Conservation of Queen Pheromones Across Two Species of Vespine Wasps

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Abstract Social insects are known for their reproductive division of labor between queens and workers, whereby queens lay the majority of the colony's eggs, and workers engage mostly in non-reproductive tasks. Queens produce pheromones that signal their presence and fertility to workers, which in turn generally remain sterile. Recently, it has been discovered that specific queen-characteristic cuticular hydrocarbons (CHCs) function as queen pheromones across multiple lineages of social insects. In the common wasp, *Vespula vulgaris*, several long-chain linear alkanes and 3-methylalkanes were shown to act as queen signals. Here, we describe similar bioassays with a related species of highly eusocial vespine wasp, the Saxon wasp, *Dolichovespula saxonica*. We show that a blend of queen-characteristic hydrocarbons that are structurally related to those of the common wasp inhibit worker reproduction, suggesting conservation of queen pheromones across social wasps. Overall, our results highlight the central importance of CHCs in chemical communication among social insects in general, and as conserved queen pheromones in these social wasps in particular.

Keywords Queen pheromone · Cuticular hydrocarbon · Social insect · *Dolichovespula saxonica*, *Vespula vulgaris*

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Introduction

Social insect colonies are well known for their marked reproductive division of labor between queens and workers, in which queens produce the majority of the colony's eggs, and workers engage mostly in non-reproductive tasks. This reproductive division of labor is regulated by the queen through the production of queen pheromones, which signal the queen's presence and fertility, and cause most workers to refrain from reproducing (Holman et al. 2010, 2013; Le Conte and Hefetz 2008; Oi et al. 2015a; Van Oystaeyen et al. 2014). In contrast to the honeybee *Apis mellifera*, in which much work has focused on the queen's mandibular pheromones and their major constituent 9-oxo-(*E*)-2-decenoic acid (9-ODA), it has been shown that cuticular hydrocarbons (CHCs) likely act as conserved queen signals across several other lineages of social Hymenoptera (Holman et al. 2013; Oi et al. 2015a; Oliveira et al. 2015; Van Oystaeyen et al. 2014). In particular, bioassays have shown that specific queen-characteristic hydrocarbons inhibit worker reproduction across a variety of social insect taxa, including the bumblebee *Bombus terrestris* (Holman 2014; Van Oystaeyen et al. 2014), the common wasp *Vespula vulgaris* (Van Oystaeyen et al. 2014), and several species of ants (Holman et al. 2010, 2013, 2016a; Smith et al. 2016; Van Oystaeyen et al. 2014). These results demonstrate that specific CHCs have evolved to become queen pheromones across at least three independent origins of eusociality (Oi et al. 2015a; Oliveira et al. 2015; Van Oystaeyen et al. 2014). Comparative analyses further showed that hydrocarbons are the most common class of compounds that appear as queen-characteristic signals in social Hymenoptera (Holman et al. 2013; Oliveira et al. 2015; Van Oystaeyen et al. 2014). It is likely that the convergent evolution of hydrocarbon queen pheromones is driven by the fact that specific CHCs correlate with reproductive state in both social and non-social insect species (Blomquist and Bagnères

2010; Liebig 2010; Oi et al. 2015a), which predisposes them to be co-opted as honest signals of fertility (Holman 2012; Monnin 2006; van Zweden 2010).

The studies cited above suggest that social insect queen pheromones show strong evolutionary conservation, even though this conclusion was based mostly on comparison of distantly related clades, and hence could be due to convergent evolution. Only a limited number of studies have tested for conservation of these signals within closely related species. Data from *Apis* bees (Plettner et al. 1997), *Temnothorax* ants (Brunner et al. 2011), and *Lasius* ants (Holman et al. 2013, 2016a) suggest that queen pheromones have remained relatively stable over evolutionary time. Some studies even have shown that queen pheromones can function interspecifically to inhibit worker reproduction (Brunner et al. 2011; Holman et al. 2013). On the other hand, there are examples where fertility signals do not appear to be conserved within a particular genus. Most notably, in *Odontomachus* trap-jaw ants, a comparison across three congeneric species showed that fertility signals were not conserved, with chemical compounds from queens and workers differing in number and type among the species analyzed (Smith et al. 2016). It also was shown that the chemical context in which the signal is perceived can be important for proper recognition of fertility signals (Smith et al. 2015). In addition, in the buff-tailed bumblebee, *Bombus terrestris*, the queen-characteristic linear alkane pentacosane induced ovary regression (Van Oystaeyen et al. 2014), whereas in the related species *B. impatiens*, pentacosane was claimed to have no activity as a queen pheromone (Amsalem et al. 2015). However, reanalysis of Amsalem et al.'s data with more powerful statistical models did indeed indicate that pentacosane reduced and delayed egg-laying by workers, thereby challenging the suggested lack of conservation (Holman et al. 2016b).

Here, as a further test of possible conservation of these signals, we describe bioassays with a related species of the highly eusocial vespine wasps: the Saxon wasp, *Dolichovespula saxonica*. The typical queen-characteristic CHCs of this species are linear and 3-methylalkanes, and thus structurally related to the queen pheromones of the common wasp, *Vespula vulgaris* (van Zweden et al. 2014). We therefore hypothesized that a synthetic blend of these queen-characteristic hydrocarbons would inhibit worker ovary development, and we found that this indeed was the case, supporting the hypothesis that queen pheromones are highly conserved among social wasps.

Methods and Materials

Shortlisting of Potential Queen Pheromone Components

To identify possible queen pheromone components, we collected three natural colonies of *Dolichovespula saxonica* in the

vicinity of Leuven, Belgium. Cuticular hydrocarbons were extracted by immersing the wasps (10 workers per colony and each of the mother queens) in 1.5 ml pentane (HPLC grade, Sigma-Aldrich). The pentane was evaporated, the extract reconstituted in 250 μ l hexane, and 2 μ l injected in a Shimadzu QP2010 Ultra GC-MS (gas chromatograph-mass spectrometer). Injections were made in split mode (10:1), using an injection temperature of 280 $^{\circ}$ C, a DB-5 MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m film thickness; J & W Scientific, Folsom CA, USA), and helium as the carrier gas with a flow rate of 1 ml/min. The oven temperature was held at 70 $^{\circ}$ C for 1 min, then increased to 220 $^{\circ}$ C at 25 $^{\circ}$ C/min, and then to 325 $^{\circ}$ C at 3 $^{\circ}$ C/min, with a final hold of 15 min at 325 $^{\circ}$ C. Peak areas of 65 CHCs were integrated using a custom script in R version 3.1.0 (Team RC 2012) (available from the authors upon request). The software AMDIS 2.71 was used in combination with NIST MS Search 2.2 and the NIST 2014 mass spectral database search and manual interpretation of mass spectra (taking into account expected fragmentation patterns and retention indices) to identify the different compounds. Alkene double bond positions were tentatively identified on the basis of measured retention indices of known alkene standards that were run on our machine on the same temperature program and whose double bond positions were checked using dimethyldisulfide (DMDS) derivatization (Carlson et al. 1989). Subsequently, we performed a principal components analysis (PCA) on Z-transformed relative peak areas (Aitchison 1986) to look at patterns of variation within our dataset, using the software packages FactoMineR (Lê et al. 2008) and psych (Revelle 2016). In addition, to short list compounds to be tested in bioassays, we fitted separate univariate linear mixed models on log₂ transformed relative peak areas for all 65 compounds present (Table S1). In these analyses, colony and caste were coded as random and fixed factors, respectively. Compounds then were ranked according to how queen characteristic they were, which was done on the basis of the mean fold difference in abundance on the cuticle of queens vs. workers (Table S2). This analysis revealed that the linear alkanes nonacosane (*n*-C₂₉), triacontane (*n*-C₃₀), and hentriacontane (*n*-C₃₁), and the 3-methylalkanes 3-methylnonacosane (3-MeC₂₉) and 3-methylhentriacontane (3-MeC₃₁) were among the most queen-characteristic compounds, as well as structurally similar to the queen pheromones of *V. vulgaris* (heptacosane *n*-C₂₇, nonacosane *n*-C₂₉, and 3-methylnonacosane 3-MeC₂₉) (Van Oystaeyen et al. 2014). The queen-characteristic alkene 7-hentriacontene (7-C_{31:1}) and dimethylalkane 3,7-dimethylnonacosane (3,7-diMeC₂₉) were not tested because they were not available to us.

Bioassays For the bioassays, we collected five *D. saxonica* colonies in the vicinity of Leuven and transferred them to closed wooden boxes (36 $\times$ 15 $\times$ 15 cm) held at approximately 28 $^{\circ}$ C, where they were fed sugar paste (Apifonda) and *Tenebrio*

mealworms ad libitum [similar to Oi et al. (2015b)]. At the time of collection, all colonies contained a healthy queen. At the start of our experiment, the queens were removed so that we could test if the queen-characteristic CHCs inhibited worker ovary development analogous to a natural queen. Because the nests were small (ca. 75 workers each), they were split into 2 approximately equal parts, of which one served as control and the other as the queen-pheromone treated group. Treatment were applied daily by pipetting 150 μ l of either pentane for the control or a pentane solution of a blend of the queen-characteristic hydrocarbons, at a concentration of two queen equivalents/day (for *n*-C₂₉ 54 μ g, 3-MeC₂₉ 25.5 μ g, *n*-C₃₀ 3.5 μ g, *n*-C₃₁ 8.1 μ g, 3-MeC₃₁ 5.3 μ g), on top of the comb for 2 wk. Absolute concentrations were quantified by using an external alkane ladder (*n*-C₇ to *n*-C₄₀, Supelco, Bellefonte PA, USA). Linear alkanes were obtained from Sigma-Aldrich, and the methylalkanes were synthesized using the procedures described in Van Oystaeyen et al. (2014). Subsequently, all workers were frozen at -20 °C for later dissection. To minimize observer bias, all dissections were carried out blind with respect to treatment. As in previous studies (van Zweden et al. 2014; Wenseleers and Ratnieks 2006), ovaries were considered activated when the largest oocyte was larger than half the size of a freshly-laid egg.

Proportions of workers with active ovaries were compared between each treatment and control using a generalized linear mixed model (GLMM) with binomial errors and logit link function, whereby colony was coded as a random intercept. Colony size was not significant, therefore was not included in the model. The chosen model had the highest explanatory power as measured by a low AIC (Akaike Information Criterion). These analyses were performed using the package lme4 in R v3.1.0 (Team RC 2012). The hydrocarbon compounds were tested only as a blend and not individually because splitting colonies into more than two treatment groups was not possible due to their small size in this study species (ca. 50–100 workers in the growth phase). In addition, an unpaired experimental design using different colonies across the different treatment groups was deemed inadvisable because this could have resulted in confounding effects linked to variation in colony size and development phase, sociogenetic structure, and genetic background, which have been suggested to have large effects on levels of worker reproduction in this species (Bonckaert et al. 2011; Foster and Ratnieks 2000).

Results

We identified 61 hydrocarbons in the CHCs of queens and workers (Fig. 1; Table S1). A principal components analysis (PCA) showed a clear separation between queens and workers, and revealed that a similar set of hydrocarbons was characteristic of queens as in a previously studied Danish

population, including the alkanes *n*-C₂₉, 3-MeC₂₉, 3,7-diMeC₂₉, *n*-C₃₁, and 3-MeC₃₁ and the alkene 7-C_{31:1} (Fig. 1) (van Zweden et al. 2014). The same compounds also emerged as characteristic for queens based on univariate tests (Table S2). Bioassays revealed that application of a blend of the queen-characteristic CHCs *n*-C₂₉, 3-MeC₂₉, *n*-C₃₀, *n*-C₃₁, and 3-MeC₃₁ indeed resulted in a significant (ca. 2 fold) reduction in the odds for workers to activate their ovaries compared to the solvent-only control (Fig. 2, Table S3; total of *N* = 223 and 153 dissected workers in the treatment and control conditions, respectively; 12.0 % vs. 20.6 %; binomial GLMM, coefficient = -0.65, odds ratio = 0.52, *z* = -2.07, *P* = 0.038). This result demonstrates that one or more compounds in the blend were bioactive queen pheromones.

Discussion

We showed that application of a blend of the queen-characteristic hydrocarbons *n*-C₂₉, 3-MeC₂₉, *n*-C₃₀, *n*-C₃₁, and 3-MeC₃₁ reduces activation of worker ovaries in the Saxon wasp, *Dolichovespula saxonica*, thereby demonstrating that one or more of these compounds acts as a queen pheromone to mediate reproductive division of labor in this species. Two of these compounds, *n*-C₂₉ and 3-MeC₂₉, have been shown to be queen pheromone components in the common wasp *Vespula vulgaris* (Van Oystaeyen et al. 2014). In addition, the queen-characteristic CHCs identified in this study were almost the same as those reported from a Danish population of the same species (van Zweden et al. 2014). These results, therefore, suggest conservation of queen pheromones in social wasps among different populations as well as across related species.

The conservation of these signals in social wasps supports the “honest signal” hypothesis for the evolution of social insect queen pheromones (Holman 2012; Keller and Nonacs 1993; Monnin 2006; Oi et al. 2015a; Peso et al. 2015; van Zweden 2010). This hypothesis proposes that workers should be selected to respond to queen pheromones only to the extent that it serves their own inclusive fitness interests (Keller and Nonacs 1993). Under this hypothesis, the alignment of the interests of the queen and workers would be predicted to lead to slow and gradual evolution of queen signals (Heinze and d’Ettorre 2009). By contrast, the alternative hypothesis that the queen uses pheromones to manipulate workers to remain sterile against their own interests (“queen control”) predicts that queen pheromones should evolve quickly due to the coercive rather than cooperative nature of such a relationship (Kocher and Grozinger 2011; Oi et al. 2015a). Thus far, experimental data from honeybees and *Temnothorax* and *Lasius* ants have demonstrated relatively strong conservation of queen pheromones (Brunner et al. 2011; Holman et al. 2013; Keller and Nonacs 1993; Oldroyd and Wongsiri 2006). Phylogenetic

Fig. 1 a Cuticular hydrocarbons (CHC) profiles of queens (top, purple) and workers (bottom, blue) of *Dolichovespula saxonica*, as determined by GC-MS analysis. Dark purple arrows indicate compounds tested in our bioassays: $n\text{-C}_{29}$, 3-MeC₂₉, $n\text{-C}_{30}$, $n\text{-C}_{31}$, and 3-MeC₃₁. Grey dotted arrows indicate compounds not tested, but abundant in queens: 3,7-diMeC₂₉ and 7-C_{31:1}. **b** Principal components analysis (PCA) of CHC profiles ($N = 3$ colonies) shows separation between queens and workers (left panel) and the squares indicate the centroids of both castes (queens in purple and workers in blue) and colonies (in grey). The right panel shows the factor loadings. Five compounds shown in dark purple were used as the treatment blend in our bioassays (Online version in color)

analyses of queen- and fertility-linked compounds in social Hymenoptera (Van Oystaeyen et al. 2014) and corbiculate bees (Oliveira et al. 2015) suggest that queen pheromones evolve slowly, thus supporting the honest signalling hypothesis [but see Smith et al. (2016)], for an example in *Odontomachus* ants where fertility signals apparently are less conserved).

Our results contrast with the hypothesis that hydrocarbon profiles would be too complex and too variably expressed (Howard and Blomquist 2005) to act as reliable caste-specific signals (Amsalem et al. 2015). Although we currently cannot determine if the *D. saxonica* worker response to queen pheromones was learned (e.g., in the form of habituation) (Amsalem et al. 2015; Wyatt 2014), all that appears to be required for workers to recognize the presence of a queen is to discriminate the relative amounts of a subset of the CHCs that are characteristic of the queen. Such discrimination does not seem unlikely, because both ants and bees are able to accurately discriminate among different hydrocarbons differing only in chain length or by the position of a methyl group, including those hydrocarbons used as fertility signals (Sharma et al. 2015) and nestmate recognition cues (Châline et al. 2005; di Mauro et al. 2015). Experimental evidence also has shown that specific hydrocarbon compounds can elicit reproducible and conserved responses as queen signals, in contrast to some of the other compounds that are present on the insects' cuticle (de Narbonne et al. 2016; Holman et al. 2010, 2016a, 2016b; Van Oystaeyen et al. 2014). In fact, the mechanism of

specific hydrocarbons acting as queen pheromones may still work even if the response to queen pheromones depends partly on chemical context (Smith et al. 2016).

Fig. 2 Percentage of workers with activated ovaries after 2 wk. of daily treatment with a blend of the queen-characteristic cuticular hydrocarbons $n\text{-C}_{29}$, 3-MeC₂₉, $n\text{-C}_{30}$, $n\text{-C}_{31}$, and 3-MeC₃₁ dissolved in pentane, compared to a pentane-only control. Bars depict means $\pm$ 95 % confidence limits as predicted from a fitted binomial mixed model. ($N = 5$ replicate colonies, $n = 223$ and 153 dissected workers in treatment and control conditions, respectively, binomial GLMM, coefficient = -0.64 , $z = -2.07$, $*P = 0.03$) (Online version in color)

In summary, our study reinforces the hypothesis that specific CHCs play a central role in regulating the reproductive division of labor in social insects, and demonstrates that one or more of these compounds act as queen pheromones. This result is in agreement with predictions of the honest signalling hypothesis, thereby suggesting that specific CHCs may act as generic queen pheromones across vespine wasps. Further work is required to determine whether only one or a subset of the compounds tested here represents the bioactive queen pheromone. In addition, comparative studies, including the study of solitary outgroups, could reveal how hydrocarbon queen pheromones originated and from which ancestral signals or cues in solitary species they might be derived (Oi et al. 2015a; Van Oystaeyen et al. 2014).

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