QUEEN SIGNALLING IN SOCIAL WASPS

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Abstract

Social Hymenoptera are characterized by a reproductive division of labour, whereby queens perform most of the reproduction and workers help to raise her offspring. A long-lasting debate is whether queens maintain this reproductive dominance by manipulating their daughter workers into remaining sterile (queen control), or if instead queens honestly signal their fertility and workers reproduce according to their own evolutionary incentives (queen signalling). Here we test these competing hypotheses using data from Vespine wasps. We show that in natural colonies of the Saxon wasp, *Dolichovespula saxonica*, queens emit reliable chemical cues of their true fertility and that these putative queen signals decrease as the colony develops and worker reproduction increases. Moreover, these putative pheromones of *D. saxonica* show significant conservation with those of *Vespula vulgaris* and other Vespinae, thereby arguing against fast evolution of signals as a result of a queen-worker arms race ensuing from queen control. Lastly, levels of worker reproduction in these species correspond well with their average colony kin structures, as predicted by the queen signalling hypothesis but not the queen control hypothesis. Altogether, this correlative yet comprehensive analysis provides compelling evidence that honest signalling explains levels of reproductive division of labour in social wasps.

Introduction

An essential step in the evolution of sociality is the maintenance of group stability through the suppression of intragroup conflicts (Bourke 2011). Hymenopteran societies, such as ants, bees and wasps, have long been a model in the study of such social conflicts and the mechanisms that can help to resolve them (Ratnieks et al. 2006). They are characterized by a reproductive division of labour, whereby the queen performs most of the reproduction and the workers generally remain sterile to help the queen reproduce (Wilson 1971), but since workers of many species have retained their ovaries, a reproductive conflict between queen and workers arises. The queen may signal her reproductive dominance and suppress worker reproduction either by means of aggression (primarily in small colony species) or the emission of pheromone signals (Kocher and Grozinger 2011; van Zweden 2010), which helps to resolve this conflict. However, there is significant variation across species in the outcome of these queen-worker conflicts over reproduction, which is reflected in large variation in the number of workers that reproduce, laying unfertilized male-destined eggs, even in the presence of the queen (Bourke 1988; Wenseleers and Ratnieks 2006a). A still ongoing debate (Keller 2009; Kocher and Grozinger 2011) is whether this variation reflects variable outcomes of a queen-worker arms race over reproduction, in which queens occasionally do not succeed in manipulating the workers to remain sterile ('queen control' hypothesis), or if instead there is variation in the workers' evolutionary incentive to respond to the queen's honest fertility signal and remain sterile ('queen signalling' hypothesis) (reviewed in Heinze and d'Ettorre 2009; Keller and Nonacs 1993; Kocher and Grozinger 2011).

In putative support of the queen signalling hypothesis (Keller and Nonacs 1993; Zahavi 1975), several studies have identified cuticular hydrocarbon (CHC) cues that accurately reflect fertility in ants (*e.g.* Cuvillier-Hot et al. 2004; d'Ettorre et al. 2004; de Biseau et al. 2004; Liebig et al. 2000), termites (*e.g.* Liebig et al. 2009; Weil et al. 2009) and wasps (*e.g.* Bhadra et al. 2010; Sledge et al. 2001). In a few cases, there is also direct experimental evidence that workers actually respond to these fertility cues, physiologically (d'Ettorre et al. 2004; Holman et al. 2010) and/or behaviourally (Endler et al. 2004; Holman et al. 2010; Smith et al. 2009; van Zweden et al. 2009), thereby supporting the role of particular CHCs as active queen- or fertility-signalling pheromones. Furthermore, in a few cases, intraspecific variation in the strength of the produced queen signals have also been linked to variation in the level of worker reproduction. In the ant *Camponotus floridanus*, for example, only workers of large established colonies effectively police eggs laid by other workers, but not in small incipient colonies. This was found to be due to the more pronounced chemical differences between queen-laid and worker-laid eggs in large colonies, where queens are more fecund, causing their eggs to contain relatively more fertility-linked hydrocarbons (Moore and Liebig 2010). A similar phenomenon was observed in *Pachycondyla inversa* (van Zweden et al. 2009), but here a lowered queen fertility and corresponding higher acceptance of worker-laid eggs was caused by moving colonies to lab conditions.

Whether or not these studies unambiguously support the queen signalling hypothesis (Keller and Nonacs 1993; Zahavi 1975) remains controversial, given that queen control is very hard to distinguish from queen signalling based solely on physiological evidence. Indeed, both hypotheses often result in identical predictions (Kocher and

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Grozinger 2011). For example, the discovery that queen mandibular pheromone (QMP), which suppresses worker reproduction in the honey bee, *Apis mellifera*, directly acts upon dopamine pathways in the worker brain (Beggs et al. 2007) has been suggested to be consistent with the queen control hypothesis. Yet, the fact that the workers as a collective also gain inclusive fitness benefits from remaining sterile (Wenseleers and Ratnieks 2006a) makes this observation consistent with queen signalling as well (Kocher and Grozinger 2011). Another example is the reversibility of the ovary development of honeybee workers observed after being reunited with the queen (Malka et al. 2007), which again is predicted under either hypothesis (Wenseleers and Ratnieks 2006a).

In our view, there are a few predictions that could more conclusively discriminate between the queen control and queen signalling hypotheses. Firstly, a queen-worker arms race, which is expected under queen control, is predicted to result in rapid divergence of queen-specific compounds among related species (Brunner et al. 2011; Heinze and d'Ettorre 2009; Keller and Nonacs 1993). The idea is that if workers would indeed gain inclusive fitness by becoming reproductive, there should be selection for workers to evade the manipulation by building up tolerance to the queen pheromone, necessitating queens to perpetually produce new compounds to keep their daughters in check. This is similar to the co-evolutionary arms race between males and females in *Drosophila*, which cause complex mixtures of seminal fluid proteins to be transferred to the female, some of which are detrimental to her fitness (Findlay et al. 2008; Swanson et al. 2001). By a similar argument, an arms race between the queens and workers would be expected to lead to species-specific and possibly complex mixtures of queen pheromones. Honest queen signalling, on the other hand,

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would be expected to result in slower evolution and hence in random or more conserved patterns of fertility-linked compounds across species. This is because the inclusive fitness of workers would be maximized by responding to this signal, thereby causing balancing selection on such signals.

Secondly, we can compare levels of worker ovary activation with theoretically predicted levels, which can be modelled for a species as a function of the average colony kin structure and the efficiency with which nestmates police worker-laid eggs (Wenseleers et al. 2004). Under the queen signalling hypothesis, workers in queenright colonies of a given species would be expected to reproduce according to this evolutionarily stable optimal proportion, where species with higher average sister-sister relatedness and lower policing efficiency are predicted to exhibit higher levels of worker reproduction. Under queen control, on the other hand, regardless of sister-sister relatedness very few reproductive workers would be expected. For queenless colonies the opposite pattern is expected: the queen signalling hypothesis predicts an evolutionarily stable optimum that is lower in species with higher sister-sister relatedness, whereas the queen control hypothesis would predict that most workers start reproducing regardless of the colony kin structure (Keller and Nonacs 1993; Wenseleers et al. 2004; Wenseleers and Ratnieks 2006b).

In order to test these predictions, we carried out a study on the Saxon wasp, *Dolichovespula saxonica*, where many workers activate their ovaries even in the presence of the queen (Bonckaert et al. 2011b; Foster and Ratnieks 2000). First, we examined to what extent information about queen fertility is encoded in her cuticular hydrocarbon (CHC) profile and whether, at an intraspecific level, increased worker

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reproduction at later stages of colony development coincides with a decrease in the strength of the putative queen signals (see also Bonckaert et al. 2011b). If the queen's egg production decreases towards the end of the colony cycle, queen signals would be expected to decrease as well if they are honest signals of the queen's true fertility. Furthermore, workers are expected to respond to this by decreasing their policing behaviour and increasing their reproduction, both because it is in their fitness interests to do so (*cf.* Ohtsuki and Tsuji 2009) and because discrimination between queen-laid and worker-laid eggs is likely to become harder (Moore and Liebig 2010). Second, we compared patterns of fertility-linked CHCs and worker reproduction of *D. saxonica* to those of *Vespula vulgaris* and several other Vespine wasps. This enabled us to assess the degree of evolutionary conservation of fertility-linked CHCs as well as to compare levels of worker reproduction against the theoretically predicted ones under a queen signalling framework (Wenseleers et al. 2004).

Methods

Study species and colony development

Young queens of *Dolichovespula saxonica* found their nests alone (and will remain the only queen) around mid May and the first workers emerge in early June. Maximally 3 - 6 combs are constructed throughout the colony's lifetime and the colony can reach up to 200 - 300 individuals. Colony development is relatively short and new queens and males can start emerging as early as the end of June, although this can be as late as mid August. At this point, the colony starts declining and eventually dies off, including the old queen. The virgin queens and males fly off to mate, after which only the queens remain to survive the winter by going into diapause.

Eight *Dolichovespula saxonica* colonies containing a single queen, 40-195 workers, eggs, larvae, and pupae, were collected on Zealand, Denmark, in July 2009 and frozen at -20 °C for further analysis. A 'Colony Stage' parameter, bound between 0 (early stage) and 1 (final stage), was calculated for each colony by measuring comb size and dividing it by the predicted comb size when the colony would be completed (see also Bonckaert et al. 2011b).

Cuticular hydrocarbon analysis

For each colony, we analysed the CHC profiles of the queen (n = 8) and 33-40 workers (n = 303, median = 38). CHCs were extracted by immersing single individuals in 1 ml HPLC-grade pentane (Sigma–Aldrich, Denmark) for 10 min. We let the pentane evaporate at room temperature and then resuspended the extract in 100 µl pentane, of which we injected 2 µl in an Agilent 6890N gas-chromatograph, equipped with a split-splitless injector set to splitless mode, an HP-5MS capillary column (30 m x 250 µm x 0.25 µm), helium carrier gas flow of 1 ml min^{-1}, and a flame ionization detector (FID). After an initial hold of 1 min at 70°C, the temperature was raised to 210°C at a rate of 30°C min^{-1}, then to 280°C at 3°C min^{-1}, and then to 320°C again at 30°C min^{-1}, with a final hold at 320°C of 5 min. Peak areas of 56 hydrocarbons (or mixtures of co-eluting hydrocarbons; Supplementary Figure S1) found on the cuticles of workers and queens were quantified using Agilent ChemStation (v. D.02.00.237). Components were identified by gas chromatography with mass spectrometry (GC-MS) using an Agilent 6890N gas chromatograph coupled with an Agilent 5375 MS with electron ionization (70eV) (Supplementary Figure S1).

Ovary development assessment

For each colony, all collected workers (n = 820, range = 40-195, median = 100) and the queen, which included all individuals used in the CHC analysis, were dissected to assess the state of their ovaries. Each individual was given an 'Ovary Score', where 0 = undeveloped ovaries, 1 = ovary development below ~20 %, 2 = ovary development between ~20% and ~50%, 3 = ovary development between ~50% and ~90%, 4 = completely developed ovaries. In addition, individuals were categorized according to their morphological and physiological status, 'Caste': queen (Q), reproductive worker (RW; Ovary Score $\geq$ 1) or sterile worker (SW; Ovary Score = 0). The percentage of reproductive workers ('% RW') was calculated for each colony.

Genotyping and worker reproduction

Male production by workers was estimated by genotyping 17–34 adult workers (n = 204, median = 25) and 19-41 male pupae and adults (n = 291, median = 39) per colony. Antennae or heads were frozen in liquid nitrogen, ground up and incubated at 95°C for 15 min in 200 µl of a 10% Biorad Chelex 100 resin solution. We used three microsatellite loci developed for *Vespula rufa*: Rufa05, Rufa13 and Rufa18 (Thorén et al. 1995). Polymerase chain reactions (PCRs) were performed in 10 µl reactions using the QIAGEN multiplex kit, with reactions containing 1 µl of template DNA, 5 µl master mix, 0.2 µl of a 10 µM solution of each primer and 2.8 µl of Rnase-free H_2O. Primers were fluorescently labelled with VIC, PET and FAM (Applied Biosystems). The PCR reaction cycle consisted of an initial denaturation at 95 °C for 15 min followed by 30 cycles of 30 s at 95 °C, 90 s at 50 °C and 60 s at 72 °C, and a final extension of 30 min at 60 °C. Genotypes were assessed using internal Genescan 500 LIZ size standards (Applied Biosystems) in an ABI-3130 Avant capillary sequencer

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and allele sizes were scored using GeneMapper software (Applied Biosystems). The percentage of worker-produced males ('% WPM') was calculated as $W_j/(P_jN_j)$, where W_j is the number of detected worker sons, P_j the probability of detecting worker sons, and N_j the total number of males analysed for the jth nest, as in Foster & Ratnieks (2001).

Statistical analysis

Principal component analysis (PCA) was based on integrated peak areas of 56 CHC compounds from 311 individuals (Figure 1). The CHC variables were first normalized using the transformation $y_{ij} = \log(x_{ij}/g(X_j))$ where y_{ij} is the transformed peak area of the ith CHC component of the jth individual, x_{ij} is the untransformed peak area of the ith component of the jth individual, and $g(X_j)$ is the geometric mean peak area of all components of the jth individual (Aitchison 1986). Transformed peak areas were then mean-centred but not scaled to unit variance. Subsequent discriminant analyses (DA) were performed with up to 6 principal components (PC1-6) as variables, since the smallest group (queens) had only 8 individuals.

PC1 was regressed against Ovary Score, Colony Stage, % RW and % WPM, using linear models and using the Akaike Information Criterion (AIC) to select among possible alternative models (Figure 2). In the first two models, PC1 was entered as the dependent variable and Colony was included as a random variable. In the case of Ovary Score the best fit was a model without an interaction with the fixed variable Caste, whereas the interaction was significant in the case of Colony Stage. In the other two models, PC1 was entered as a fixed explanatory variable, in interaction with Caste. Here, the random variable Colony was left out, since the dependent variables

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(% RW, % WPM) were colony-level traits so that Colony did not add any information. We corrected p-values for multiple testing using the Benjamini-Hochberg false discovery rate correction (Benjamini and Hochberg 1995).

To test the association between fertility and single CHC components we used general linear mixed models, with 1000 Markov Chain Monte Carlo simulations to estimate p-values (Figure 3). Each of the 56 components was entered as a dependent variable, Colony was entered as a random effect and Caste (Q, RW or SW) as a fixed effect. Again, p-values were Benjamini-Hochberg corrected to control for multiple testing (Benjamini and Hochberg 1995) and we only regarded $p < 0.01$ as being significant. This level of stringency was used because our variables are non-independent due to normalisation to relative values. For effect size measures of each CHC component, we sum-normalized to relative concentrations (%) before calculating the log2 ratios for Q vs. SW and Q vs. RW (Figure 3). All analyses were performed using R 2.14.0 (packages: FactoMineR, lme4, languageR) and MS Excel 2007.

Comparative analysis of Vespine wasps

Data on log2 ratios between queen and worker CHC profiles were compared between *D. saxonica* and those of *Vespula vulgaris* (Bonckaert et al. 2012) and four other Vespine wasps (Butts et al. 1991) (Figure 2). Data on levels of reproductive workers were compared between *D. saxonica* and *V. vulgaris* (Table 1) based on several studies (Supplementary Table S3; Bonckaert et al. 2011b; Foster and Ratnieks 2000; Foster and Ratnieks 2001; Helanterä et al. 2006, and unpublished data J.S. van Zweden, W. Bonckaert, T. Wenseleers). Evolutionary stable optimum levels of worker reproduction were calculated using formula 1.8 and 2.8 in Wenseleers et al.

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(2004; Supplementary Methods; Supplementary Tables S3-S5; Supplementary Figure S2). This formula calculates the ESS proportion of laying workers per colony as a function of colony size, the relative survival of worker-laid eggs compared to queen-laid eggs, the effect of worker reproduction on colony productivity, species-level average sister-sister relatedness and the relative fecundity of the queen compared to workers in terms of male eggs (see further details in Supplementary Information).

Results

Fertility-linked CHCs of Dolichovespula saxonica

We identified 55 of the 56 CHC peaks found on the cuticles of all individuals (Supplementary Figure S1), which we used as variables in a principal component analysis (PCA; see Supplementary Table S1 for diagnostic details). We found a clear distinction between the reproductive castes queens (Q), reproductive workers (RW), and sterile workers (SW) (Figure 1), and each of PCs 1-5 could discriminate among both colonies and reproductive castes (Supplementary Table S1). In a discriminant analysis (DA) based on PCs 1-6 we could classify 95.5% of the individuals correctly (overall: 297/311; Q: 5/8 (= 62.5 %), RW: 21/30 (= 70.0 %), SW: 271/273 (= 99.3 %). Using PCs 1 and 2 alone, however, already resulted in a strong discrimination among the individuals belonging to different reproductive castes, with PC1 separating reproductives (Q and RW) from non-reproductives (SW) (ANOVA, $F_{1,309} = 254.42$, p < 0.001) and PC2 discriminating between Q and RW ($F_{1,36} = 23.90$, p < 0.001). A discriminant analysis (DA) based on PCs 1 and 2 alone could correctly classify 93.6 % of the individuals (overall: 291/311; Q: 2 out of 8 (= 25.0 %), RW: 18 out of 30 (= 60.0 %), SW: 271 out of 273 (= 99.3 %). Incorrectly classified Q and SW were in all

cases classified as RW instead, whereas 2 incorrect RW were consistently classified as Q and the rest as SW.

We found a positive relationship between the fertility-linked PC1 and the ovary development (Ovary Score) for both Q and RW, with the effect being comparable in the two castes (Figure 2a). Interestingly, the PC1 score of queens also decreased at later stages of colony development, whereas the opposite was true for RW (Figure 2b). For Q, PC1 also had a negative relationship with the percentage of reproductive workers (% RW; Figure 2c) and the percentage of worker-produced males (% WPM; Figure 2d), but these relationships were positive for RW, if anything. This parallels the decrease in queen fecundity and increase in levels of worker reproduction observed near the end of colony development (Bonckaert et al. 2011b).

PC1 was strongly associated with CHC chain length. Nearly all compounds with a backbone of 28 or more carbon atoms had positive loadings and those with less than 28 had negative factor loadings. The alkane compounds n-C_{31}, 3-MeC_{31}, and 3,7-diMeC_{29} had the highest positive loadings (Figure 1; Figure 3; Supplementary Table S2) and thus distinguished reproductive from non-reproductive individuals. PC2 gave high loadings for the alkanes n-C_{28}, n-C_{29}, and 3-MeC_{29}, which thus were more characteristic for queens than for reproductive workers, whereas all the unsaturated hydrocarbons (alkenes) loaded most negatively on PC2 (Figure 3; Supplementary Table S2). Correspondingly, the log2 ratios further confirmed that n-C_{31}, 3,7-diMeC_{29}, 3-MeC_{29}, 3-MeC_{31} and n-C_{29} were most strongly increased in both Q and RW relative to SW (Figure 3; Supplementary Table S2).

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Comparative analysis of Vespine wasps

When we compared *D. saxonica* to other Vespine wasps, we found that several CHCs are consistently increased in Q relative to W across species (Figure 3). In particular, the species *Vespula vulgaris*, *Vespa crabro*, and *Vespula maculifrons* (and to a certain extent *Vespula squamosa* as well) show higher abundance of the alkanes n-C_{29}, 3-MeC_{29} and n-C_{31} on Q compared to workers. In *D. maculata*, however, the alkenes are characteristic of the Q profile (Figure 3). When comparing only *D. saxonica* and *V. vulgaris*, we find that for the former Q and RW profiles are very similar to each other, meaning that in *D. saxonica* CHC profiles are mostly fertility linked, whereas in *V. vulgaris* profiles of RW are very similar to those of SW, meaning that CHC profiles are mostly caste specific and have a much weaker link with fertility (Figure 3). Nonetheless, there is a significant correlation between the fertility-linked compounds found on Q and RW of *D. saxonica* and the Q-specific profiles of *V. vulgaris* (Spearman rank correlation of log2 ratios of overlapping compounds, *D. saxonica* Q/SW vs. *V. vulgaris* Q/SW, Rho = 0.614, df = 41, p < 0.001; *D. saxonica* RW/SW vs. *V. vulgaris* Q/SW, Rho = 0.472, df = 41, p = 0.001), showing a significant conservation of the expression levels of these cues/signals across the two species. The data of other Vespine species were not statistically compared with *D. saxonica* and *V. vulgaris*, because the data were obtained with too divergent methods and machinery and, as far as we are aware, there is currently no species-level calibrated phylogeny available to correct for phylogenetic non-independence. Visual inspection, however, also indicates high abundance of n-C_{29} and 3-MeC_{29} on queens compared to workers in *Vespa crabro*, *Vespula squamosa* and *Vespula maculifrons*, thereby reinforcing the idea of phylogenetic conservation of queen-specific cues across Vespine wasps.

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Levels of worker reproduction of *D. saxonica* and *V. vulgaris* were found to fit well with theoretically predicted values as expected under the queen signalling hypothesis (Table 1; Supplementary Methods; Supplementary Tables S3-S5; Supplementary Figure S2). *D. saxonica* shows a higher species-level average sister-sister relatedness and lower policing efficiency, and observed values of worker reproduction were higher for *D. saxonica* than for *V. vulgaris* in queenright colonies (6.5 % vs. 1.0 %), whereas the opposite was true for queenless colonies (10.0 % vs. 29.7 %),

Discussion

Our results demonstrate that fertile and sterile individuals of the Saxon wasp, *Dolichovespula saxonica*, can be reliably distinguished based on their cuticular hydrocarbon (CHC) profiles. Particular CHCs that load high on the first principal axis of variation moreover correlate well with the queens' levels of ovary development, and the relative abundance of these fertility-linked CHCs decreases and worker reproduction increases with the development of their colonies. Next, when comparing fertility-linked CHCs of *D. saxonica* to those of *Vespula vulgaris* and other Vespine wasps, we found similar components to be increased in queens compared to workers across species. Finally, we found that levels of worker reproduction in *D. saxonica* and *V. vulgaris* followed the prediction of the queen signalling hypothesis, with workers reproducing according to their own best inclusive fitness interests. In particular, in *D. saxonica*, where nestmates are on average more related to each other and policing of worker-laid eggs is less efficient, there is a smaller difference in worker reproduction between queenright and queenless colonies. Therefore, all lines of evidence point towards the validity of the queen signalling hypothesis in *D. saxonica* wasps.

The CHCs that were most specific of queens and reproductive workers in *D. saxonica*, and thus appeared mostly fertility-linked, were several 3-(di)methyl and linear alkanes of long chain length: n-C_{29}, 3-MeC_{29}, 3,7-diMeC_{29}, n-C_{31}, and 3-MeC_{31}. Amongst these, the compounds n-C_{29}, 3-MeC_{29}, and n-C_{31} are also queen-specific in most of the other Vespine wasps investigated here (Figure 3), as well as for example in the wasp *Polistes dominulus* (Sledge et al. 2004) and in the ant *Pachycondyla inversa* (van Zweden et al. 2009). On the other hand, different compounds have been observed to correlate with fertility in other social Hymenopteran species (*e.g.* Liebig et al. 2000; Monnin et al. 1998; Smith et al. 2008). Reproductive individuals of *D. saxonica* generally also appear to shift to longer CHC chain lengths compared to non-reproductives (Figure 3). Such shifts to longer or shorter chain length in relation to fertility have also been observed in other ants and wasps (e.g. Bonckaert et al. 2012; Hannonen et al. 2002; Liebig et al. 2000; Moore and Liebig 2010; van Zweden et al. 2009), suggesting that some of the physiological pathways involved in the production of fertility-linked CHCs are conserved across species. From a biochemical point of view, chain length specificity is, at least in Dipteran species, controlled by a fatty acyl-CoA elongase reaction, which in turn is influenced by ecdysteroid hormones produced by the female ovaries and fat bodies (Blomquist 2010; Chertemps et al. 2007). CHC fertility cues in these species thus appear to arise as a by-product of the shuttling of hydrocarbons to the ovaries. Similar ecdysteroid hormones, and genes involved in the production thereof, are produced in developed *Apis mellifera* ovaries and fat bodies (Robinson et al. 1991; Yamazaki et al. 2011), so that the resulting expression of CHC patterns, even if simple by-products

of ovary development, could be co-opted to function as an honest signal and mediate reproductive division of labour in social insects.

Dolichovespula species are characterised by a high percentage of reproductive workers in queenright colonies (*D. norwegica*, Bonckaert et al. 2011a; *D. saxonica*, Bonckaert et al. 2011b; *D. sylvestris*, Wenseleers et al. 2005). This pattern of worker reproduction appears strong in species with annual life cycles, such as Vespine wasps and bumble bees (Duchateau and Velthuis 1988), although the eventual percentage of worker-produced males varies considerably across species and across stages of colony development (Alaux et al. 2004; Wenseleers and Ratnieks 2006a). In a previous study of the same population but with a more extended data set, we observed that colony development was a strong determinant for the proportion of worker-produced males (Bonckaert et al. 2011b), which is in line with the notion that there should be less worker reproduction and more worker policing in the early ergonomic stages of the colony (Ohtsuki and Tsuji 2009). Indeed, workers may gain fitness benefits by increasing their own reproduction and decreasing efforts towards policing the reproduction of other workers when enough new queens have been produced, when the production of males has started, or the queen's fecundity has dropped due to old age (Alaux et al. 2004; Ohtsuki and Tsuji 2009). Therefore, we expect that worker responses to cues, whether directly from the queen or not, that allude to such an increase in inclusive fitness are selected for amongst most social Hymenoptera, but are most easily observed in annual species such as those of Vespinae and Bombini.

The correlational evidence in this study suggests that the increase in worker reproduction as a function of colony development is caused by the decreasing

fecundity of the queen and the consequential decrease in the queen's CHC fertility signal, which in turn can inform the workers when to start developing their ovaries (Figure 2). Alternatively, the CHC profiles of *D. saxonica* individuals could simply reflect their reproductive status, without necessarily functioning as informative signals. In this case, workers could for example simply be responding to environmental change using a different set of queen-derived or environmental cues that we did not investigate. Under this scenario we might observe a similar pattern as when the CHCs do function as informative signals. However, we deem this unlikely, as we now have evidence from the related species *V. vulgaris* that the CHCs most characteristic for queens can induce worker sterility, suggesting that these compounds do indeed act as queen signalling pheromones (Van Oystaeyen et al. submitted). Based on this, it is therefore reasonable to assume that queen-specific CHCs are also informative signals central in the regulation of division of labour in *D. saxonica*.

Comparison of CHC patterns amongst the different Vespine species indicates that there is strong conservation of the queen-specific compounds between these species, arguing for the validity of the queen signalling hypothesis in social wasps (*cf.* Heinze and d'Ettorre 2009; Keller and Nonacs 1993). Similar to *Lasius* ants, where 3-methyl alkanes characterize the queens' cuticular profiles and in at least two species have a proven function as queen signal (Holman et al. 2013), the linear and 3-methyl alkanes are generally characteristic for queens in the species studied so far in the genera *Dolichovespula*, *Vespula*, and *Vespa* (with the notable exception of *D. maculata*). Focusing only on *D. saxonica* and *V. vulgaris* we further notice that, whereas in the first species CHCs differences are most strongly linked to differences in fertility, similar compounds show caste-specific differences in the latter species (Figure 3).

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This means that in *D. saxonica* the difference lies in the fertility of an individual, almost regardless of it being a worker or a queen, whereas in *V. vulgaris* the difference lies more in the caste of an individual, regardless of their fertility. This is in line with the greater queen-worker size dimorphism observed in *V. vulgaris* (Wenseleers and Ratnieks 2006b) and argues for the evolution of 'queen signals' through co-option of pre-existing 'fertility cues', assuming at least that *D. saxonica* represents the more primitive state. This is interesting, especially considering the idea that CHCs are produced as mere by-products of the shuttling of hydrocarbons towards developing oocytes (see also Blomquist 2010; Smith et al. 2009). What it could mean is that while in a species such as *D. saxonica* the CHC profile is a direct index of an individual's fertility, the CHCs and ovary development of *V. vulgaris* became decoupled and the former could evolve to become a proper signal, with the possibility of individuals hiding or exaggerating their fertility status and/or a different evolutionary mechanism to ensure the signal's honesty. These inferences still require further testing, both at a molecular level and in a phylogenetic setting using a more complete data set of the pheromone profiles of different eusocial species and their reproductive castes.

Lastly, the level of worker reproduction in *V. vulgaris* shows a much larger difference between queenright and queenless colonies than in *D. saxonica*, in line with the queen signalling hypothesis rather than queen control, since this pattern follows the workers' optimum given their differences in species-level average sister-sister relatedness and policing efficiency (Table 1; Wenseleers et al. 2004; Wenseleers and Ratnieks 2006b). An earlier test, based on the comparison of levels of worker reproduction in nine species of Vespine wasps and the honeybee, provided similar support for the

queen signalling hypothesis (Wenseleers and Ratnieks 2006b). In particular, fewer workers attempted to reproduce in queenright colonies of species with low average sister-sister relatedness, because of the stronger selection to effectively police each others' eggs (Ratnieks 1988; Ratnieks et al. 2006), thereby supporting the idea that workers respond to queen signals in their own best interest (Keller and Nonacs 1993; Kocher and Grozinger 2011). To conclude, our study provides several lines of evidence showing that the queen signalling hypothesis is the most parsimonious hypothesis to explain levels of reproductive division of labour in social wasps, thereby helping to resolve a long-standing controversy in evolutionary sociobiology.

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Figure Legends

Figure 1. A PCA based on cuticular hydrocarbon (CHC) profiles of queens (n = 8), reproductive workers (n = 28) and sterile workers (n = 273) of eight colonies of *Dolichovespula saxonica*. PC1 separates egg laying (queens and reproductive workers) and non-egg laying individuals (sterile workers), while PC2 separates queens and reproductive workers. The lower panel shows the factor loadings and has six compounds highlighted that most strongly correlated with fertility (see also Figure 3). PCA diagnostics are given in Supplementary Table S1 and exact loadings are given in Supplementary Table S2. Two outlier reproductive workers with negative scores on PC1 were left out.

Figure 2. The scores of queens (Q; black dots; n = 8) and reproductive workers (RW; grey dots; n = 28) on the fertility-associated axis PC1 (see Figure 1) regressed against **a)** their Ovary Score, and **b)** the stage at which colonies were in their development (Colony Stage). **c)** The percentage of reproductive workers in their colonies (% RW), and **d)** the percentage of worker-produced males in their colonies (% WPM) regressed against PC1 scores. Two outlier reproductive workers with negative scores on PC1 were left out.

Figure 3. Heat map of how components of the cuticular hydrocarbon (CHC) profile correlate with fertility in *Dolichovespula saxonica*, *Vespula vulgaris*, and other Vespinae (red = positive values, green = negative values, black = zero). Exact values are given in Supplementary Table S2. Details of the GLMM and PCA are given in the text. Log2 ratios are calculated as the $\log_2$ of the ratio of the means. References for *Vespula vulgaris*: (Bonckaert et al. 2012), *Vespa crabro*, *D. maculata*, *Vespula*

squamosa, and *Vespula maculifrons*: (Butts et al. 1991). The colour version of this figure only appears online.

a
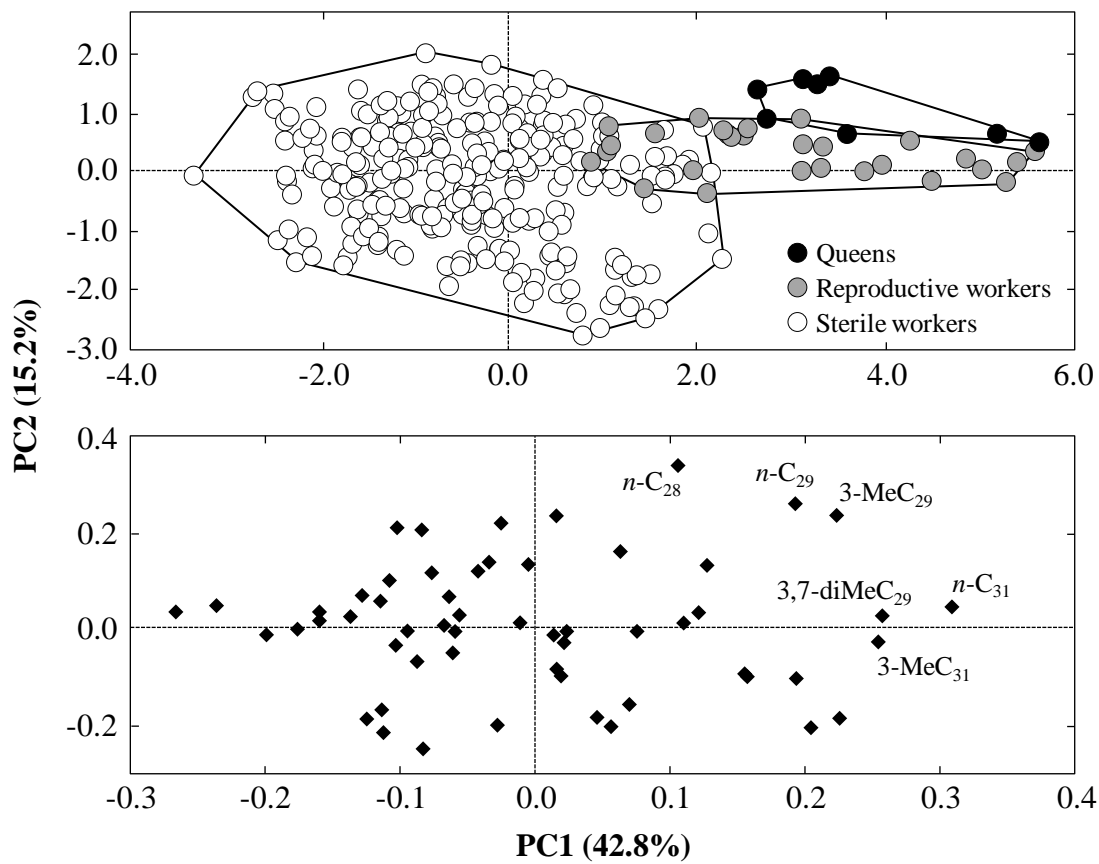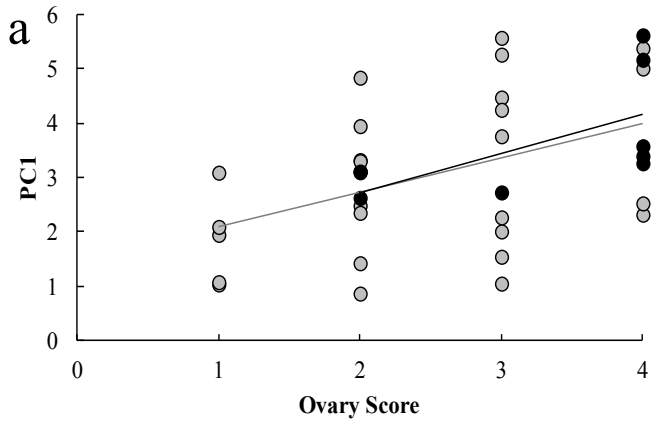
Variable	Estimate	t	p
(Intercept)	0.50	0.88	0.384
Ovary Score	0.89	5.81	**< 0.001 *****

b
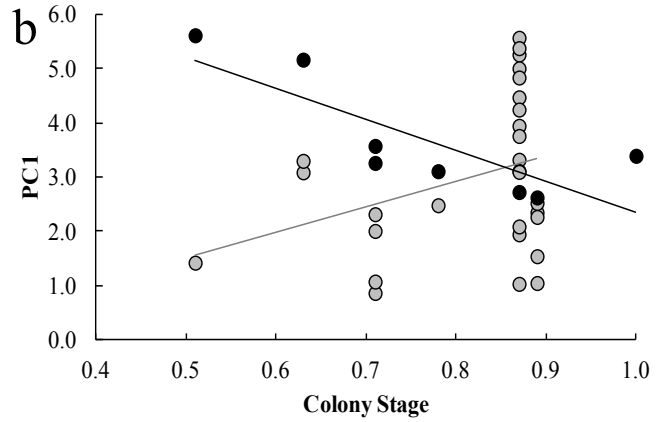
Variable	Estimate	t	p
(Intercept) Q	8.03	3.26	**0.003 ****
Colony Stage	-5.68	-1.79	0.083
Caste RW	-8.47	-3.02	**0.005 ****
Colony Stage * Caste RW	9.62	2.67	**0.012 ***

c
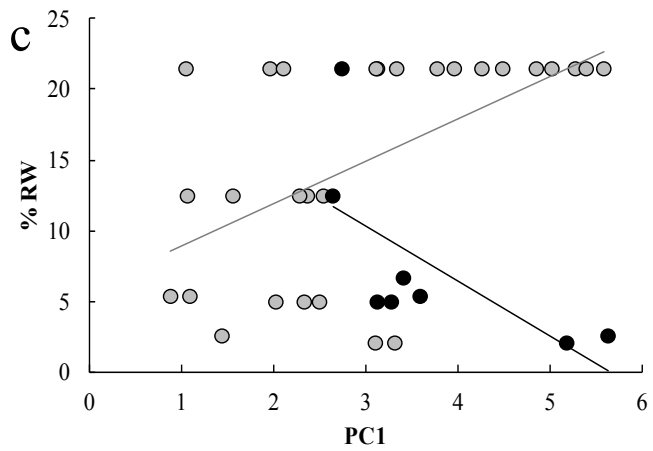
Variable	Estimate	t	p
(Intercept) Q	21.96	2.63	**0.013 ***
PC1	-3.88	-1.78	0.084
Caste RW	-16.06	-1.82	0.078
PC1 * Caste RW	6.89	2.95	**0.006 ****

d
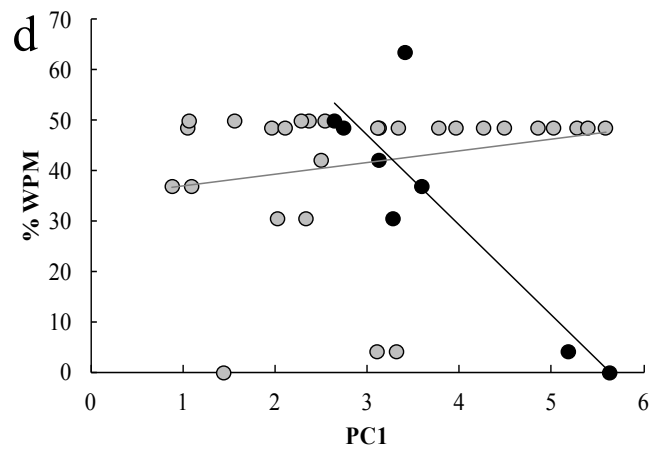
Variable	Estimate	t	p
(Intercept) Q	100.66	5.44	**< 0.001 *****
PC1	-17.87	-3.71	**< 0.001 *****
Caste RW	-65.97	-3.38	**0.002 ****
PC1 * Caste RW	20.17	3.90	**< 0.001 *****

		D. saxonica				V. vulgaris		Other Vespinae			
			PCA		Log2 ratio		Log2 ratio		Log2 ratio			
Peak #	Component	GLMM: Q vs. SW, p < 0.01 / GLMM: RW vs. SW, p < 0.01 / Average Percentage	PC1 loading	PC2 loading	Q vs. SW	RW vs. SW	Q vs. SW	RW vs. SW	Q vs. W - V. crabro	Q vs. W - D. maculata	Q vs. W - V. squamosa	Q vs. W - V. maculifrons

Peak #	Component	GLMM Q	GLMM RW
1	n-C$_{23}$	✓	✓
2	7-,9-,11-MeC$_{23}$	✓	✓
a	5-MeC$_{23}$		
3	3-MeC$_{23}$	✓	✓
b	5,x -diMeC$_{23}$		
4	n-C$_{24}$	✓	✓
5	3,7-diMeC$_{25}$	✓	✓
6	8-MeC$_{24}$	✓	✓
c	6-MeC$_{24}$		
d	4-MeC$_{24}$		
7,8	x-C$_{25:1}$ (+isomer)	✓	✓
9	4,8-diMeC$_{24}$	✓	✓
10	n-C$_{25}$	✓	✓
11	7-,9-,11-,13-MeC$_{25}$	✓	✓
12	5-MeC$_{25}$	✓	✓
13	3-MeC$_{25}$	✓	✓
14	5,9-diMeC$_{25}$	✓	✓
15	n-C$_{26}$		
16	3,9-diMeC$_{25}$		
17	10-,11-,12-,13-MeC$_{26}$		✓
18	5-MeC$_{26}$	✓	✓
19	4-MeC$_{26}$	✓	✓
20,21	x-C$_{27:1}$ (+isomer)	✓	✓
22	4,y-diMeC$_{26}$	✓	✓
23	n-C$_{27}$		✓
24	9-,11-,13-MeC$_{27}$	✓	✓
25	7-MeC$_{27}$	✓	✓
26	5-MeC$_{27}$	✓	✓
27	11,15-diMeC$_{27}$	✓	✓
28	3-MeC$_{27}$	✓	✓
29	5,11-diMeC$_{27}$		
30	5,y-diMeC$_{27}$ + x-C$_{28:1}$	✓	✓
31	n-C$_{28}$	✓	✓
32	3,11-, 3,13-diMeC$_{27}$	✓	✓
33	3,7-diMeC$_{27}$	✓	✓
34	11-,12-,13-,14-MeC$_{28}$		✓
35	6-MeC$_{28}$		
36	4-MeC$_{28}$	✓	✓
37,38	x-C$_{29:1}$ (+isomer)		
e	4,x-diMeC$_{28}$		
39	n-C$_{29}$	✓	✓
40	9-,11-,13-,15-MeC$_{29}$	✓	✓
41	7-MeC$_{29}$	✓	✓
42	5-MeC$_{29}$	✓	✓
43	9,13-, 11,15-, 13,17-diMeC$_{29}$		
44	3-MeC$_{29}$	✓	✓
45	5,y-diMeC$_{29}$	✓	✓
f	n-C$_{30}$		
46	3,9-, 3,11-, 3,13-, 3,15-diMeC$_{29}$	✓	✓
47	3,7-diMeC$_{29}$	✓	✓
48	12-, 13-, 14-, 15-MeC$_{30}$	✓	✓
49	4,y-diMeC$_{30}$	✓	✓
50	x-C$_{31:1}$	✓	✓
51	unidentified	✓	✓
52	n-C$_{31}$	✓	✓
53	13-, 15-MeC$_{31}$		
54	11,15-diMeC$_{31}$		
55	3-MeC$_{31}$ + 7,13-, 7,15-diMeC$_{31}$	✓	✓
56	5,y-diMeC$_{31}$	✓	✓
g	13-, 15-MeC$_{33}$		

Legend — Percentage: >13 / 0; PCA: >0.3 / 0.0 / <-0.3; Log2 ratio: >3 / 0 / <-3

Table 1. Data on the average colony kin structure, the presence or absence of policing by the queen or workers and the observed and predicted (evolutionarily stable) percentage of reproductive workers in queenright and queenless colonies of the two Vespine wasps, *Dolichovespula saxonica* and *Vespula vulgaris* (see Methods S1), where chemical fertility cues have been characterized in detail. Sample sizes (colonies) are shown in brackets.

	D. saxonica	*V. vulgaris*	References
Effective queen mating frequency (# of colonies)	1.4 (35)	1.9 (17)	Bonckaert et al. 2011b; Foster and Ratnieks 2000; Foster and Ratnieks 2001
Worker-worker relatedness (# of colonies)	0.61 (35)	0.51 (17)	Bonckaert et al. 2011b; Foster and Ratnieks 2000; Foster and Ratnieks 2001
Average colony size in terms of workers (# of colonies)	113 (25)	2042 (46)	Bonckaert et al. 2011b; Helanterä et al. 2006
Queen policing present?	yes	no	Foster and Ratnieks 2001), T. Wenseleers, unpublished data
Worker policing present?	perhaps[a]	yes	Bonckaert et al. 2011b; Foster and Ratnieks 2000; Foster and Ratnieks 2001
Effectiveness of policing[b]	0.88	0.99-1[c]	reviewed in Wenseleers and Ratnieks 2006a
Observed % RW in queenright colonies (# of colonies)	6.5% (22)	1.0% (6)	Table S3
Expected ESS % RW in queenright colonies[d]	7.9%	0.0%	Supplementary Methods; Wenseleers et al. 2004
Observed % RW in queenless colonies (# of colonies)	10.0% (19)	29.7% (10)	Table S3
Expected ESS % RW in queenless colonies[d]	16.9%	23.4%	Supplementary Methods; Wenseleers et al. 2004

[a] Foster & Ratnieks (2000) and Bonckaert et al. (2011b) provided evidence that the proportion worker-laid eggs and the proportion worker-produced males surviving to adulthood differ significantly, but there was no direct behavioural data on worker policing. Behavioural data from other *Dolichovespula* species have shown that although workers sometimes cannibalize worker-laid eggs, this policing is not very effective, since these workers often lay eggs themselves (Bonckaert et al. 2011a; Wenseleers et al. 2005).

[b] Defined as $(1-S_w)$, where S_w is the probability that worker-laid eggs survive to adulthood relative to queen-laid eggs (Wenseleers et al. 2004).

[c] In policing bioassays all of the 120 introduced worker-laid eggs were removed within 16 hours, which would imply a policing efficiency of 1. Nevertheless, a more extensive study on the related species *V. germanica* has estimated the effectiveness of policing at 0.99 (Bonckaert et al. 2008), and this is probably quite a realistic estimate also for *V. vulgaris*.

[d] Predicted evolutionarily stable percentage of reproductive workers in queenright and queenless colonies, as described in the Methods S1.