Formica rufibarbis*. We first established that nestmate recognition is strongly affected by the phenotypes expressed by young adoptee workers, and that there is nearly no influence of physical environment, which demonstrates that most likely heritable hydrocarbons are of particular importance as nestmate recognition cues. Furthermore, for each cuticular hydrocarbon of adoptees, we calculated the strength of direct (synthesis) and indirect effects (propensity of hydrocarbons to be transferred among nestmates). These results show that a subset of methyl branched alkanes, but not linear alkanes, have both strong direct and indirect genetic components, so that these are most suitable to function as nestmate recognition cues.

Adult workers updated their nestmate recognition template, with non-nestmate workers eclosing in their nest. Most likely, the eclosing workers were blank slates without a significant chemical profile upon eclosion (Dahbi *et al.*, 1998; Breed *et al.*, 2004), after which they

Table 1 Variance component estimates, average percentage and diagnostic power (DP) for each of the hydrocarbons found on the cuticle of workers of *Formica rufibarbis*, and for the first five principal components, extracted from a principal component analyse using all stock and experimental colonies. See Materials and methods for exact calculation of variance components and DP. Average percentage and DP were calculated across stock colonies only. High values (> 0.70 for variance components; > 4.00 for percentage; > 5.00 for DP) are indicated in bold. The values presented here are not true heritabilities, but measures that are proportional to the relative heritabilities of direct and indirect effects of hydrocarbons.

	Proportion of total phenotypic variance				
Compound	Total among-colony component	Synthesis component	Transfer component	Average %	DP
C_{25}	0.74	0.19	0.59	0.34	1.72
5-MeC_{25}	0.80	0.55	0.35	0.18	2.50
C_{27}	0.69	–0.17	0.19	2.34	1.24
13-, 11-, 9-MeC_{27}	**0.99**	0.34	0.60	1.67	3.45
7-MeC_{27}	**0.92**	0.64	**0.88**	0.67	4.12
5-MeC_{27}	**0.98**	0.30	0.65	1.31	3.53
3-MeC_{27}	**0.95**	**0.76**	0.69	1.22	4.02
5,15- 5,17-diMeC_{27}	**0.94**	0.09	0.32	0.55	2.32
C_{28}	0.65	0.01	0.19	0.28	1.27
3,15-, 3,9-, 3,7-diMeC_{27}	**0.95**	**0.79**	**0.88**	0.80	3.00
4,12-, 4,10-, 4,8-diMeC_{28}	0.84	0.63	0.66	0.50	2.11
C_{29}	0.01	0.11	-0.03	1.82	1.28
15-, 13-, 11-, 9-MeC_{29}	**0.97**	0.51	0.73	**5.76**	3.63
7-MeC_{29}	0.67	0.42	0.25	1.19	3.42
5-MeC_{29}	**0.96**	0.35	**0.87**	1.75	4.90
13,17-, 9,15-, 9,19-diMeC_{29}	**1.00**	**0.93**	**0.87**	**4.79**	**7.61**
7, 12-diMeC_{29}, 3-MeC_{29}	**0.97**	**0.79**	0.74	2.95	**5.48**
5,15-, 5,17-, 5,19-diMeC_{29}	**0.98**	0.57	**0.86**	**4.63**	**5.95**
3,11-, 3,9-, 3,7-diMeC_{29}	**0.97**	**0.92**	**0.93**	3.62	**5.03**
15-, 14-, 13-, 12-, 11-, 10-MeC_{30}	**0.98**	**0.77**	**0.85**	1.75	1.99
8-MeC30	**0.98**	**0.86**	**0.92**	1.65	3.78
4-MeC_{30} 13,17-, 12,14-diMeC_{30}	**0.97**	0.67	**0.71**	2.07	2.95
6,14-, 6,12-diMeC_{30}	0.70	0.42	0.54	1.47	2.62
4,14-, 4,12-, 4,10-diMeC_{30}	**0.93**	**0.72**	**0.74**	1.03	3.92
C_{31}	0.52	0.19	0.39	1.01	1.59
15-,13-,11-,9-MeC_{31}	**0.99**	**0.74**	**0.85**	**11.72**	**6.21**
7-MeC_{31}	0.48	-0.24	0.24	0.87	2.28
5-MeC_{31} 13,17-diMeC_{31}	**1.00**	**0.79**	**0.93**	**11.94**	**8.47**
7,17-, 7,15-diMeC_{31}	**0.98**	0.49	**0.89**	3.30	4.69
5,19-, 5,17-, 5,15-, 5,9-diMeC_{31}	**0.97**	-0.24	**0.73**	**7.14**	**5.31**
3,y-diMeC_{31}	**0.98**	0.61	**0.93**	3.22	**5.10**
16-,15-,14-, 13-MeC_{32}	**0.97**	**0.79**	**0.92**	1.51	4.45
8-MeC_{32}	**0.93**	**0.80**	**0.91**	1.63	3.51
6,16-, 6,14-diMeC_{32}	**0.98**	0.56	**0.85**	1.52	4.49
17-,15-,13-MeC_{33}	**0.96**	**0.75**	**0.91**	**4.32**	3.75
5-MeC_{33} 13,17-, 15,19-diMeC_{33}	**0.98**	**0.75**	**0.94**	**4.89**	**5.45**
7,y-diMeC_{33}	0.75	–0.01	0.66	0.86	4.06
5,19-, 5,17-, 5,15-diMeC_{33} + 3-MeC_{33}	**0.90**	–0.06	0.73	1.73	2.38
PC#1	**1.00**	**0.94**	**0.96**	–	–
PC#2	**0.96**	–0.42	0.65	–	–
PC#3	**0.98**	0.39	0.72	–	–
PC#4	**0.86**	–0.05	0.45	–	–
PC#5	0.63	–0.11	0.21	–	–

started to synthesize their own hydrocarbon profiles and to acquire hydrocarbons from their foster nestmates (Dahbi *et al.*, 1998; Lenoir *et al.*, 1999). Because this process is rather slow, foster workers could possibly habituate to the odour of adoptees being mixed in the colony *Gestalt* and thus gradually came to regard the alien young workers as nestmates. When the foster workers subsequently encountered kin of their adoptees, the typical relative abundance of hydrocarbons expressed was similar enough to that of the adoptees to not elicit

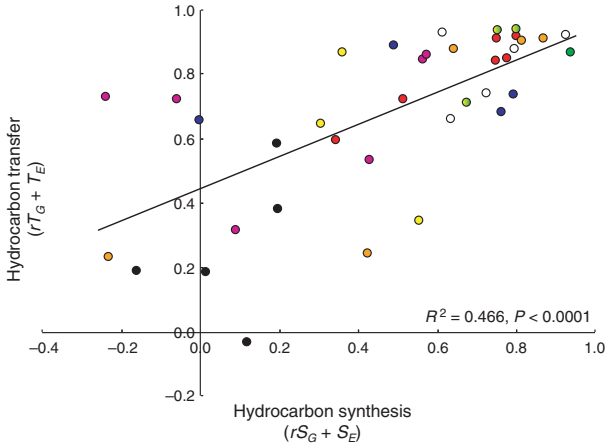

Fig. 5 Transfer of heritable compounds creates a colony *Gestalt* and prevents within-colony nepotism. There is a significant correlation between hydrocarbon synthesis and hydrocarbon transfer ($R^2 = 0.456$, $P < 0.0001$). Compounds in the upper right corner of the graph are highly heritable and transferred extensively among ant workers, and thus likely nestmate recognition cues. The lack of compounds showing a strong synthesis component but a low transfer component indicates a low potential for within-colony kin recognition. Colours correspond to those in Fig. 2 and Table 1.

aggressive reactions. This suggests that the nestmate recognition template is influenced by exogenous factors, especially the social environment (Errard & Hefetz, 1997; Errard *et al.*, 2006). Our results clearly show that the label of an individual worker is highly influenced by both hydrocarbon transfer (indirect genetic effects of nestmates; cf. Linksvayer, 2006) and hydrocarbon synthesis (direct genetic effects). We cannot entirely exclude other phenotypic cues, but the wealth of evidence pointing towards cuticular hydrocarbons functioning as nestmate recognition cues in ants (van Zweden & d'Ettorre, 2010) and the foster workers responding differentially towards freshly killed kin and non-kin strongly suggests that heritable cuticular hydrocarbons are the prime phenotypic cues mediating nestmate recognition in *F. rufibarbis*.

The results obtained from the quantitative analyses suggest that heritable nestmate recognition cues are predominantly encoded in the relative abundances of a subset of methyl branched alkanes (especially 13,17-diMeC$_{29}$ and 13,17-diMeC$_{31}$), whereas linear alkanes show low synthesis and transfer components. Moreover linear alkanes are almost equally variable within colonies as between colonies, whereas some methyl branched alkanes are as much as eight times more variable between colonies than within colonies (Table 1). This finding is consistent with previous work showing that branched alkanes and alkenes are more important than linear alkanes in nestmate recognition of the wasp *Polistes dominulus* (Dani *et al.*, 2001), the honeybee *Apis mellifera* (Dani *et al.*, 2005) and the ant *Camponotus aethiops* (Guerrieri *et al.*, 2009). Using the ant species *F. exsecta* and *F. fusca*, which are congeneric to *F. rufibarbis*, Martin *et al.* (2008a) recently found that a worker could be correctly classified in its own colony using either a series of alkenes or a series of dimethyl alkanes, respectively, whereas linear alkanes were too variable within colonies for accurate colony assignment. Quantitative variation in linear alkanes has been suggested to function as cues for worker task allocation (e.g. Wagner *et al.*, 2001; Greene & Gordon, 2003) or fertility assessment (Smith *et al.*, 2009), which would be consistent with these compounds having low heritabilities of direct effects (relative to other hydrocarbons), as variation should be age and context related for the former purpose and condition related for the latter. Our results therefore imply that the classification of recognition compounds in categories that serve different purposes may well follow the same rules across social Hymenoptera.

Our study shows that classes of potential recognition cues vary considerably in their relative synthesis heritability (direct effects), but that not a single compound ends up having kin-specific distinctness within colonies because transfer among nestmates (indirect effects) increases proportionally with the relative synthesis heritability of compounds (Fig. 5). This exchange of recognition cues among nestmates serves the joint interests of all colony members, because the ensuing heritable colony *Gestalt* allows for efficient discrimination between nestmates and non-nestmates, while at the same time removing the potential usefulness of these compounds for within-colony kin recognition. In other words, the strong correlation between relative synthesis and transfer rate enhances the efficiency of nestmate recognition and precludes the use of heritable compounds for nepotistic purposes.

Our finding that certain groups of compounds (e.g. some of the linear alkanes) seem to combine relatively low synthesis heritability (direct effects) and low transfer rate among nestmates (indirect effects) may also have interesting implications. This combination of characteristics implies that these compounds are the only ones expressing genetic variation among individuals within colonies, which is the type of noisy, low-heritability-of-direct-effects variation that has been hypothesized to characterize cues that workers use when making cumulative assessments of colony-level genetic variation (Boomsma *et al.*, 2003). Such assessments would, for example, apply when workers bias the sex ratio based on assessments of variable relatedness asymmetry, as is know to happen in two other *Formica* species, *F. truncorum* and *F. exsecta* (Sundström, 1994; Sundström *et al.*, 1996). For *F. truncorum*, a previous study has confirmed that patriline-level variation for a subset of hydrocarbons dominated by linear alkanes is indeed low but positive (Boomsma *et al.*, 2003), which is consistent with our present results of compounds like this having low exchange among nestmates. The hydrocarbon profile of *F. exsecta* consists of linear alkanes and (Z-)9-alkenes, but

only the latter have been implicated as the nestmate recognition cues (Martin *et al.*, 2008b). It appears that linear alkanes in this species vary more according to the genetic heterogeneity of colonies, whereas alkenes are equally homogeneous across colony kin structures (J.S. van Zweden, E. Vitikainen, P. d'Ettorre, L. Sundström, unpublished results), which would be consistent with the inferences above.

Our results confirm conceptual notions that the evolution of informative recognition cues can be understood from kin selection and levels of selection theory (Keller, 1999; Boomsma *et al.*, 2003). They provide general confirmation for the idea that individual compounds or profiles do not express sufficient genetic variation to allow nepotistic discrimination to be efficient. They also clarify the cue-scrambling mechanism by which these informational constraints apply throughout a range of possible recognition compounds and suggest that future studies would benefit from targeting specific classes of hydrocarbons depending on the hypothesis being tested. Finally, our results underline that an explicit distinction between direct and indirect genetic effects on the maintenance of genetic variation for recognition cues is helpful to understand the evolution of colony-level traits that help resolve conflicts at lower levels of selection.

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Supporting information

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

Data S1 Materials.

Figure S1 Principal component analyses of hydrocarbon profiles of *Formica rufibarbis* ants.

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