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Evolution of Caste-Specific Chemical Profiles in Halictid Bees

Iris Steitz[1] · Callum Kingwell[2,3] · Robert J. Paxton[4,5] · Manfred Ayasse[1]

Abstract

Chemical communication is crucial for the maintenance of colony organization in eusocial insects and chemical signals are known to mediate important aspects of their social life, including the regulation of reproduction. Sociality is therefore hypothesized to be accompanied by an increase in the complexity of chemical communication. However, little is known about the evolution of odor signals at the transition from solitary living to eusociality. Halictid bees are especially suitable models to study this question as they exhibit considerable variability in social behavior. Here we investigated whether the dissimilarities in cuticle chemical signals in females of different castes and life stages reflect the level of social complexity across halictid bee species. Our hypothesis was that species with a higher social behavior ergo obligate eusocial species possess a more distinct chemical profile between castes or female life stages. We analyzed cuticular chemical profiles of foundresses, breeding females and workers of ancestrally solitary species, facultative and obligate eusocial halictid species. We also tested whether social complexity was associated with a higher investment in chemical signals. Our results revealed higher chemical dissimilarity between castes in obligate than in facultative eusocial species, especially regarding macrocyclic lactones, which were the single common compound class overproduced in queens compared with workers. Chemical dissimilarities were independent of differences in ovarian status in obligate eusocial species but were dependent on ovarian status in facultative eusocial species, which we discuss in an evolutionary framework.

Keywords Sweat bees · Communication · Social organization · Eusociality · Sociobiology · Chemical distance · Fertility signals · Macrocyclic lactones

Introduction

The origin and development of complex social societies, as seen in ants, bees, termites and wasps, has become a central topic of evolutionary research. An increase in social

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complexity is hypothesized to require more diverse and complex communication systems to mediate interactions among group members (Leonhardt et al. 2016). Indeed, key factors for the success of eusocial insects, the most complex form of animal society (Kocher and Paxton 2014), are well developed communication systems, and chemical signaling is the most prominent communication mode (Leonhardt et al. 2016). Solitary insects are thought to use chemical signals primarily for mate and species recognition (Wyatt 2014), whereas chemical profiles of eusocial species encode several more signals, e.g., colony membership, caste recognition, or the regulation and coordination of colonial living including nest defense, brood care, and reproduction division of labor via fertility signals of queens or egg-laying workers (Blum 1996; Howard and Blomquist 2005; Kocher and Grozinger 2011). Therefore, the evolution of social complexity is accompanied by a need for an increase in the complexity of chemical communication. An increased complexity is not necessarily correlated with an increased complexity of the chemical composition of the signals, but may rather be correlated with the diversity of chemical signals themselves (Kather and Martin 2015; Leonhardt et al. 2016). However, most studies dealing

with the evolution of chemical communication systems focus on highly eusocial insects and only little is known about the evolution of odor signals at the transition from solitary living to eusociality, the point at which one might expect greater differences in communication than among extant social species differing in their degree of social complexity.

One hallmark of eusocial insect colonies is the maintenance of a reproductive hierarchy via chemicals produced by fertile females in which queens monopolize egg laying and workers forego their own reproduction to focus on tasks such as brood care, foraging, nest maintenance, and defense, a process that has been thoroughly studied throughout the last few decades (Le Conte and Hefetz 2008; Wilson 1971). However, discussions continue as to whether queen chemicals function as 'real queen pheromones' with a primer effect enforcing worker sterility or whether they are 'honest' fertility signals of the queen, inducing self-regulation in workers (Keller and Nonacs 1993; Le Conte and Hefetz 2008). Regardless of the mechanisms underlying the actions of queen fertility signals on workers, the evolution of those chemicals that signal reproductive status remains mostly unexamined. The most plausible hypothesis is that these fertility signals evolved from by-products of ovary development (reviewed in: Oi et al. 2015). The hypothesis is primarily based on the finding that ovary development is accompanied by characteristic changes in cuticular lipids including cuticular hydrocarbons (CHCs) in both solitary (e.g., housefly, Blomquist and Bagnères 2010) and social insects. Several studies dealing with highly eusocial species have suggested that related CHCs act as a conserved class of queen pheromone, as most of the species chemically analyzed to date have revealed caste-specific differences in chemical profiles mainly attributable to an increase in the amount of CHCs in fertile compared with non-fertile females (Oi et al. 2015; Van Oystaeyen et al. 2014). However, more recent studies have clearly indicated that compounds other than CHCs are also involved in fertility signaling. One of these studies is a comparative analysis of fertility signals in three different *Odontomachus* trap-jaw ants, which employ diverse compound classes in signaling, e.g., the non-hydrocarbons dialkyl-tetrahydrofurans in the species *O. ruginodis* (Smith et al. 2016). In order to shed more light on the evolutionary history of fertility signals and the complexity of communication systems in social insects, investigations dealing with species at the transition from solitary living to eusocial behavior are necessary.

Halictid bees are especially suitable for studying the evolution of chemical communication systems regulating reproduction as they comprise a wide variety of social behaviors that range from solitary to primitively eusocial species (Schwarz et al. 2007). Phylogenetic studies of the family Halictidae have also revealed several independent evolutionary origins of eusociality with various reversals from obligate eusociality back to more flexible modes of eusociality (facultative eusocial species) or even complete solitary behavior

(Brady et al. 2006; Danforth 2002; Gibbs et al. 2012; Wcislo and Danforth 1997). This enables comparable studies with a variety of species covering a large evolutionary framework (Kocher and Paxton 2014).

The cuticular chemical profile of one obligate eusocial halictid species, *Lasioglossum malachurum*, has been well characterized and indicates the occurrence of compounds mainly belonging to the substance classes of n-alkanes, n-alkenes, macrocyclic lactones, isopentenyl esters of unsaturated fatty acids, and ethyl esters (Ayasse et al. 1993, 1999; Soro et al. 2011). Whereas isopentenyl esters of unsaturated fatty acids are potentially used as a female sex pheromone (Ayasse et al. 1999), macrocyclic lactones have been shown to mediate aggressive interactions between nest-founding gynes and are potentially used as dominance signals (Smith and Weller 1989). Moreover, n-alkanes and macrocyclic lactones have been found in larger amounts in breeding queens compared with workers in *L. malachurum* (Ayasse et al. 1993, 1999) and are therefore potentially involved in the communication of fertility and consequently in the regulation of reproduction. In most of the so far investigated halictid bees and also in many other insects the glandular source of the cuticular chemical profiles is unknown and a combination of the secretion from several glands might generate the behaviorally active bouquet on the cuticle surface. Insect pheromones are generally produced by exocrine glands that are modifications of the epidermal cells of the integument; all exocrine glands are associated with the cuticle (Ayasse et al. 2001). In case of the halictid bees, n-alkanes and macrocyclic lactones are produced in large amounts in the Dufour's gland and are thought to contribute to the cuticular chemical profile (Ayasse et al. 1993; Soro et al. 2011).

A recent study comparing obligate eusocial, facultative eusocial, and ancestrally solitary halictid species has revealed that solitary species invest less in communication than do social species; this is mainly expressed as a reduced density of antennal sensilla (Wittwer et al. 2017). Moreover, solitary and social populations of a single, facultative eusocial species could be separated by their chemical profiles indicating a shift in the production of chemical signals (Wittwer et al. 2017). Such a shift may also be caused by a change in chemical signal investment via a higher signal diversity, as social living might demand a more complex chemical communication system. This might be especially true within social halictid nests in their social phase, when breeding queens and workers occur simultaneously in a single nest. Therefore, we hypothesize that a higher social level, ergo a higher social complexity in halictid bees (obligate vs. facultative eusocial species vs. ancestrally solitary species), requires a more distinct chemical profile between castes or female life stages (nest foundresses/ breeding queen).

To test this hypothesis, we analyzed cuticular chemical profiles of foundresses, breeding females, and workers of

ancestrally solitary, facultative eusocial and obligate eusocial halictid species and determined their chemical dissimilarities. We did not only analyze the whole chemical odor bouquet, but also focused on those substance classes, namely n-alkanes and macrocyclic lactones, that in former studies have been shown to be produced in large quantities in queens versus workers and are potentially used as fertility signals. Furthermore, we tested whether social complexity (i.e. obligate eusociality) is also associated with a higher caste-dependent dissimilarity in these potential fertility signals. We did so by analyzing whether greater chemical dissimilarity is linked to a greater difference in ovarian development between castes in obligate versus facultative eusocial species.

Material and Methods

Bee Collection Halictid bees of seven species, their castes, and female life stages (social nest foundresses, social breeding queens, workers, solitary nest foundresses, and solitary breeding females) were collected from natural nests at sites in Germany, Austria, Northern Ireland, Panama, and USA (Table 1). We collected all females at their nest sites by using an insect net or a vacuum suction device as described in Soro et al. (2009). Using this technique, all bees were carefully sucked up when appearing at their nest entrance without having to excavate the nests. The bees were individually placed in small plastic vials (Eppendorf tubes: 1.5 ml) and killed by freezing at −40 °C for further use in chemical analyses.

Chemical Analyses Frozen females of all species were individually rinsed for 15–60 s in n-pentane (Uvasol, 99.5%, Merck, Germany) to extract cuticle surface compounds. Species collected in former years where extracted for 60 s in n-pentane,

however, our following chemical analyses revealed that 15 s are also sufficient to extract the entire cuticle surface profile. As the various species differ in size, the volumes of solvent used ranged from 0.2 ml (*L. pauxillum*) to 2 ml (*M. genalis*). All extracts were concentrated under a gentle stream of nitrogen to 25% of its primarily used volume. For quantitative analysis, 10 μl n-undecane (*M. genalis*, *M. centralis*) or n-octadecane (for all other species, stock solution: 100 μg/ml in n-hexane) were added to each extract as an internal standard.

Chemical analyses were performed on an Agilent 7820 A Series gas chromatograph (Agilent Technologies, Germany) equipped with a non-polar DB-5 MS capillary column (30 m × 0.25 mm inner diameter, J&W) and a flame ionization detector by using hydrogen as a carrier gas (constant flow, 2.0 ml/min). One microliter of each sample was injected splitless into the gas chromatograph (injector temperature: 310 °C), operating at 50 °C for 1 min, after which the split valve was opened, and the temperature was increased continuously by 10 °C/min to a final temperature of 310 °C. The structural elucidation of individual compounds was based on gas chromatography/mass spectrometry (HP 6890 series, Hewlett-Packard, Germany; method as described above for GC, carrier gas: helium) and by comparisons of mass spectra using references from the NIST11 library, a database of the Institute of Evolutionary Ecology and Conservation Genomics and GC retention times with those of authentic reference samples by using AMDIS 2.71 (Automated Mass Spectral Deconvolution and Identification System). The absolute amounts of all substances were determined using Agilent ChemStation Software (Agilent Technologies, Germany) and the internal standard as a reference. In order to estimate relative proportions for further downstream analyses, absolute amounts of individual compounds were divided by the sum of the absolute amounts of all compounds and multiplied by 100.

Table 1 Species names, degree of sociality, life stages collected, collection sites, and collection years of all halictid bees used in the study

Species name	Social level	Life stages	Site	Year
Lasioglossum malachurum	Obligate primitively eusocial	Foundresses (n = 8), workers (*n* = 8), breeding queens (n = 8)	Ulm, Germany	2013
Lasioglossum pauxillum	Obligate primitively eusocial	Foundresses (n = 8), workers (*n* = 8), breeding queens (n = 8)	Ulm, Germany	2015
Lasioglossum zephyrum	Obligate primitively eusocial	Workers (n = 8), breeding queens (*n* = 7)	Ithaca, New York, USA	1998
Halictus rubicundus	Facultative eusocial, social nests	Foundresses (n = 8), workers (*n* = 8), breeding queens (n = 8)	Hayingen, Germany	2013
	Facultative eusocial, solitary nests	Foundresses (n = 8), breeding females (n = 8)	Belfast, UK	2011
Megalopta genalis	Facultative eusocial, social nests	Foundresses (n = 5), workers (n = 8), breeding queens (n = 8)	Barro Colorado Island, Panama	2014, 2016
Megalopta centralis	Facultative eusocial, social nests	Workers (n = 8), breeding queens (n = 5)	Barro Colorado Island, Panama	2014
Systropha curvicornis	Ancestrally solitary	Foundresses (n = 8), breeding females (n = 8)	Mödling, Austria	2015, 2016

Measurement of Physiological State After extraction of the cuticle surfaces, all bees were dissected under a stereomicroscope to check for ovarian stage, which was classified into five categories based on Duchateau and Velthuis (1989): (1) totally undeveloped, empty; (2) small, all follicles beyond vitellogenic phase; (3) enlarged, vitellogenic follicle growth initiated; (4) large, ovarioles containing eggs; (5) oocyte fully developed and ready to lay eggs.

Statistics To check whether chemical dissimilarity between castes (workers vs. breeding queens) or various female life stages (nest foundresses vs. breeding females) was linked with social status in halictid bee species, we analyzed cuticular odor bouquets of 5–8 replicates per caste, female life stage, and species. Relative amounts [%] of each compound were calculated with respect to the total concentration of the whole bouquet, and peaks with a concentration < 0.1% were excluded from downstream analyses (*L. malachurum*: 65 peaks, *L. pauxillum*: 79 peaks, *L. zephyrum*: 62 peaks, *H. rubicundus* social: 72 peaks, *H. rubicundus* solitary: 74 peaks, *M. genalis*: 61 peaks, *M. centralis*: 60 peaks, *S. curvicornis*: 68 peaks). Despite this, we checked whether we could find a link between the number of compounds produced by a species and its social level using a generalized linear model (GLM) with a quasi-Poisson error distribution implemented using the *stats* package of R v. 3.3.1 (R Core Team 2016).

To visualize dissimilarities between species, castes and female life stages, we performed non-metric multidimensional scaling (NMDS) based on Bray-Curtis square root transformed distances as implemented in Primer (Clarke and Gorley 2006). Stress values (which indicate the level of reliability) below 0.15 indicate a good fit between the statistical ordination and the observed matrix. Furthermore, we performed a one-way ANOSIM (analysis of similarities, permutations: 10,000) following post-hoc SIMPER analysis to check for the relative contributions of certain compounds to species-specific, caste-specific, or life-stage-specific differences.

All analyses were run in R v. 3.3.1 (R Core Team 2016). To compare chemical differences between caste or female life stages and social complexity (obligate eusocial, facultative eusocial, or solitary), we calculated Bray-Curtis dissimilarities based on the square-root-transformed cuticle odor bouquet datasets using the *vegan* package (Oksanen et al. 2017). For each species, each caste, and each female life stage comparison (breeding queens versus workers, nest foundresses versus breeding females), we obtained 40–64 pairwise dissimilarity values that were compared using generalized linear models (GLMs) with a Gamma error distribution followed by a Tukey post-hoc test of pairwise comparisons with the *stats* and *multcomp* packages (Hothorn et al. 2008). The same analyses were performed on subsets of the chemical profiles: only the n-alkanes or only the macrocyclic lactones. Overall, model

choice was based on a high explanatory power measured by low AIC values (Aikaike Information Criterion). As bee size and nest were never significant, we did not include them in the models.

To check whether the relative amounts of n-alkanes or macrocyclic lactones differed between female life stages and social statuses, we used the sum of the relative amounts of either n-alkanes or macrocyclic lactones as the dependent variable in GLMs with quasi-Poisson error distribution following a Tukey post-hoc test for pairwise comparisons using the *stats* and *multcomp* packages (Hothorn et al. 2008)

In order to investigate whether chemical dissimilarities were related to differences in ovarian development between castes, we compared various categories of ovarian stage differences (queen's ovarian category minus worker's ovarian category) by employing GLMs with a quasi-Poisson error distribution and chemical distance as the response variable with the *stats* package of R.

Results

Odor Profiles In total we found 233 different chemical compounds in seven analyzed halictid bee species mainly belonging to the compound classes of n-alkanes, n-alkenes, saturated and unsaturated macrocyclic lactones, saturated and unsaturated isopentenyl esters, and ethyl esters (Table S1). Not all chemical structures of compounds could be identified. However, we used characteristic molecular ions that have been described in former investigations (Duffield et al. 1981; Francke 2010) and in addition, if available, retention indices to exclude the occurrence of further lactones or alkanes beside the ones we describe. Although all species produced n-alkanes with chain lengths ranging from C_{23} to C_{31}, some also exhibited n-alkanes with shorter (C_9 to C_{22}) or longer (C_{32} to C_{35}) chains. With regard to the saturated lactones, all species other than the solitary *S. curvicornis* produced 20-eicosanolide, 22-docosanolide, and 24-tetracosanolide, whereas some species also exhibited 16-hexadecanolide, 18-octadecanolide, or even 26-hexacosanolide. However, we found the main species-specific differences to lie in the relative amounts of n-alkanes (mainly tricosane and pentacosane), n-alkenes (mainly *(Z)*-9-pentacosene, *(Z)*-9-heptacosene, and *(Z)*-9-nonacosene), and saturated macrocyclic lactones (18-octadecanolide, 20-eicosanolide, and 22-docosanolide; SIMPER analyses, each compound contributed more than 1.8% to the total Bray-Curtis dissimilarity). The occurrence or relative amounts of specific compounds were important for separating certain species from all other species (*L. pauxillum*: *(Z)*-12-hentriacontene; *L. zephyrum*: unsaturated isopentenyl ester, 18-octadecenolide with an unknown double bond position; *M. genalis*: tritriacontadiene with unknown double bond positions; *M. centralis*: 18-octadec-*(Z)*-11-enolide; *H.*

rubicundus: (Z)-11-heptacosene, 24-tetracos-(Z)-11-enolide; *S. curvicornis*: (Z)-7-heptacosene and tetracosene with an unknown double bond position). Therefore, all studied halictid bee species had a distinctive cuticular chemical profile (ANOSIM, *global R* = 0.967, *P* < 0.001, all pairwise comparisons: *P* < 0.001; Fig. 1, Fig. S1-S7). The most distinct species was *S. curvicornis*, which was the solitary outgroup, whereas all obligate and facultative eusocial species were more clustered.

Moreover, all female groups (social nest foundresses, breeding queens, workers, solitary nest foundresses, and solitary breeding females) within each species could be separated by their cuticular chemical profile, except for the breeding queens and workers of *M. centralis* (ANOSIM, *global R* = 0.95, *P* < 0.001, all pairwise comparisons: *P* < 0.05, but *M. centralis* queens and workers: *P* = 0.148; Fig. 1). The main compounds separating female groups within species were n-alkanes (tricosane, pentacosane, heptacosane, and nonacosane), n-alkenes ((Z)-9-heptacosene, (Z)-9-nonacosene), and saturated macrocyclic lactones (20-eicosanolide, 22-docosanolide; SIMPER analyses, each compound contributed more than 1.8% to the total Bray-Curtis dissimilarity). The total number of chemical compounds produced by each species was not linked to its social level (obligate eusocial vs. facultatively eusocial vs. ancestrally solitary species; quasi-Poisson GLM, $F_{2,5}$ = 0.05, *P* = 0.95).

Caste-Specific Chemical Dissimilarities We used the dissimilarities in chemical profiles between castes to calculate differences between obligate eusocial (*L. malachurum, L. pauxillum, L. zephyrum*) and facultative eusocial (*H. rubicundus, M. genalis, M. centralis*) species. With regard to breeding queens and workers of obligate eusocial species, we found a significantly higher chemical distinction between castes than that in facultative eusocial species (Gamma GLM, $F_{1,350}$ = 115,99, *P* < 0.001; Fig. 2a).

As macrocyclic lactones and n-alkanes are typically found in greater amounts in queens compared with workers in the obligate eusocial halictid bee species *L. malachurum* (Ayasse et al. 1993; Steitz and Ayasse, unpublished), we further checked whether we could find similar caste-differences across our halictid species. In particular, in the obligate eusocial species *L. malachurum* and *L. zephyrum*, macrocyclic lactones were significantly higher in queens than in workers (*L. malachurum*: quasi-Poisson GLM, $F_{1,14}$ = 10.77, *P* = 0.005; *L. zephyrum*: quasi-Poisson GLM, $F_{1,13}$ = 11.284, *P* = 0.02; Fig. 3a), whereas we could find no caste-specific differences in the relative amounts of macrocyclic lactones in facultative eusocial species (*H. rubicundus*: quasi-Poisson GLM, $F_{1,14}$ = 0.0868, *P* = 0.77; *M. genalis*: quasi-Poisson GLM, $F_{1,14}$ = 1.876, *P* = 0.19; *M. centralis*: quasi-Poisson GLM, $F_{1,11}$ = 0.0095, *P* = 0.92; Fig. 3a). With regard to n-alkanes, only *L. malachurum* exhibited significantly more n-alkanes in queens (quasi-Poisson GLM,

$F_{1,14}$ = 4.6251, *P* = 0.049; Fig. 3b), whereas in *L. zephyrum* workers possessed even higher amounts than queens (quasi-Poisson GLM, $F_{1,13}$ = 7.2142, *P* = 0.02; Fig. 3b). In a comparison of chemical dissimilarities considering only chemical profiles of macrocyclic lactones or n-alkanes, we found greater chemical differences between castes in obligate eusocial than in facultative eusocial species (macrocyclic lactones: Gamma GLM, $F_{1,350}$ = 175.93, *P* < 0.001; n-alkanes: Gamma GLM, $F_{1,350}$ = 49.755, *P* < 0.001; Fig. 2b, c). Overall, when considering only macrocyclic lactones, the highest chemical distinction between castes was in obligate eusocial species, whereas the lowest distinction was in facultative eusocial species (Fig. 2).

We furthermore tested whether the chemical differences between castes were influenced by ovarian development. Our results indicated that the chemical distinction between queens and workers was indeed related to the difference in ovarian development between castes, but only in facultative eusocial species (quasi-Poisson GLM, $F_{1,166}$ = 22.422, *P* < 0.001; Fig. 4b) and not in obligate eusocial species (quasi-Poisson GLM, $F_{1,182}$ = 0.0908, *P* = 0.76; Fig. 4a). However, when considering only macrocyclic lactones or n-alkanes, we could not find any link between caste-specific chemical distinction and difference in ovarian development, neither for obligate eusocial species (macrocyclic lactones: quasi-Poisson GLM, $F_{1,182}$ = 4.7285, *P* < 0.31; n-alkanes: quasi-Poisson GLM, $F_{1,182}$ = 3.1207, *P* = 0.08), nor for the facultative eusocial species (macrocyclic lactones: quasi-Poisson GLM, $F_{1,166}$ = 0.5936, *P* < 0.44; n-alkanes: quasi-Poisson GLM, $F_{1,166}$ = 0.2203, *P* < 0.64).

Female-Life-Stage-Specific Chemical Dissimilarities We also investigated whether the chemical distinction between non-breeding females (foundresses of nests that later become social or solitary) and breeding females (queens, breeding solitary females) differed between obligate eusocial, facultative eusocial (solitary and social), and solitary species. The chemical distinction between nest foundresses and breeding queens did not differ markedly between obligate and facultative eusocial species (Gamma GLM, $F_{3,356}$ = 70.602, *P* < 0.001, following Tukey post-hoc tests, *P* = 0.67; Fig. 5a). In solitary nests of the facultative eusocial species (*H. rubicundus* and *M. genalis*), non-breeding nest foundresses and breeding females showed a higher distinction than those in social nests (Gamma GLM, $F_{3,356}$ = 70.602, *P* < 0.001, following Tukey post-hoc tests, *P* < 0.001; Fig. 5a), whereas in the solitary outgroup species *S. curvicornis* we found the lowest distinction (Gamma GLM, $F_{3,356}$ = 70.602, *P* < 0.001, following Tukey post-hoc tests, *P* < 0.001; Fig. 5a). We found a similar pattern with regard to chemical distances of a subset including only macrocyclic lactones (Gamma GLM, $F_{3,356}$ = 49.251, *P* < 0.001, following Tukey post-hoc tests, *P* < 0.05; Fig. 5b). However, when we considered only n-alkanes, non-breeding

Fig. 1 Species-specific and female-function-specific differences in cuticular chemical profiles based on the relative amounts of all compounds (NMDS, Bray-Curtis similarity measures, 3D Stress: 0.11)
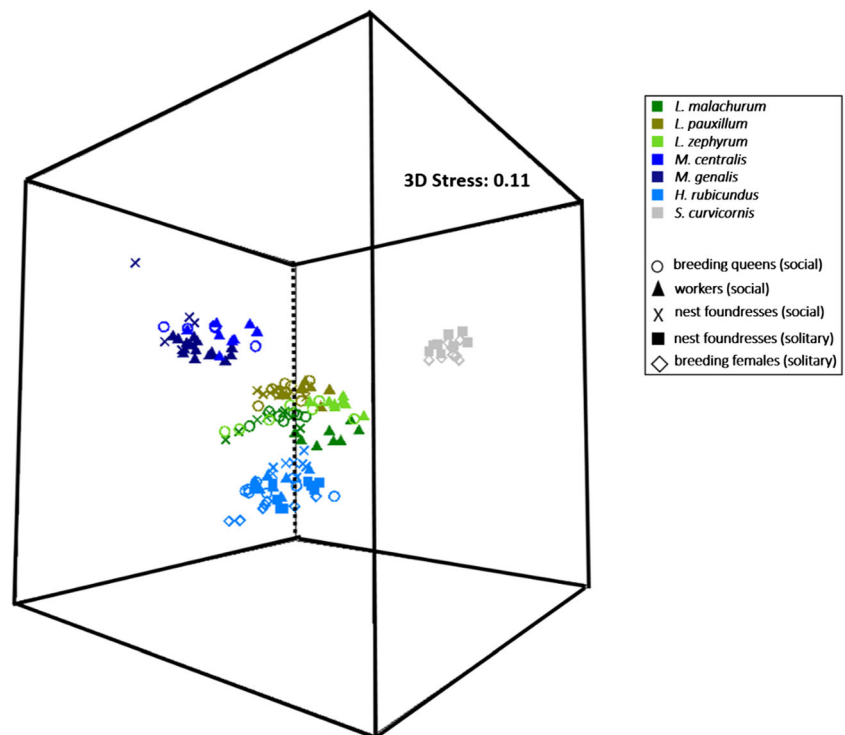
and breeding females of obligate eusocial species were more chemically distinct than those in social and solitary nests of facultative eusocial species, whereas the lowest distinction was again found in the solitary outgroup (Gamma GLM, $F_{3,356} = 104.53$, $P < 0.001$, following Tukey post-hoc tests, $P < 0.05$; Fig. 5c).

Discussion

All of the halictid bee species examined in this study produce a unique cuticular odor profile, mainly because of the different relative amounts of common compounds such as n-alkanes, n-

alkenes, or macrocyclic lactones. Interestingly, we found these compounds to commonly differentiate females based on caste and life stages (nest foundresses and breeding females) in all studied species, except for the solitary outgroup. Despite this, we found that higher amounts of macrocyclic lactones are characteristic of queens, especially in obligate eusocial species. Macrocyclic lactones occur in large amounts in the Dufour's glands of halictid bees (Ayasse et al. 1993; Soro et al. 2011) and are known to be species-specific with regard to their relative amounts and chain lengths (Johansson et al. 1982). They are involved in mediating aggressive interactions between nest-founding gynes (Smith and Weller 1989) and probably also contribute to the sex pheromones of halictids (Smith et
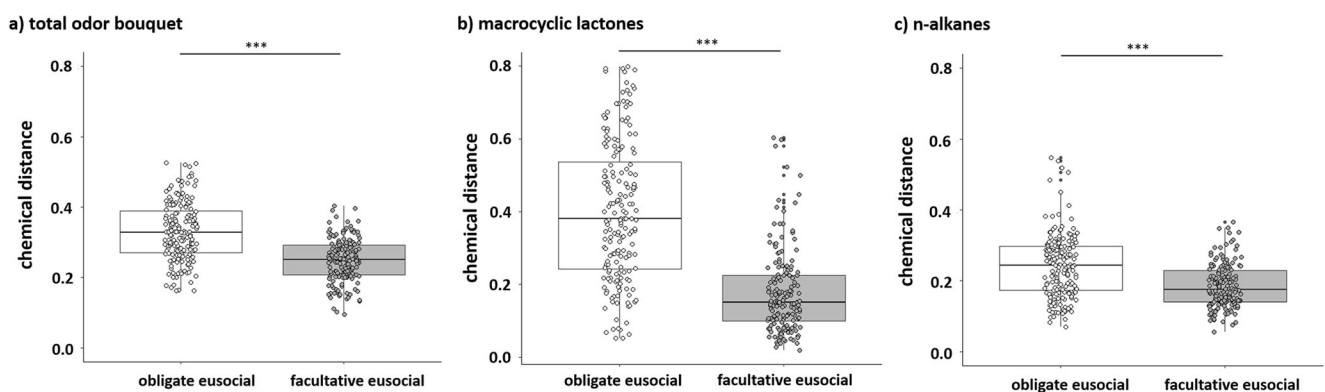

Fig. 2 Comparison of chemical distances (Bray-Curtis dissimilarities) between queens and workers of obligate eusocial and facultative eusocial halictid bee species with regard to (**a**) the complete cuticular chemical profile, (**b**) a subset consisting of macrocyclic lactones, or (**c**) a subset consisting of n-alkanes. In all three cases, obligate eusocial

species exhibited significantly higher chemical differences between castes than did facultative eusocial species (Gamma GLMs, $P < 0.001$). Boxes represent the median (bar) and 25th and 75th percentile, and the overlaying dots represent all calculated data points

Fig. 3 Comparison of relative amounts of (**a**) macrocyclic lactones or (**b**) n-alkanes between queens (white bars) and workers (gray bars) of three obligate eusocial and three facultative eusocial species. Asterisks indicate significant differences between castes (quasi-Poisson GLMs, * $P < 0.05$, ** $P < 0.001$). Boxes represent the median (bar) and 25th and 75th percentile, and the overlaying dots represent all calculated data points
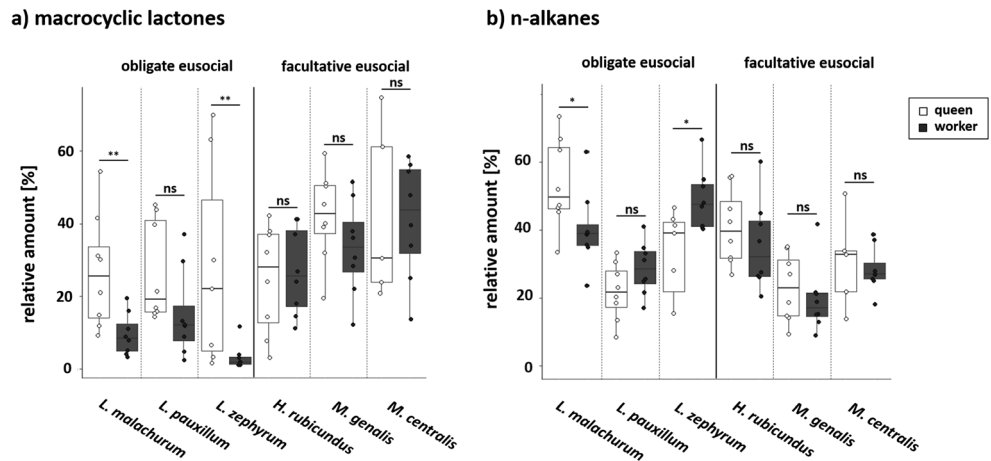
al. 1985). Former chemical analyses of *L. malachurum* have revealed an increase of macrocyclic lactones in fertile or breeding females compared with non-breeding females (Ayasse et al. 1993, 1999), and they function as fertility signals affecting worker behavior and ovarian activation (Steitz and Ayasse, unpublished). Most studies dealing with pheromones in social insects have, in contrast, demonstrated that cuticular hydrocarbons are responsible for the communication of colony membership and fertility across various species (Holman et al. 2013, 2016; Howard and Blomquist 2005; Leonhardt et al. 2016; Monnin 2006; Oi et al. 2015; Smith et al. 2012; Van Oystaeyen et al. 2014; van Zweden and d'Ettorre 2010). Our results suggest that halictid bees use macrocyclic lactones and not cuticular hydrocarbons in fertility signaling.

Among halictid bees, CHCs alone do not appear to explain the chemical dissimilarities of life stages or castes, a result that should be viewed in its evolutionary context. Most insect pheromones seem to have evolved from non-communicative precursors that are, for instance, a by-product of physiological processes (Stökl and Steiger 2017). This is consistent with the prominent hypothesis that queen pheromones have evolved from chemicals produced as by-products of ovarian development (Holman 2012; Oi et al. 2015). A recently hypothesized

evolutionary trajectory suggests that, with increasing colony size, specific chemicals became associated with fertility and may therefore have evolved into consistent learned fertility signals or even innate queen pheromones (Smith and Liebig 2017). With regard to these hypotheses, compounds other than CHCs may evolve into fertility signals or even queen pheromones as long as they are reliably associated with fertility or breeding behavior. Macrocyclic lactones are primarily used by halictid bees to line the inner surface of brood cells and nest entrances, generating a hydrophobic layer as protection against fungi or microorganisms (Cane 1981; Duffield et al. 1981; Hefetz et al. 1986; Hefetz 1987). They are produced in large amounts in the Dufour's gland of breeding females (Ayasse et al. 1993, 1999). Thus, the production of macrocyclic lactones is indeed correlated with nesting behavior and consequently also with breeding and fertility. They may therefore be good candidate compounds that have evolved into a fertility signal or even a queen pheromone. Dufour's gland compounds are known to differentiate reproductive individuals also in other groups of social insects such as ants (Monnin et al. 2002).

As our results indicate a common pattern of an overproduction of saturated macrocyclic lactones in fertile females or

Fig. 4 Effect of a higher queen-worker difference in ovarian development on the chemical distinction between castes of (**a**) obligate eusocial species and (**b**) facultative eusocial species. The chemical distance increased with a higher difference in ovarian development between castes (queen's ovarian category minus worker's ovarian category) in facultative eusocial species (quasi-Poisson GLM, $P < 0.001$) but not in obligate eusocial species (quasi-Poisson GLM, $P = 0.76$)
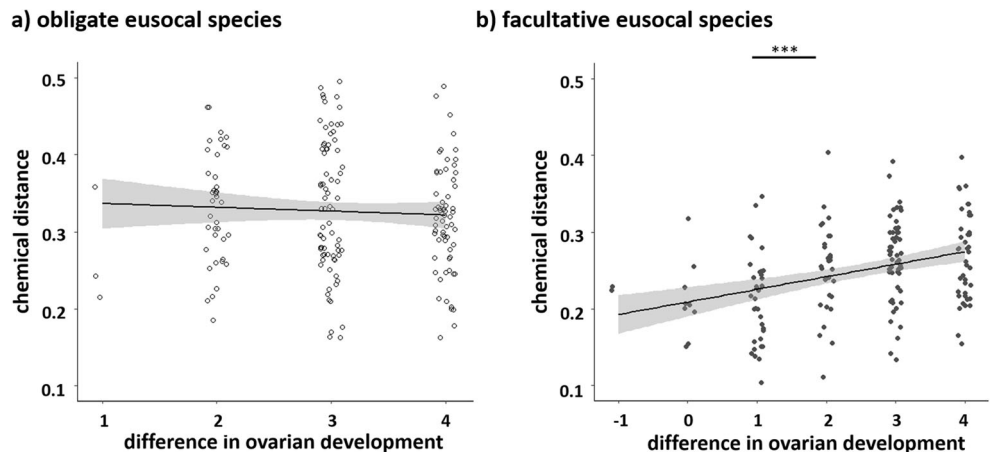
queens compared with workers, they are most likely used as fertility signals across several halictid species. However, more studies incorporating behavioral and physiological assays with additional halictid species are needed to test this idea. Indeed, our results presented here may also be influenced by phylogenetic aspects including different origins of eusociality within the Halictidae. *Lasioglossum* and *Halictus* may in fact share a single origin of eusociality, however, *Megalopta* belonging to the Augochlorini represents another origin (Gibbs et al. 2012). Therefore, we would suggest to include these aspects in further comparative studies to examine if these different origins also affect the evolution of chemical signals used for the regulation of reproduction. As halictid bees exhibit a wide variety of social behaviors, they are ideal model organisms for testing hypotheses that deal with the evolution of fertility signals or queen pheromones.

We found that chemical differences between castes were higher in obligate than in facultative eusocial halictid species, a result which became even clearer when we restricted our analyses to macrocyclic lactones alone. Wittwer et al. (2017) have documented a change in sensilla density associated with changes in social behavior across halictid bee species, suggesting a link between the evolution of social behavior and investment in chemical communication systems. Interestingly, they reported a similarly high sensilla density in ancestrally solitary and obligate eusocial species, but a significantly lower density in facultative eusocial species. Our results complement these findings, as we found higher investment in caste-specific compounds in obligate eusocial species. We hypothesize this to reflect higher investment in intra-colonial communication driven by, or associated with, a higher social complexity. However, we could not find a link between social complexity and an increase in the number of chemical compounds produced as obligate eusocial and even *S. curvicornis*,

the ancestrally solitary species, indicated a rather complex odor profile which function we are not able to explain yet. Therefore, our results suggest that a higher social complexity is not necessarily linked to a higher number or diversity of chemical compounds as suggested by Kather and Martin (2015), but rather reflects a higher investment in caste-specific communication signals.

Our findings also suggest that the chemical distinction between castes is linked to differences in ovarian development and is therefore linked to fertility, but only in facultative eusocial species and not in obligate eusocial species. These data are consistent with the hypothesis for the evolution of fertility signals and queen pheromones as proposed by Smith and Liebig (2017). We hypothesize that cuticular chemical caste-differences of facultative eusocial species may arise in part because of the production of fertility signals that are closely linked to fertility and ovarian activation, whereas chemical distinctions between castes in obligate eusocial species can be explained by a specific queen odor that is probably independent of ovarian activation. These results were not consistent when considering macrocyclic lactones or n-alkanes alone, indicating that more compounds from different compound classes are synergistically linked to fertility and ovarian activation in facultative eusocial species, even if macrocyclic lactones evolved into a queen signal. However, further studies with additional halictid species across levels of social complexity are necessary to support this evolutionary hypothesis. Thus, we suggest that future studies pay close attention to odor differences of castes by using breeding queens in their social phase, and not only at the nest foundress stage as in Wittwer et al. (2017). We suggest that the necessity to produce chemicals with a function in communication in social interactions is higher in breeding queens that have direct contact with their workers than in nest foundresses. Therefore, odor
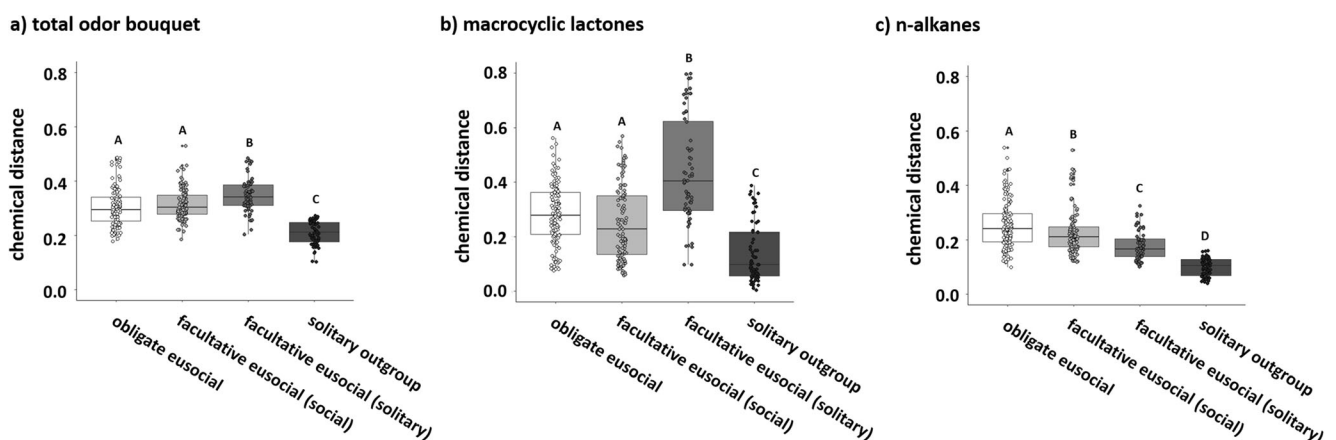

Fig. 5 Comparison of chemical distances (Bray-Curtis dissimilarities) between non-breeding nest foundresses and queens/breeding females of obligate eusocial, social, and solitary nests of facultative eusocial and solitary halictid bee species with regard to (**a**) the complete cuticular chemical profile, (**b**) a subset consisting of macrocyclic lactones, or c) a subset consisting of n-alkanes. Different letters indicate significant differences (Gamma GLMs, $P < 0.05$). Boxes represent the median (bar) and 25th and 75th percentile, and the overlaying dots represent all calculated data points

comparisons between castes are more meaningful when using breeding queens and workers, especially if the study is aimed at examining chemical differences between castes.

Our findings also reveal marked chemical differences between female life stages of non-breeding and breeding females/queens. Solitary females of facultative eusocial species such as *H. rubicundus* exhibit the highest differences compared to conspecifics, whereas the ancestrally solitary species *S. curvicornis* show the lowest chemical distinction with regard to the total odor bouquet or a subset comprising macrocyclic lactones. However, when we consider only n-alkanes, obligate eusocial species exhibit the highest chemical difference among conspecifics. Cuticular hydrocarbons such as n-alkanes or n-alkenes have previously been shown to correlate strongly with the reproductive activity of various social insects, a relationship that is endocrine-mediated (Blomquist and Bagnères 2010; Liebig et al. 2009; Monnin 2006; Peeters and Liebig 2009; Van Oystaeyen et al. 2014). As obligate eusocial halictid species usually produce larger colonies and have higher reproductive activity compared with facultative eusocial or solitary species, queens of obligate species are indeed expected to exhibit greater differences in their CHC profiles when starting to reproduce due to a higher fecundity. However, when considering the complete odor profile or only macrocyclic lactones, we found the greatest difference between non-breeding and breeding females in the solitary nests of facultative eusocial species. Despite their function in fertility signaling (Steitz and Ayasse, unpublished), macrocyclic lactones are used to line brood cells and therefore are involved in nesting functions, not only in eusocial but also in solitary nests of facultative eusocial halictid species (Cane 1981; Duffield et al. 1981; Hefetz et al. 1986; Hefetz 1987). These nesting functions in the social nests of facultative eusocial species are also performed by workers, whereas they are performed solely by the single breeding female in solitary nests of facultative eusocial species. This may promote a greater need for macrocyclic lactones by solitary-nesting females of these species. Interestingly, the extent of the chemical dissimilarity between non-breeding and breeding females was the same in obligate and facultative eusocial species, indicating a lack of evolutionary shift to a higher chemical distinction at higher social complexity. Consequently, we suggest that a higher investment in chemical communication during the evolution of social living is mainly in the greater chemical dissimilarity between castes. The pivotal shift is attributable to the chemical profile of workers whose profile is more distinct from that of queens in species with higher social levels. The higher chemical similarity between castes in facultative eusocial species is probably also attributable to the higher proportion of workers with developed ovaries. Indeed, workers with more developed ovaries have previously been shown to have a more queen-like odor in bumble bees (Ayasse et al. 1995; Sramkova et al. 2008), *Polistes* wasps (Dapporto et al.

2005) and ants (Dietemann et al. 2003; Liebig et al. 2000; Peeters et al. 1999).

In conclusion, our results clearly show the presence of species-specific chemical profiles across several halictid species. All species have common compounds that nevertheless differ between castes and life stages, with macrocyclic lactones being the single common compound class overproduced in queens compared with workers. Caste dissimilarities are also highest with regard to macrocyclic lactones, especially among obligate eusocial species. Therefore, we suggest that macrocyclic lactones are common fertility signals across halictid bee species. As obligate eusocial species demonstrate a higher caste-specific chemical dissimilarity than facultative eusocial species, we hypothesize that there is a link between the investment in intra-colonial communication and social complexity. Moreover, the link between chemical dissimilarities and ovarian activation is found solely in facultative eusocial species, indicating a potential queen-specific odor signal independent of the reproductive stage in obligate eusocial species. In summary, our findings support the hypothesis of the evolution of queen signals from previous fertility-linked signals, as outlined by Smith and Liebig (2017). These results emphasize the strong link between chemical communication and the evolution of social living and illustrate the great opportunities that halictid bees offer for further studies dealing with the evolution of social complexity and chemical communication.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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