

Original Article

# Cuticular hydrocarbons as potential kin recognition cues in a subsocial spider

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In animal societies, recognition of group members and relatives is an important trait for the evolution and maintenance of social behavior. In eusocial insects, nest mate recognition is based on cuticular hydrocarbons and allows colony members to reject competitors and parasites. The study of recognition cues in subsocial species can provide insights into evolutionary pathways leading to permanent sociality and kin-selected benefits of cooperation. In subsocial spiders, empirical evidence suggests the existence of both kin recognition and benefits of cooperating with kin, whereas the cues for kin recognition have yet to be identified. However, cuticular hydrocarbons have been proposed to be involved in regulation of tolerance and interattraction in spider sociality. Here, we show that subsocial *Stegodyphus lineatus* spiderlings have cuticular hydrocarbon profiles that are sibling-group specific, making cuticular hydrocarbons candidates for kin recognition cues. Our behavioral assays indicate that spiderlings can discriminate between cuticular cues from kin and nonkin: In a choice set-up, spiderlings more often chose to reside near cuticular chemical extracts of siblings compared with nonsiblings. Furthermore, we show that cuticular chemical composition changes during development, especially around the stage of dispersal, supporting the hypothesis that cuticular cues are involved in regulating conspecific tolerance levels. Lastly, our results indicate that the potential kin recognition cues might be branched alkanes that are influenced very little by rearing conditions and may be genetically determined. This indicates that a specific group of cuticular chemicals, namely branched alkanes, could have evolved to act as social recognition cues in both insects and spiders. *Key words*: chemical communication, cooperation, Eresidae, evolution of sociality, kin discrimination. [*Behav Ecol* 22:1187–1194 (2011)]

## INTRODUCTION

Social groups share valuable resources that can be exploited both from the inside by selfish individuals and from the outside by competitors and parasites. To face the latter, recognition systems enabling discrimination of group members from strangers have evolved and been exhaustively documented in many social animals (Fletcher and Michener 1987; Vander Meer and Morel 1998; Lenoir et al. 1999; Liebert and Starks 2004). Insect societies, for instance, show complex recognition abilities based on the perception of the variation encoded in multicomponent chemical cues. Individuals belonging to the same colony share a common colony odor, which usually consists of a specific blend of cuticular hydrocarbons whose proportions vary among colonies. Discrimination occurs by comparing the chemical profile of an encountered individual with the own colony odor, with dissimilarity leading to rejection. The neural substrate responsible of information coding has yet to be identified, although there is some evidence that the process might be decentralized, that is, not necessarily involving the higher brain centers (van Zweden and d’Ettorre 2010). Discrimination between nest mates and nonnest mates

allows social insects to share the benefits of cooperation only with colony members, which are usually closely related (Howard and Blomquist 2005; d’Ettorre and Lenoir 2010). Within groups, individuals that exploit a common resource may be prone to cheating through the “tragedy of the commons” (Hardin 1968), which results in a reduction in group fitness (Rankin et al. 2007). This social dilemma can be resolved by directing cooperation toward close kin because selfish acts toward kin are costly owing to the loss of inclusive fitness that could otherwise be realized through the propagation of shared genes by related group members (Hamilton 1964). Cooperation among relatives can evolve through the ability to discriminate group members based on genetic relatedness (Hamilton 1987).

Social animals that show preferential cooperation with kin are ideal for studying the underlying mechanisms of kin recognition, and the social spiders provide a good example. In contrast to the majority of spiders that are solitary hunters intolerant of both hetero- and conspecifics, a few genera of spiders have evolved cooperative group living characterized by tolerance and interattraction (Kullmann 1972). These traits require the ability to recognize, directly or indirectly, individuals with whom to cooperate. Social spiders cooperate in feeding, prey capture, and brood care but seem to lack reproductive division of labor and overlapping generations (Lubin and Bilde 2007), which are 2 key features characterizing eusocial societies. Spider sociality evolved by the subsocial pathway, namely via extended maternal care and a prolonged

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coexistence of spiderlings in the maternal nest where the young cooperate in nest activities, such as foraging, before dispersal (Avilés 1997; Agnarsson et al. 2006).

Empirical evidence shows that subsocial spiders benefit from cooperation among kin, and there is good evidence for kin discrimination abilities. Within the genus *Stegodyphus*, Ruch et al. (2009) found that subsocial *S. tentoriicola* young extracted more mass from prey when feeding in groups of siblings compared with nonsiblings, and Schneider and Bilde (2008) found that subsocial *Stegodyphus lineatus* young foraging in sibling-groups showed higher feeding efficiency and higher individual weight gain compared with both familiar and unfamiliar nonsib groups. The use of cross-fostering in the latter study suggests that kin-mediated benefits of cooperation resulted from genetic similarity within the group and hence true kin recognition, rather than through association with familiar individuals. Indeed, kin recognition has been shown in the permanently social spiders *Diaea ergandros* (Evans 1999) and *Delena cancerides* (Beavis et al. 2007), as well as in the subsocial *S. lineatus* (Bilde and Lubin 2001) in experiments where juveniles, when starved, showed higher levels of cannibalism toward nonsiblings than toward siblings.

The recognition systems and cues that spiders use for kin discrimination have not previously, to our knowledge, been investigated. However, Pasquet et al. (1997) found that social *Anelosimus eximius* showed quantitative differences in cuticular chemical profiles between colonies, suggesting that cuticular chemical composition in social spiders may contain some information of colony identity, despite a lack of obvious discrimination against noncolony members in this species. Furthermore, a number of studies have suggested that important traits in social spider evolution, namely tolerance and interattraction of group members, may be based on cuticular chemical cues, similar to the social insects. Kullmann (1972) found that chemosensory perception alone allows social *Stegodyphus* species to discriminate between prey and conspecifics and suggested that tolerance is based on chemical cues. Similarly, cuticular chemicals are thought to act as cues in regulating agonistic versus tolerant behavior in *Tegenaria atrica* (Trabalon et al. 1996, 1998; Pourie and Trabalon 1999; Pourie et al. 2005). Young *T. atrica* stay for a while in the maternal nest and are tolerated by their mother until they disperse. The composition of cuticular compounds in these spiders changes during development (Trabalon et al. 1996), and cuticular extracts from postdispersal solitary young induce higher levels of aggression in adult females compared with cuticular extracts from predispersal gregarious young (Pourie et al. 2005). Hence, evolutionary changes shaping the cuticular compound composition according to age could be important steps in the evolution of spider sociality, prolonging nonaggressive life stages by allowing recognition of individuals that belong to the maternal nest (Pourie and Trabalon 2001).

In the present study, we investigated the possible role of cuticular hydrocarbons in communication and kin recognition in the spider *S. lineatus* that shows adaptive kin discrimination abilities (Bilde and Lubin 2001; Schneider and Bilde 2008). Our aim was to address the following questions: 1) Can cuticular hydrocarbons act as family-specific recognition cues in cooperative predispersal spiderlings? 2) Do the cuticular hydrocarbon profiles of spiderlings change during development, indicating that these chemical compounds could act as cues in regulating tolerance levels? 3) Are cuticular chemical profiles influenced by rearing conditions? Potential cues for true kin recognition should be determined mostly by genetic factors, independently of environmental factors, such as rearing conditions. To address these questions, we compared the cuticular hydrocarbon profiles of same-aged pre-

dispersal spiderlings from family groups of siblings and tested whether families could be differentiated based on variation in chemical profiles. In behavioral assays, we tested whether spiderlings could discriminate between cuticular extracts of siblings and nonsiblings and thus use cuticular chemicals as recognition cues. We also characterized and compared chemical profiles in 10-day interval age groups through the first 50 days of development, covering the predispersal and dispersal stages of spiderlings. Finally, we tested whether spiderlings bred in the laboratory showed cuticular chemical patterns that differed from those of field-bred spiderlings to investigate environmental influence on cuticular hydrocarbon composition.

## MATERIALS AND METHODS

### Study organism

The genus *Stegodyphus* (Eresidae) includes 18 subsocial and 3 social spider species (Kraus and Kraus 1988). Phylogenetic evidence indicates that the social species represent 3 independent events of sociogenesis, making *Stegodyphus* a prime genus for the study of evolution of spider sociality (Kraus and Kraus 1988; Johannesen et al. 2007). The subsocial *S. lineatus* is thought to resemble a transitional stage between solitary and social spiders because it shows considerable behavioral plasticity in social tendencies. This species is able to extend the period of tolerance and cooperation under circumstances of unlimited food availability or prevention from dispersal (Schneider 1995).

*Stegodyphus lineatus* inhabit arid environments in Southern Europe, Northern Africa, the Mediterranean islands, and across to Kazakhstan and West Asia (Kraus and Kraus 1988). In Israel, *S. lineatus* spiders are found in dry river beds where they build capture webs and tubular retreats in low shrubs. Males start maturing in March, females in April or May, and the mating season and egg-laying period may extend into June (Bilde et al. 2005). Eggs are laid approximately 4 weeks after mating, and clutches of 40–140 eggs hatch after another 4 weeks. The mother spider feeds her offspring by regurgitation, and after about 2 weeks of maternal care, spiderlings consume their mother (matrphagy). Hereafter follows a period of group living and cooperation in the natal nest for approximately 4 weeks until juvenile dispersal is gradually initiated (Kullmann 1972).

### Collection and housing

In April and June 2008, mated *S. lineatus* females were collected in Israel from 2 different populations located approximately 70 km apart: near Lehavim (lat 31° 21.800' N, long 34° 50.000' E) and near Kfar Adumim (lat 31° 49.000' N, long 35° 21.200' E), which is a settlement located within the occupied Palestinian territories, and young from these females were used for behavioral assays.

In March 2009, juvenile and subadult *S. lineatus* of both genders were collected in 2 different populations located about 30 km apart, near the villages Lehavim and Har Amasa (lat 31°20.500' N, long 35°07.100' E). When sexually mature, females were mated to same population males in the laboratory by placing a male with a female for 2 days, assuming mating would take place (Schneider and Lubin 1996). The females produced young that we henceforth refer to as 'laboratory-bred' young used for cuticular chemical analyses.

In June 2009, females with brood (eggs or hatched young) were collected in Lehavim. The offspring of these females are henceforth referred to as 'field-bred' young and these were used for both chemical analyses and behavioral assays. (For

a schematic overview of time and place of collections and allocation of spiders for experiments, see Supplementary Material.)

Each collected spider was kept in a plastic box (size in mm: 80 × 60 × 50) with a mesh on the side and provided with its original tubular silk retreat. Boxes allowed for construction of small prey capture webs. Spiders were kept at room temperature (25 ± 5° C) with a natural day/night light cycle, and water was sprayed in the boxes twice a week. Spiders were fed nymphs of small migratory locusts (*Locusta migratoria*) and crickets (*Acheta domestica*) 3 times a week until spiderlings emerged. After matrophagy (2 to 3 weeks after hatching), spiderlings were fed fruit flies (*Drosophila melanogaster*) twice a week.

### Chemical analysis

To examine whether cuticular chemical composition in *S. lineatus* spiderlings varies sufficiently among genetic families to contain potential cues for kin discrimination, we quantified variation in cuticular hydrocarbon profiles among sibling groups of same-aged spiderlings. Furthermore, we analyzed the chemical profile of individual spiderlings at different ages in order to characterize possible developmental changes in compound composition at a temporal scale.

For the analysis of between-family variation of the chemical profile, 6 field-bred spiderlings of the same age (30 ± 2 days) from each of 6 different families collected in 2009 were individually sampled (36 individuals in total). At this age, matrophagy had usually occurred, and spiderlings were relatively mobile yet still in their cooperative stage before dispersal. A family refers to a group of siblings. In order to analyze developmental changes in the chemical profile, 2 or 3 laboratory-bred spiderlings from each of 5 different families were sampled at intervals of 10 days, starting at the age of 10 days and ending when a nest was depleted or at age 50 d at which age young have usually dispersed (a total of 49 individuals). All same-aged spiderlings were pooled into each age group in the subsequent statistical analysis. This sampling method ensured representation of chemical profiles from 5 discrete age groups covering the full social cooperative period and the stage of dispersal.

All sampled spiderlings were freeze killed by placing them in a -18 °C freezer for ≥3 h. Spiders were then immersed individually in clean glass vials (Supelco) containing 100 µl of pentane (HPLC grade; Sigma-Aldrich, Chromasolv) for 5 min. The animal was removed, the solvent was allowed to evaporate under a fume hood at room temperature, and the total cuticular extract from each animal was stored at -18 °C until chemical analysis. For analysis, the extract was redissolved in 10 µl of pentane, and 4 µl of this mixture was injected into an Agilent Technologies 6890N gas chromatograph (GC, capillary column: HP5MS 30 m × 250 µm × 0.25 µm; split-splitless injector; carrying gas: helium at 1 ml/min). The initial temperature was 70 °C and was increased at a rate of 30 °C/min to 230 °C, then to 300 °C at 4 °C/min, then to 320 °C at 10 °C/min, and held for 6 min. The GC was coupled to a 5975 Agilent Technologies Mass Spectrometer (MS, 70 eV electron impact ionization). Chemical compounds were identified on the basis of their retention time (compared with standards) and by inspecting diagnostic ions in their mass spectra.

### Behavioral assay

*Stegodyphus lineatus* spiderlings show no aggression or other obvious discriminatory behavior toward nonsiblings in their gregarious phase. Accordingly, choice tests rather than aggression tests were conducted in order to examine whether cuticular chemicals can be used as kin recognition cues by

*S. lineatus* spiderlings. We recorded where spiderlings settled when they were placed individually in a T-like maze and presented with cuticular chemical extracts from siblings and non-siblings. We expected spiderlings to be more attracted to siblings (i.e., sibling chemical extract) than to unrelated conspecifics due to the documented kin-mediated fitness benefits in this species (Schneider and Bilde 2008).

Mazes were constructed from three 20-ml plastic vials (40 × 27 mm each) glued together constituting a middle compartment with access to 2 choice compartments through holes drilled in the plastic (see Supplementary Material for a diagram of the maze). In each choice compartment of a clean maze, we placed a clean pentane-washed cotton ball (diameter 6–8 mm) to which we applied the cuticular chemical extract of 4 siblings to one side and 4 nonsiblings to the opposite side. Chemical extracts were made by washing 4 30 ± 2 days old spiderlings in 200 µl of pentane for 5 min.

After application of chemical extracts, the solvent was allowed to evaporate for ≥1 h, allowing only the cuticular compounds to remain on the cotton balls. Hereafter, one focal spiderling was placed in the middle compartment and left for one night (from 17:30 ± 1 h to the next morning at 10:00 ± 1 h) after which settlement choice was recorded. Most spiders settled directly on or in close proximity of a cotton ball. Focal spiders were tested only once, at the age of 30 ± 2 d. Similarly, cotton balls containing chemical extracts were used only once. Mazes were washed in water and odorless detergent before use to eliminate chemical cues from previous trials.

In October 2008, 32 spiderlings were tested in the laboratory with a natural day/night light cycle. Nonsibling extracts originated from populations different from the focal individual and were placed randomly in the right or left compartment in each maze. Seven spiderlings did not make a choice (i.e., they were still residing in the middle compartment in the morning) and were excluded from further analysis. In July and August 2009, 29 spiderlings were tested using the same apparatus but in a dark room to eliminate any other possible cues, and nonsibling extracts, originating from the same population as the focal individual, were placed to the right in half of the trials and to the left in the other half. All 29 spiderlings made a choice in the tests performed in 2009.

### Statistical analysis

The 2 data sets on behavioral assays were compared with Fisher's exact test. Subsequently, we pooled the data sets and analyzed for effect of kinship on settling choice using the binomial test with the null hypothesis of equal preference for sibling and nonsibling cuticular extract.

In order to analyze the chemical profile of spiderlings, 38 regularly occurring gas chromatography–mass spectrometry peaks, representing identified hydrocarbons, were integrated using Agilent Technologies ChemStation software. The normalized peak areas within each profile were calculated according to Aitchinson (1986) using the formula:

$$Z_{ij} = \ln \left[ \frac{Y_{ij}}{g(Y_j)} \right],$$

where  $Z_{ij}$  is the transformed area of peak  $i$  for individual  $j$ ;  $Y_{ij}$  is the area of peak  $i$  for individual  $j$ ; and  $g(Y_j)$  is the geometric mean of the areas of all peaks for individual  $j$ .

These normalized peak areas were used as variables in principal component analyses (PCAs). The reduced number of variables (principal components [PCs]) were used in subsequent discriminant analyses (DAs), performed by STATISTICA 7.1 (StatSoft Inc.). For a more conservative test of chemical differences between family groups, we performed a permutation test using

the MASS package (Venables and Ripley 2002) in R (<http://www.R-project.org>) according to Nehring et al. (2010). After reducing the number of variables to 8 PCs, we randomly allocated the individual profiles to 6 arbitrary groups in a DA 10 000 times. If the success rate (i.e., the percentage of correctly assigned samples to their group) from the original DA was higher than the upper 95% confidence interval from the permutation test, the family groups had significantly different chemical profiles.

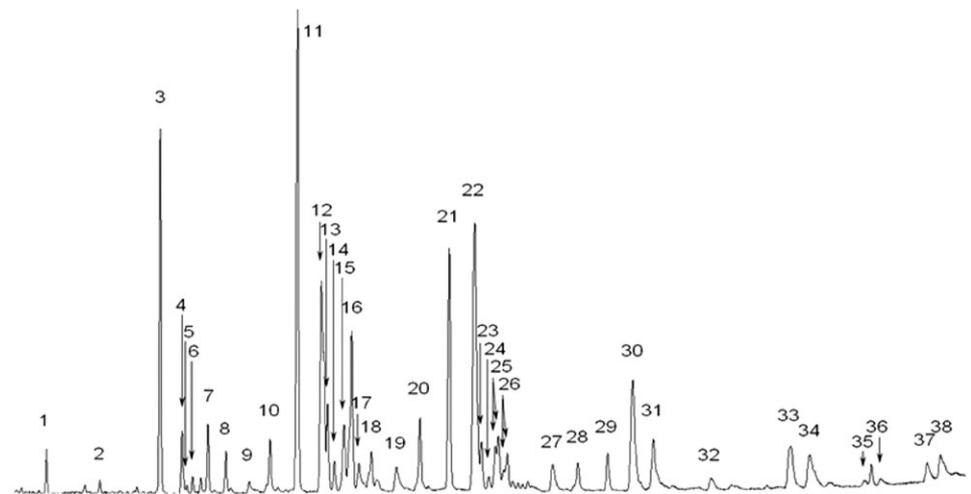
Chemical compounds that vary more between groups of individuals than within groups can carry information about group identity and hence may represent potential recognition cues. In order to identify the best candidates for kin recognition cues, the diagnostic power (DP) of each GC-MS peak was calculated in accordance with van Zweden et al. (2009). Peaks with higher-than-average DP were considered as "high DP" compounds.

As a measure for chemical distance, squared Mahalanobis distances (SMDs) between age groups were calculated to determine at which developmental stage (i.e., age interval), the largest change in cuticular compound composition occurred. The SMD measures normalized distances between points in a multivariate space; it differs from the simple Euclidean dis-

tance by taking into account the covariance among the variables in calculating distances and by being independent of the scale of measurements. A mixed-model analysis of variance (ANOVA), followed by a post hoc Tukey Honestly Significant Difference (HSD) test, was used to compare chemical changes at different developmental stages, with age interval (e.g., 10–20 days or 20–30 days) as a fixed factor and family as a random factor. Chemical distances between age groups (i.e., the chemical changes for each age interval) were calculated as SMDs from each individual sample to the centroid of the following age group (10 days older) in order to determine which 10-day age interval was associated with the largest change in cuticular chemical composition. For instance, the SMD from age 20–30 days was calculated as the mean of the SMDs from each of the samples from age group 20 days to the centroid of age group 30 days.

## RESULTS

The cuticular chemical profile of *S. lineatus* was characterized by 38 regularly occurring peaks, which could be identified consistently as hydrocarbons. These hydrocarbons belonged to 3 different classes: linear alkanes and mono- and di-methyl-branched



Peak no.	Peak ID	DP	Peak no.	Peak ID	DP
1	<i>n</i> -C23	1.88	20	4-MeC28	1.21
2	<i>n</i> -C24	1.49	21	<i>n</i> -C29	1.07
3	<i>n</i> -C25	1.63	22	15-, 13-, 11-, 9-MeC29	1.89
4	13-, 11-, 9-MeC25	3.19	23	7-MeC29	1.29
5	7-MeC25	1.58	24	5-Me29	1.21
6	5-MeC25	1.78	25	13.17-, 11.15-, 9.19-, 9.15-, 9.13-diMeC29	2.20
7	3-MeC25	1.70	26	7.15-, 7.11-diMeC29 + 3-MeC29	2.15
8	<i>n</i> -C26	1.12	27	13-, 12-, 11-, 10-MeC30	2.49
9	11-, 10-, 9-MeC26	1.36	28	4-MeC30	2.49
10	4-MeC26	1.16	29	<i>n</i> -C31	1.30
11	<i>n</i> -C27	1.30	30	15-, 13-, 11-, 9-, 7-MeC31	2.60
12	13-, 11-, 9-MeC27	2.78	31	13.17-, 11.15-, 9.13-diMeC31	3.16
13	7-MeC27	2.28	32	16-, 13-, 12-, 11-, 10-MeC32	1.22
14	5-MeC27	2.74	33	17-, 15-, 13-, 11-MeC33	1.62
15	11.15-, 9.13-diMeC27	3.78	34	15.17-, 13.17-, 11.15-, 9.y-diMeC33	1.92
16	7.11-diMeC27 + 3-MeC27	4.08	35	14-, 13-, 12-, 11-, 10-MeC34	1.06
17	Unknown	1.07	36	Unknown	1.06
18	<i>n</i> -C28	1.09	37	17-, 15-, 13-, 11-MeC35	1.22
19	14-, 13-, 12-, 11-, 10-, 9-MeC28	1.66	38	15.19-, 13.17-, 11.15-diMeC35	2.62

**Figure 1**

A gas chromatogram showing the cuticular hydrocarbon profile of a 30-day-old *Stegodyphus lineatus* spiderling. The panel shows peak identification and the variation in chemical profiles between family groups expressed as the DP of each compound.

alkanes with chain lengths ranging between  $n\text{-C}_{23}$  and  $n\text{-C}_{35}$  (Figure 1). Unlike studies of the cuticular chemical profile of solitary *T. atrica* (Trabalon et al. 1996; Pourie et al. 2005), we did not find any fatty acids on the cuticle of *S. lineatus* young.

### Between-family variation of the chemical profile and behavioral assays

Field-bred spiderlings showed family-specific cuticular hydrocarbon profiles (Figure 2). Chemical variation among families was due to quantitative differences between chemical profiles, that is, differences in relative abundances of the same set of compounds. A PCA produced 8 PCs with eigenvalues greater than 1, accounting for 89.0% of the total variance. A DA on the 8 PCs significantly differentiated the 6 families (Wilks'  $\lambda = 0.0000009$ ;  $F_{40,103} = 60.4$ ;  $P < 0.00001$ ) and 100% of the samples were correctly assigned to their family (i.e., the success rate was 100%). The highest success rate from the permutation test with random groups was 80.6% and the mean 52.2%. The upper 95% confidence interval was a success rate of 63.9%, confirming the statistical significance of our 100% success rate from the original DA using the real family groups.

The DP of the different compounds ranged from 1.06 to 4.08, with a  $DP_{\text{average}} = 1.88$ . All 16 peaks with high DP values represented branched alkanes (with the exception of  $n\text{-C}_{23}$ ), and all the 7 di-methyl alkanes present in the chemical profile had high DP values. (A list of all compounds ranked according to their DP is available as Supplementary Data.) The peak with the highest DP was peak number 16 (Figure 1): a mixture of 7,11-diMeC<sub>27</sub> and 3-MeC<sub>27</sub> ( $DP_{\text{peak 16}} = 4.08$ ).

Families could be significantly separated based on individual cuticular hydrocarbon composition also for laboratory-bred spiderlings. A DA significantly separated the families, correctly assigning 100% of the samples to their family, even though individuals of different ages were included for each family (Wilks'  $\lambda = 0.00452$ ;  $F_{28,138} = 17.2$ ;  $P < 0.00001$ ). The DP of compounds were generally low, ranging from 0.15 to 0.39, with a  $DP_{\text{average}} = 0.23$ . However, the peak with the highest DP was again peak number 16: 7,11-diMeC<sub>27</sub> and 3-MeC<sub>27</sub> ( $DP_{\text{peak 16}} = 0.39$ ), suggesting that these compounds in par-

ticular may contain information of family identity, independent of age and rearing conditions of spiderlings.

The behavioral results indicated that spiderlings were able to use the between-family variation in chemical profiles as cues for discrimination between siblings and nonsiblings. There was no difference between the 2 data sets from different years with respect to settling choice (Fisher's exact test,  $P = 0.395$ , degrees of freedom [df] = 1); therefore we pooled the data. We found a significant effect of kinship on choice in the mazes as spiderlings preferred to reside near sibling cuticular extract compared with nonsibling extract (binomial test;  $P = 0.0402$ , df = 1,  $N = 54$ , Figure 3).

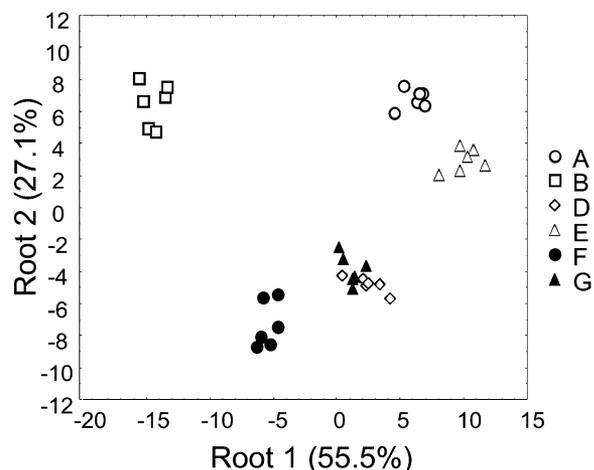
### Developmental changes of the chemical profile

The composition (i.e., relative abundances) of hydrocarbons in the cuticular profile of spiderlings changed significantly with age (see Supplementary Material for exact changes in relative abundance for each compound according to age). A PCA was conducted on 6–14 laboratory-bred individuals from each of 5 age groups, from age 10 to 50 days (a total of 49 individuals from 5 different families). The PCA produced 7 PCs with eigenvalues higher than 1, which together explained 87.9% of the total variance. A subsequent DA significantly separated the age groups (Wilks'  $\lambda = 0.0183$ ;  $F_{28,138} = 10.1$ ;  $P < 0.00001$ ) and correctly assigned 83.7% of the samples to their age group (see Supplementary Material for a graph of the DA).

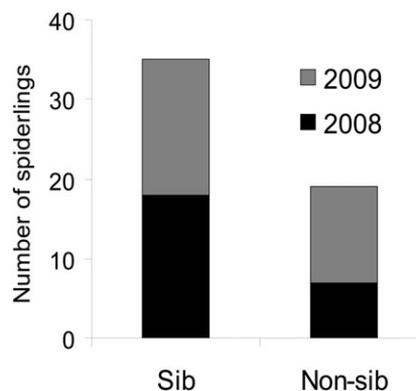
Chemical distances between adjacent age groups, calculated as Mahalanobis distances, differed significantly among age intervals (mixed-model ANOVA; age interval [fixed effect]:  $F = 38.03$ ,  $P < 0.0001$ , df = 3; family [random effect]:  $Z = 0.26$ ,  $P = 0.397$ ,  $N = 4$ ). The chemical distance increased substantially in age interval 40–50 days (see Supplementary Material for a graph showing the chemical distances between age groups). This age interval, 40–50 days, differed significantly from each of the other 3 age intervals (Tukey HSD;  $P < 0.0001$  for each of the 3 comparisons), whereas the 3 early age intervals (10–20, 20–30, and 30–40 days) did not differ significantly from each other ( $P > 0.05$  for each of the 3 comparisons).

### Influence of rearing conditions on the chemical profile

Spiderlings bred in the laboratory could be significantly separated from those bred in the field based on their cuticular hydrocarbon profiles (see Supplementary Material for a graph of the PCA). Chemical data from 9 laboratory-bred 30-day



**Figure 2** Between-family variation of the chemical profiles: a plot of the first 2 roots of the DA based on cuticular hydrocarbons from 30-day-old field-bred spiderlings (36 individual chemical profiles in total). Each symbol represents a family, that is, a group of siblings from the Lehavim population. The percentage of the variance explained by each root is given in parenthesis.



**Figure 3** Behavioral assays: results from choice tests performed in 2008 (black) and 2009 (gray), showing the number of spiderlings residing by cuticular chemical extracts of siblings and nonsiblings.

spiderlings from 3 different families (available from the “developmental chemical changes” assay) and 36 field-bred 30-day spiderlings from 6 different families (from the “between-family chemical variation” assay) were used for this analysis. A PCA produced 7 PCs with eigenvalues higher than 1 that together explained 87.5% of the total variance. The main function (PC 1; explaining 44.1% of the variance) clearly separated the 2 groups. A subsequent DA significantly separated the laboratory and field groups (Wilks'  $\lambda = 0.0240$ ;  $F_{7,37} = 215$ ;  $P < 0.00001$ ), assigning 100% correctly to their group.

Twenty peaks had high factor loadings on PC 1 (factor loading  $>|0.70|$ ) and these were all linear or mono-methyl alkanes. The compounds with relatively high abundance in field-bred young were all shorter hydrocarbons (chain lengths between 24 and 27 carbon atoms), whereas the compounds more abundant on the laboratory-bred spiders were longer hydrocarbons (chain lengths between 28 and 35 carbon atoms). Only 5 of the 20 peaks that were important in separating field- and laboratory-bred young were peaks that had high DP in separating families (see between-family chemical variation results). All di-methyl alkanes (including peak number 16) had low factor loadings on PC 1 (all factor loadings  $<|0.52|$ ) in the analysis on rearing conditions. A list of all compounds ranked according to their factor loadings is available in the Supplementary Material.

## DISCUSSION

Our study shows that young *S. lineatus* have complex cuticular chemical profiles consisting mainly of long-chain hydrocarbons, namely linear and branched alkanes with 1 or 2 methyl groups. We found that the cuticular chemical profiles of pre-dispersal spiderlings varied significantly among sibling groups, regardless of age and rearing conditions. This suggests that cuticular hydrocarbon profiles carry information about family identity and thus can potentially be used as cues in kin recognition. Our behavioral results support this hypothesis, indicating that spiderlings can discriminate between kin and nonkin solely by means of cuticular hydrocarbon cues. We also show that the cuticular hydrocarbon profiles of *S. lineatus* spiderlings change significantly at the age of dispersal, which supports the hypothesis that cuticular chemicals may act as cues regulating the level of tolerance toward siblings in the nest (Trabalon et al. 1996; Pourie and Trabalon 2001). Furthermore, we show that spiderlings bred under different conditions express significantly different hydrocarbon profiles, yet the potential kin recognition cues identified by our analysis (a subset of branched alkanes) appear to be less affected by environmental conditions, indicating that they might be determined mostly by genetic factors. Thus, cuticular hydrocarbon cues might have the potential to be used for genetic kin recognition.

Group living and cooperation are associated with certain costs, including having to share limited resources. In *S. lineatus*, costs of feeding in groups involve decreased individual growth rates due to competition for prey (Schneider 1995). However, cooperating with genetic kin increases feeding efficiency and growth rates, thereby decreasing costs of cooperation (Schneider and Bilde 2008). Direct benefits of cooperating with kin exerts a selective pressure on the ability to distinguish between family and nonrelatives, and kin recognition has indeed been shown in *S. lineatus* in cannibalism experiments (Bilde and Lubin 2001). Our study shows that spiderlings have family-specific cuticular hydrocarbon profiles, that is, profiles specific enough for them to potentially be used in recognition of siblings. Indeed, when given the choice of cuticular chemical cues from siblings versus non-siblings, spiderlings preferred to reside by sibling cues.

Hence, our study indicates that the cues *S. lineatus* uses for kin recognition may be cuticular hydrocarbons. True kin recognition based on cuticular hydrocarbons has previously been found in cockroaches (*Blattella germanica*) that discriminate between sibling and nonsibling, independent of previous social experience (Lihoreau and Rivault 2009), and chemical cues may be involved in genetic kin recognition in the primitively social sweat bees (*Lassioglossum zephyrum*) that accept unfamiliar bees in their nest at an increasing rate with increasing genetic similarity (Greenberg 1979).

Arthropods have a large range of different cuticular hydrocarbons present on their cuticle. The original purpose of these hydrocarbons was to protect the animal from desiccation, but a subset of these compounds have evolved to act as recognition cues in inter- and intraspecific communication in different arthropods (Gibbs et al. 1991; Lahav et al. 1999; Akino et al. 2004; Dani et al. 2005; d'Etorre and Moore 2008). Because cuticular hydrocarbons have been shown to play a role in communication in both social and non-social insects, such as fruit flies (Ferveur 2005), as well as in some spiders (Pourie et al. 2005), the communication function of these chemicals may be quite old. Nest mates recognition in social insects based on cuticular hydrocarbons often relies on branched alkanes and alkenes as recognition cues, whereas linear alkanes have little influence on nest mate recognition (Dani et al. 2001, 2005; van Zweden and Dreier 2009). Interestingly, our results indicate that this might be the case also in subsocial spiders. We found that all di-methyl-branched alkanes present on the cuticle of spiderlings had high DP in separating families, as did several mono-methyl alkanes. Almost all linear alkanes had low DP and therefore contained little information of family identity. Although we do not have any direct experimental evidence that branched alkanes play a key role in communication, our data indicate that linear alkanes might be less important in social recognition in *S. lineatus*, similarly to what has been found in many social insects where alkenes and branched alkanes, rather than linear alkanes, are important recognition cues.

Spiders communicate with chemicals during many different activities in life, such as identification of prey, brood (Pourie et al. 2005), and sexual partners (Schulz and Toft 1993), and possibly chemical cues play a role in behavioral transitions like the one from gregarious to solitary living (Pourie and Trabalon 2001). Our study shows that the cuticular hydrocarbon profile of *S. lineatus* changes significantly during the first 50 days of development. The relative proportions of longer alkanes increased with age. Similarly, some ants produce heavier (i.e., longer) hydrocarbons during specific seasons (e.g., *Camponotus aethiops* in van Zweden and Dreier (2009)). Heavier compounds are less volatile and have higher melting points and are therefore produced more by some insects in seasons or geographical areas that are dry and hot, in order to provide better protection from desiccation (Gibbs et al. 1991). Thus, the increase with age in the abundance of longer hydrocarbons in *S. lineatus* could occur mainly to prepare the cuticle for the risky dispersal stage, at which the spiders may be exposed to different environmental conditions in their arid habitat than those they have experienced in the maternal nest.

Nevertheless, some of these long-chained compounds may be involved in triggering the onset of developmentally specific behaviors, such as matrophagy or dispersal, by acting as cues for the regulation of tolerance levels and interattraction. Indeed, we found a dramatic change in spiderlings' cuticular compound composition between age 40 and 50 days, where dispersal often occurs. Hence, it is possible that developmental changes in cuticular compound composition play a role in decreased acceptance and tolerance of nest mates, leading to

dispersal of young, similar to in *T. atrica* (Trabalon et al. 1996). Evolutionary modifications of developmental changes in chemical composition like these could have been important in extending the cooperative stage of tolerance toward siblings from temporary, in subsocial spiders, to permanent in social spider species.

Both genetic and environmental factors can contribute to the composition of the cuticular chemical profile of arthropods, and diet, especially, can influence the ratios of compounds in a chemical blend (Jutsum et al. 1979; Liang and Silverman 2000; van Zweden et al. 2009). Thus, family-specific cuticular hydrocarbon profiles may exist either simply because siblings are exposed to a similar environment, because genetic similarities between siblings are responsible for the cuticular compound composition, or because of influence by both factors.

We examined the cuticular hydrocarbon profiles of laboratory-bred spiderlings whose mothers had been mated in the laboratory and of spiderlings from broods collected in the field. During the first stages of life, young spiders are fed by maternal regurgitation. The liquid food that the young from the field ingested likely originated from a broad source of prey items caught by the mother. In contrast, the mothers that were kept in the laboratory (for a minimum of 2 months longer than the freshly collected ones) had a uniform diet that could have influenced the diet of their young. This differential diet, together with other environmental factors, could be the cause of the clear distinction between the cuticular profiles of field- and laboratory-bred spiderlings. Field-bred spiderlings had higher relative proportions of short linear and mono-methyl-branched alkanes. The di-methyl alkanes that were important in chemical separation of families, however, had very low power in separating spiderlings bred under the 2 different conditions (some branched alkanes have been shown to be heritably synthesized in ants, see van Zweden et al. 2010). This could indicate that the relative amounts of the potential kin recognition cues in the chemical profiles are little influenced by the environment and may therefore be determined more by genetic factors.

In conclusion, our results show that subsocial *S. lineatus* young have complex cuticular hydrocarbon profiles that are family specific throughout the social stage and change during development especially at the age of dispersal. Cuticular chemical profiles can thus carry information of family identity and developmental stage of spiderlings. Our behavioral results indicate that spiderlings are able to use the information encoded in the chemical profiles to recognize siblings. Bioassays accounting for genetic relatedness and familiarity are now needed to investigate what our present results suggest; that is, kin discrimination in *S. lineatus* as reported by Bilde and Lubin (2001) and Schneider and Bilde (2008) may depend on cuticular hydrocarbons as cues.

## SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.beheco.oxfordjournals.org/>.

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