Cuticular hydrocarbon cues of immune-challenged workers elicit immune activation in honeybee queens

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Abstract
Recently, evidence has shown that variations in the cuticular hydrocarbons (CHCs) profile allow healthy honeybees to identify diseased nestmates, eliciting agonistic responses in the former. Here, we determined whether these ‘immunologic cues’ emitted by diseased nestmates were only detected by workers, who consequently took hygienic measures and excluded these individuals from the colony, or whether queens were also able to detect these cues and respond accordingly. Healthy honeybee queens were exposed to (i) healthy, (ii) Ringer-injected and (iii) lipopolysaccharide (LPS)-injected nestmates by allowing direct body contact. Quantitative differences in the CHC profiles of these three groups were measured using GC-MS. The transcript levels of the products of four genes that encode for antimicrobial peptides (AMPs), which are part of the queen’s immune response, were measured in bees exposed to direct contact using qPCR. A significant increase in the transcript levels of these AMP genes over baseline levels in queens was observed when body contact was allowed between the queens and the Ringer- and LPS-injected nestmates. These results provide the first evidence that the detection of CHCs contributes to the initiation of an immune response in insects. In an additional experiment, CHCs were extracted from diseased workers and directly presented to queens, which also evoked a similar immune response. A potential mechanism that relied on volatile compounds could be ruled out by conducting a distance experiment. The study helps to expand our knowledge of chemical communication in insects and sheds light on a likely new mechanism of social immunity.

Keywords: antimicrobial peptides, chemical communication, cuticular hydrocarbons, honeybee queens, immune system

Introduction
Chemical cues are commonly used to facilitate communication among social insects such as honeybees. These cues are crucial for effective colony function because they play essential roles in nestmate recognition, discrimination and recognition of individuals and caste determination (Howard 2005; Le Conte & Hefetz 2008). As an example of a volatile cue, adult worker bees exposed to honeybee queen mandibular pheromone (QMP) display temporal changes in the expression of hundreds of genes in their brains, the chronic activation of a small subset of genes involved in nursing and the repression of genes involved in foraging (Grozinger et al. 2003). In a more recent study, QMP and (E)-beta-ocimene (eβ) were reported to suppress ovary activation among honeybee workers and positively influence hypopharyngeal gland (HPG) development (Traynor et al. 2014). Similarly, brood pheromone (BP) evokes changes in the expression of several genes that are upregulated in nurse bees and, consequently, delays the onset of foraging (Alaux et al. 2009).

Another type of signalling and chemical communication in social insects is mediated by cuticular hydrocarbons (CHCs). These components have no or little
volatility and, therefore, direct contact is required for their perception (Howard 2005). Hydrocarbons function as recognition pheromones, and the temporary expression, quantity and production of these substances can be altered in individuals in order to influence communication (Dani et al. 2005; Richard & Hunt 2013). These cuticular lipids are complex mixtures, which primarily consist of long-chain hydrocarbons such as alkanes and alkenes. Individuals that belong to each honeybee colony produce a unique CHC profile, the nature of which is primarily dependent on the quantitative proportions of individual hydrocarbons (Howard 2005). CHCs act as chemical cues and have been shown to be involved in nestmate recognition, intracolony and species recognition, fertility and sex recognition in ants, termites, wasps and bees (Smith & Breed 1995; Lorenzi et al. 1996, 1997; Singer 1998; Cullivier-Hot et al. 2002; Dietemann et al. 2003; Greene & Gordon 2003; Dani et al. 2005; Richard et al. 2007).

In addition, CHC profiles can serve to discriminate between healthy and sick nestmates of a colony (Salvy et al. 2001; Richard et al. 2008; Baracchi et al. 2012). Richard et al. (2008) characterized CHC profiles of naïve honeybee workers and compared these with the CHC profiles of bees that had been injected with saline solution or LPS. The authors reported that substantial alterations in the profiles of the latter groups were observed, with more significant changes seen in LPS-injected bees. Furthermore, healthy (untreated) nestmates were observed to respond differently to treated individuals than untreated individuals. When healthy individuals were coated with extracts containing the CHCs of injected individuals, the uncoated nestmates displayed antagonistic behaviour towards these individuals. In a complementary study, honeybee workers were injected with saline solution, beads or bacteria in an attempt to stimulate an immune response. Subsequent genetic analyses of these injected bees revealed major alterations in gene expression patterns in the fat bodies and epithelial tissues, including the oenocytoids (i.e. immune cells that are primary biosynthetic sites for hydrocarbons) (Richard et al. 2012). Several genes that were involved in immune responses, spermatogenesis and tissue development were upregulated, as well as two genes involved in lipid metabolism. These genes are of particular interest as fatty acids are the biosynthetic precursors of cuticular hydrocarbons in insects. Parasitism of honeybee larvae by *Varroa* mites or infection by DWV has also been reported to change the relative proportion of cuticular hydrocarbons produced by pupae and emerging adult bees. These parasitized or infected individuals displayed significantly different CHC profiles as compared to unparasitized or healthy individuals (Salvy et al. 2001; Baracchi et al. 2012).

Social insects such as honeybees generally live in small spaces, creating ideal conditions for both the high levels of social and physical contact and the transmission of pathogens and parasites among colony individuals (Comman et al. 2012). Within the colony environment, the pathogenic pressures might vary between different castes and groups of workers (Baracchi & Cini 2014). Only one-third of the genes that have been identified to be involved in immunity in the *Anopheles* or *Drosophila* genomes have been identified in honeybees (Evans et al. 2006; Sadd et al. 2015). Nonetheless, the four pathways (Toll, Imd, Jak-STAT and JNK) that are known to be involved in immune responses in invertebrates are functional in honeybees. Social immunity, which comprises cooperative activities performed to control, diminish or avoid the risk of infection, has been assumed to compensate for this lack of genes (Cremer et al. 2007; Wilson-Rich et al. 2009). Our previous research has shown that honeybee queens that have been previously exposed to an immune challenge can influence the immune competence of subsequently produced progeny, namely, increasing their resistance to infectious agents that may prevail in the colony (Hernández López et al. 2014). This phenomenon, which is known as trans-generational immune priming (TgIP), has also been reported in other species of invertebrates (Little et al. 2003; Sadd et al. 2005; Moret 2006; Freitak et al. 2009; Roth et al. 2010; Tidbury et al. 2011; Zanchi et al. 2011). A plausible molecular mechanism for the transfer of immunity to offspring has been proposed (Salmela et al. 2015). Briefly, the immune priming of the honeybee larvae seems to be mediated by the active transfer of bacterial cell wall fragments from the haemocoel of the queen to the eggs she lays. This mechanism implies that queens must come into direct contact with the infectious agent (e.g. receive it orally), but little is known about the occurrence of apparent infections of honeybee queens in colonies.

Initially, we posed the question of whether queens could distinguish between healthy and diseased nestmates (due to the presence of an infectious agent in the colony) by detecting their emitted chemical cues without becoming infected themselves. Previous research had clearly indicated that diseased nestmates display CHC profiles that differ from those of healthy nestmates and that the latter recognize these profile differences and display altered behavioural responses (Richard et al. 2008; Baracchi et al. 2012). Diseased nestmates rely on humoral responses such as the production of antimicrobial peptides (AMP) or phenoloxidase and cellular responses such as nodulation, encapsulation and melanization to cope with infections (Schmid-Hempel 2005; Evans et al. 2006). In this study, we immune-challenged worker bees and examined their chemical profiles as well as the genetic expression levels.
of four AMPs to determine whether alterations in the CHC profiles and an initiation of an immune activation could be observed. Subsequently, we exposed queens to immune-challenged workers to determine whether a humoral response was initiated in the former and whether this response was dependent upon direct physical contact. Finally, we prepared ablations that contained CHCs from differently treated workers and presented them on paper discs to queens to collect additional evidence that CHCs contributed to the immune activation observed in queens.

Materials and methods

Rearing of honeybee queens

All honeybees used in this study (Apis mellifera subsp. carnica) were kept in the garden apiary of the University of Graz under normal living conditions. New waxless comb frames were used to rear queens; each frame contained two stacked wooden rows, each with 10–15 queen cups. Queenless colonies were used to introduce frames that had been previously grafted with larvae <12 h old, to be reared as queens. Each week, frames with grafted larvae were introduced into the colonies, and frames from the previous week that contained already-capped queen cells were taken out. These were then kept in an incubator (34.5 °C, approx. 60% humidity). Queen cells were placed in individual plastic cages, which were stacked on top of a plastic box that contained ~200 nurses, which were separated from the queen cages by a metal mesh. The nurses were supplied with pollen and sugar solution (50%) ad libitum. This set-up ensured that all newly emerged queens were kept apart from one another and could be nursed immediately after emerging by workers at any time. One- to two-week-old virgin queens were used in this study.

Queen contact with immune-challenged workers

This experiment was carried out to test the hypothesis of whether queens could detect immune-challenged workers and, consequently, whether the immune response would be activated (indicated by an upregulation in the production of AMP transcripts as compared to baseline values). Every week, queens were separated into different experimental groups. Similarly, 5-day-old worker bees that had emerged from combs maintained in an incubator were used in the immune challenge experiment. These bees were assigned to one of the following groups: naïve workers, 2 μL Ringer-injected workers or 2 μL LPS-injected workers. The concentration of LPS injected was 0.5 mg/mL. Emerged queens were introduced in separate plastic cages containing thirty naïve, Ringer-injected or LPS-injected worker bees and supplemented with sugar solution and pollen ad libitum. Queens were permitted to have direct contact with each of the three groups of worker bees (which functioned as nurses to feed queens) for 48 h prior to being snap-frozen in liquid N2 for subsequent genetic analyses. Two sets of worker bees were also frozen either 24 or 48 h after injection of Ringer or LPS to study the dynamics of the four selected AMPs. To exclude diet-related effects, the same fresh pollen and sugar solution was fed to the workers in all experimental groups.

Queen kept under spatial separation from immune-challenged workers

This experiment was designed to test the hypothesis of whether direct contact between the immune-challenged workers and queens positively correlated with immune activation in queens, which would support the involvement of nonvolatile CHC cues in immune activation. This experiment required a special design; specifically, a spatial barrier was provided that prevented direct contact between the queens and LPS-treated bees. Again, 5-day-old worker bees were assigned to either a naïve group or an LPS-injected group. Thirty naïve or LPS-injected workers were introduced into the lower compartment of plastic cages. This lower compartment was split into two smaller compartments, which were separated by 4–5 cm of empty space in the middle. In the upper compartment, 5–6 nurse bees that had previously nursed queens in the incubator directly after their emergence (prior to the experiments) were introduced to each cage. In this way, any physical contact between queens and injected bees or between nurse bees and injected bees was prevented. 48 h later, queens were snap-frozen in liquid N2 until genetic analyses could be conducted. As preliminary results had indicated that this hypothesis would not be supported (i.e. direct contact was required for immune activation to take place), the spatial separation experiment was not additionally conducted with Ringer-injected workers.

Hydrocarbon extraction and presentation to queens

HCs were extracted from the cuticle of naïve or immune-challenged workers and presented to naïve queens in order to test the hypothesis of whether chemical changes in worker CHC profiles could be detected by queens and elicit an immune activation response. 5-day-old worker bees were injected with either Ringer or LPS solution. 24 h later, HCs were extracted. For this purpose, thirty frozen naïve, Ringer or LPS bees were placed in a small beaker that contained 6 mL of n-hexane (HPLC analytical grade) and agitated gently at RT.
for 10 min to extract the nonpolar compounds from the cuticle surface. By following this extraction method, we could ensure that only hydrocarbons were present in the extract (Richard et al. 2008, 2012). The workers were then removed from the n-hexane solution, and one paper disc per queen (n = 7 for the naïve, n = 5 for the Ringer and n = 6 for the LPS group) was soaked in the extract and allowed to air-dry until the solvent had evaporated. These paper discs impregnated with CHCs were presented to queens. Each disc was gently rubbed against the antennae of one queen to ensure direct physical contact and, subsequently, queens were returned to their individual cage with the paper disc. All queens were kept in individual cages in the incubator for 24 h, after which they were snap-frozen in liquid N₂ until genetic analyses were conducted.

Hydrocarbon analyses

Hydrocarbon profile analyses were conducted using the GC-MS method of (Richard et al. 2008). Briefly, naïve, Ringer-injected and LPS-injected honeybees from the same colony were individually soaked in n-hexane (HPLC grade) for 8 min, and the eluate was dried under a flow of nitrogen. After the solvent had evaporated, the residue was redissolved in 100 µL n-hexane and injected (1 µL, splitless) onto a GC-MS column.

EI-MS were recorded using an Agilent Technologies HP 7890A instrument fitted with a detector HP 5975C VL MSD (70 eV, ion source 250 °C, quadrupole temperature 150 °C). The column used was an Agilent HP-5MS (30 m, ID 0.25 mm, film 5% phenyl: 95% methylpolysiloxane 0.25 µm). The oven temperature was held at 45 °C for 2 min and programmed to climb up to 200 °C at a rate of 15 °C/min, then increase up to 300 °C at a rate of 5 °C/min and then held at this temperature for 20 min. Helium was used as the carrier gas. Hydrocarbons were analysed using the NIST atomic spectra database 4.0 and a series of alkane standards.

Genetic analyses of genes related to immune activation

RNA extraction and cDNA synthesis. An RNA isolation kit was used to extract total RNA from individual queens and workers following the manufacturer’s protocol (NucleoSpin RNAII; Macherey-Nagel). A DNase incubation step is already included in the protocol. First-strand cDNA was generated from ~250 ng of total RNA from queens (500 ng from workers) using an M-MLV Reverse Transcriptase kit (Invitrogen). cDNA products were diluted 1:5 in Milli-Q H₂O, and 3 µL of the solution was used as starting material for qPCR. Real-time quantitative PCR. Gene amplification was performed in a 12-µL reaction mixture using a KAPA SYBR FAST Universal qPCR Kit (KAPA Biosystems). The oligonucleotide primers used in this study are shown in Table 1. Four antimicrobial peptides (AMPs) related to immune response in insects were analysed: abaecin, defensin1, hymenoptaecin and apidaecin. Actin was used as ‘housekeeping gene’ to normalize the AMP gene transcript levels. PCRs were carried out using a Rotor-Gene RG-3000 (RCorbett) instrument. The cycling program had an initial pre-incubation step of 95 °C for 3 min followed by 40 cycles of 95 °C for 3 s, 59 °C for 20 s and 72 °C for 20 s. Fluorescence was measured during each cycle at the end of the elongation step. This procedure was followed by a melt curve analysis over an increasing temperature range from 55 to 95 °C to confirm the product size. qPCR products of some samples were randomly run in an electrophoresis gel to confirm the presence of a single amplicon.

Statistical analysis

The software package SPSS v. 22 was used to conduct statistical analyses. Genetic data presented a normal distribution and were tested for statistical significance by conducting a one-way ANOVA test followed by post

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Table 1 Primers used for amplification of AMP genes

| Primer name | Sequence 5’–3’ | References |
|-------------|----------------|------------|
| Actin-F     | TTGTATGCAACACTGTCCATT | (Simone et al. 2009) |
| Actin-R     | TGGCGGCGATGATCTTAAATT | (Simone et al. 2009) |
| Abaecin-F   | CACGATCGATCTGATCAACA | (Evans 2006) |
| Abaecin-R   | GACACAGAAAACTTGGAAAC | (Evans 2006) |
| Defensin1-F | TGGCCTCTTAACTTCGTCAGC | (Evans 2006) |
| Defensin1-R | AATGCGACCTTAAACGAAACG | (Evans 2006) |
| Hymenopt-F  | CTCTTCTCTGCCGGTCATA | (Evans 2006) |
| Hymenopt-R  | CGCTTCTCTGCATCTCCATT | (Evans 2006) |
| ApidNT-F    | TTTTGCCTTACGAAATCTCCGG | (Simone et al. 2009) |
| ApidNT-R    | GTAGTGCGAGTAGCGGATCT | (Simone et al. 2009) |

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hoc Tukey’s HSD or Dunnett (two-sided) tests. The Kruskal–Wallis test was applied in order to compare the chemical profiles of worker bees (data were not normal distributed), and pairwise comparisons were made by applying the Mann–Whitney U-test. The Bonferroni correction was used (due to multiple testing) to obtain significances. After detection of compounds, we selected the 26 most abundant compounds for analysis as they were detected in all analysed samples. A further discriminant analysis was conducted in order to test whether groups could be separated with regard to their treatment (discriminant variables). In this analysis, all compounds with a relative proportion below 1% were discarded. To perform a cross-validation, the leave-one-out technique was performed.

Results

Immune activation was detected in queens that had direct contact with immune-challenged workers, but not in queens that were spatially separated from immune-challenged workers

The expression levels of four AMP genes in queens were determined. The normalized gene transcript levels of abaecin, defensin1, hymenoptaecin and apidaecin of queens that had either had direct contact with Ringer- or LPS-injected workers or had been prevented from coming into direct contact (i.e. through spatial separation) with LPS-injected workers were compared to the transcript levels of these AMPs in naïve queens. To conduct statistical analysis, we initially grouped all queens that had had contact with workers (regardless of their treatment) and queens that had been prevented from contact with workers and compared the AMP transcript levels of members of these groups to those of control queens. A significant increase in the mRNA levels of three of four AMPs was observed in queens that had had direct contact with immune-challenged workers, whereas the transcript levels of all four AMPs in queens that had had no contact with immune-challenged workers were similar to those measured in the controls. The one-way ANOVA revealed significant differences between the gene expression levels of abaecin, defensin1 and hymenoptaecin, but not of apidaecin, as measured in queens that had had contact with workers ($n = 25$), and the levels that were measured in the controls ($n = 13$) (Tukey’s HSD; $F$ ratio = 8.16, $P = 0.001$, $F$ ratio = 5.43, $P = 0.030$, $F$ ratio = 1.60, $P = 0.002$ and $F$ ratio = 7.60, $P = 0.349$, respectively). No significant differences were found in the gene expression levels measured for any of the AMPs in queens that had been prevented from contact with LPS-treated workers ($n = 8$) and the levels that were measured in the controls ($n = 13$) (Tukey’s HSD; $F$ ratio = 8.16, $P = 0.719$ for abaecin, $F$ ratio = 5.43, $P = 0.926$ for defensin1, $F$ ratio = 1.60, $P = 0.694$ for hymenoptaecin, and $F$ ratio = 7.60, $P = 0.965$ for apidaecin) (Fig. 1). Our results provide clear support for the hypothesis that honeybee queens are capable of recognizing diseased nestmates (i.e. distinguishing them from healthy nestmates), that this recognition is facilitated through physical contact and that a positive correlation between this direct contact and the immune activation response can be observed.

Queens that were allowed contact with Ringer-injected workers displayed significantly higher transcript levels

![Fig. 1](image-url) Mean gene transcript levels of four genes related to immune response: (a) abaecin, (b) defensin1, (c) hymenoptaecin, (d) apidaecin. Error bars represent standard error. Control, $n = 13$; queens with no contact to LPS-injected workers, $n = 8$; queens with contact to Ringer-injected workers, $n = 12$; queens with contact to LPS-injected workers, $n = 13$. © 2017 The Authors Molecular Ecology Published by John Wiley & Sons Ltd
for the genes abaecin (threefold) and hymenoptaecin (3.9-fold) \((n = 12)\), as revealed by an ANOVA of the data, as compared to the control queens (Dunnett post hoc test; \(F\) ratio = 7.99, \(P = 0.039\) and \(F\) ratio = 7.66, \(P = 0.024\), respectively). The transcript levels for defensin1 and apidaecin, however, did not significantly differ between these groups \((P > 0.05)\). In contrast, queens that had had direct contact with LPS-injected workers \((n = 13)\) displayed significantly higher transcript levels for abaecin (7.5-fold), defensin1 (2.6-fold) and hymenoptaecin (7.3-fold) as compared to control queens (Dunnett post hoc test; \(F\) ratio = 7.99, \(P = 0.001\), \(F\) ratio = 3.69, \(P = 0.025\), \(F\) ratio = 7.66, \(P = 0.001\), respectively). Again, the transcript levels of apidaecin did not differ between these groups \((P > 0.05)\).

### Queen immune response activation observed upon detection of the hydrocarbon profile

The exclusive extraction of hydrocarbons from the cuticle of adult bees (Richard et al. 2012) allowed us to obtain cuticular hydrocarbon samples that could either be presented to the queen on a paper disc or analysed by GC-MS.

The CHCs of thirty adult bee workers that had either been injected with Ringer or LPS solutions were extracted 24 h after challenging the bees. These CHCs were presented to new virgin queens on paper discs 1 day before genetic analyses were conducted. The normalized gene transcript levels of all four AMP genes for these queens were compared to the transcript levels measured for control queens using a one-way ANOVA test. Queens that had come into direct contact with CHCs extracted from Ringer-injected workers \((n = 5)\) displayed higher transcript levels of hymenoptaecin as compared to controls \((n = 7)\) (Dunnett post hoc test; \(F\) ratio = 4.31, \(P = 0.016\)), but the abaecin, defensin1 and apidaecin levels did not differ from those of the controls \((P > 0.05)\). In contrast, queens that had come into direct contact with CHCs extracted from LPS-injected workers \((n = 6)\) displayed higher transcript levels of defensin1 as compared to controls \((n = 7)\) (Dunnett post hoc test; \(F\) ratio = 7.06, \(P = 0.002\)) (Fig. 2), but abaecin, hymenoptaecin and apidaecin transcript levels did not differ from those of the controls \((P > 0.05)\). These results are particularly interesting in the light of the results of the previous experiment and provide additional evidence that supports the hypothesis that queens are capable of recognizing immune-challenged nestmates through either direct contact with immune-challenged workers or CHC extracts from the immune-challenged worker. It is significant that a positive correlation between this contact and immune activation responses in queens could be observed.

### Immune activation in workers 24 and 48h after challenge with Ringer solution or LPS

The immune activation in workers used for experimentation was confirmed by measuring the gene expression levels of the four selected AMPs 24 and 48 h after challenge with Ringer or LPS (Fig. 3). A significant increase in the mRNA levels of three of four or in all four AMPs was observed 24 h after workers were injected with Ringer solution or LPS, respectively (Tukey’s HSD; \(F\) ratio = 14.59, \(P = 0.003\) for abaecin, \(F\) ratio = 22.99, \(P < 0.000\) for defensin1, \(F\) ratio = 13.61, \(P = 0.010\) for...
hymenoptaecin, and \( F \) ratio = 10.81, \( P = 0.450 \) for apidaecin in 24 h Ringer workers and \( F < 0.000 \) for abaecin, defensin1 and hymenoptaecin and \( P = 0.010 \) for apidaecin in 24 h LPS workers). Two days after being injected with Ringer solution, the mRNA transcript levels of the three AMP genes that had been upregulated in workers injected with Ringer solution returned to their baseline level (i.e. no significant differences could be found as compared to naïve workers (\( P > 0.05 \)). In contrast, the transcript levels of all four AMPs still showed significant differences as compared to controls 48 h after workers were injected with LPS (Tukey’s HSD; \( F \) ratio = 14.59, \( P = 0.002 \) for abaecin, \( F \) ratio = 22.99, \( P < 0.000 \) for defensin1, \( F \) ratio = 13.61, \( P = 0.001 \) for hymenoptaecin, and \( F \) ratio = 10.81, \( P = 0.026 \) for apidaecin). The observed effect of immune activation in worker bees with Ringer solution was weaker as compared to the immune activation observed with LPS.

**Hydrocarbon profiles of naïve, Ringer-injected and LPS-injected workers**

The statistical analyses of the cuticular profiles of differentially treated worker bees revealed significant differences in the proportions of the cuticular hydrocarbons among groups (see Table S1, Supporting information for statistics and list of compounds). Due to multiple testing and the number of variables (26 compounds were selected for analysis), a Bonferroni correction was used, and only \( P \)-values below 0.0019 (alpha-value) were considered to be statistically solid. An additional discriminant analysis was carried out, and significant differences in the cuticular profiles of the naïve, Ringer-injected or LPS-injected workers were revealed [test function 1 through 2, Wilk’s \( \lambda = 0.008 \), \( \chi^2(46) = 97.32 \), \( P < 0.0001 \); test function 2, Wilk’s \( \lambda = 0.129 \), \( \chi^2(22) = 40.93 \), \( P < 0.008 \); 70.6% of cross-validated cases were correctly classified] (Fig. 4).
Discussion

Honeybees are capable of modulating complex social interactions such as gender and caste recognition, intracolony divisions of labour, the reproductive status of individuals or distinction between healthy and diseased nestmates by detecting chemical cues. These processes have been shown to be frequently mediated via volatile [e.g. pheromones such as queen mandibular pheromone (QMP) and brood pheromone (BP)] and nonvolatile (e.g. CHC) cues (Howard 2005; Alaux et al. 2009; Traynor et al. 2014). In this study, we carefully studied interactions between queens and workers, focusing the contribution of CHCs to chemical communication. Wounding honeybee workers by injecting them with Ringer solution or LPS has been shown to correlate with alterations in the CHC profile and immune responses. These changes have been demonstrated to be greater in bees injected with LPS as opposed to Ringer solution, and the behaviour of healthy towards immune-stimulated bees has been observed to alter drastically (Richard et al. 2008, 2012). We hypothesized that honeybee queens could detect infected nestmates through direct contact and that this contact would positively correlate with the activation of the transcription of key genes involved in the humoral immune response. Our experiments demonstrated that the transcript levels of these genes did not alter in queens that were spatially separated from LPS-injected workers (i.e. direct contact prevented). This finding allowed us to rule out the hypothesis that volatile pheromone-like compounds acted as chemical cues, contributing to an immune response.

By focusing specifically on the expression levels of four effector genes for AMPs of the Toll and Imd pathways of the honeybee’s immune response, the results of our study allowed us to conclude that the biosynthesis of specific products of these two immune pathways was triggered when queens were allowed direct physical contact with immune-challenged nestmates that presented altered CHC profiles or presented with paper discs saturated with CHCs from immune-challenged nestmates. Our findings led us to conclude that contact with the worker bees’ CHCs (i.e. nonpolar compounds with no or little volatility) positively correlated with immune activation in the queen. It is important to note that the contact experiments with immune-challenged workers were carried out for 48 h, which meant that queens were exposed to the regularly produced, specific profile of thirty workers challenged with LPS for a relatively long period of time. In the last experiment, in contrast, CHCs extracted from thirty Ringer- or LPS-injected workers were adsorbed on several small paper discs and presented to queens for a shorter period of time, resulting in a lower exposure frequency. In addition, as the paper discs were soaked only once during the 24 h experimental time, the hydrocarbon bouquet may have faded over the time of the experiment, presenting the queens with weaker stimuli than occurred with direct contact between the queen and workers. The fact that changes in CHC profiles are already detected by nestmates as shortly as 4 h after the workers have been immune-challenged (Richard et al. 2008), and that we extracted the CHCs from our workers 24 h after the challenge, may contribute to the differences between the transcript levels of the AMP genes observed in queens that had direct contact with immune-challenged worker bees and paper discs saturated with CHC extracts of immune-challenged workers.

The CHC profiles of bees that had been injected with either Ringer solution or LPS were significantly altered after 24 h as compared to naive workers. Other studies have shown that wounding produces short-term immune reactions in insects, whereas infections cause immune reactions that are stronger and maintained over longer periods of time (Lemaitre et al. 1997; Korner & Schmid-Hempel 2004; Haine et al. 2008). Our experiments showed that queens displayed elevated transcript levels for two of four AMP genes (contact with Ringer-injected workers) or three of four AMP genes (contact with LPS-injected workers). Queens clearly could distinguish these immune-challenged individuals from healthy bees, but the chemical cues emitted by the LPS-injected individuals (mimicking the effects of infection) provided stimuli over a longer period of time and, therefore, had the potential to elicit an immune response from queens over a longer period of time.

The ability of honeybee queens to modulate their immunologic status in response to infections present in the hive has profound implications for colony health, especially when considering that the maternal immune experience that is acquired during pathogen exposure is passed on to progeny (Hernández López et al. 2014). A molecular mechanism has been proposed to explain this transfer of immunity (TgIP) (Salmela et al. 2015). According to this study, the egg yolk protein vitellogenin (Vg) carries the immune elicitors present in bacterial cell walls from the hemolymph of queens into the eggs (in addition to other molecules necessary for egg development). However, the fact that antigens derived from bacteria are transferred by Vg to honeybee offspring and mediate priming implies that queens must come into direct contact with the infectious agent through ingestion. This hypothesis has not yet been supported by experimental evidence.

However, the social transfer of immune elicitors present in cell walls of bacteria or fungi between individuals has been demonstrated in social insects (Vodova...
honeybee worker's gut flora, which could then promote LPS solution either directly or indirectly influences a... tion, it seems unlikely that the injection of Ringer or... nurses to queens seems more likely to co-occur in... in queens. The direct feeding of contaminated food by... CHCs act as cues and contribute to immune activation... in queens, which cannot be explained by a pos... experiments correlated positively with immune activation... ing infected themselves, and pass resistance on to their... queens can detect infections, without necessarily beco... quee breast milk, which queens regulate the immunity of... or trophallaxis) in a colony results in the transfer of... pathogens, which may lead to an enhanced collective... resistance to infections as a mechanism of social immun... the mechanism for a transfer of pathogenic material... between diseased workers and queens is still unknown; however, it might seem unlikely considering that unhealthy nestmates are excluded from tasks that are related to feeding the brood and queen in social groups (Cremer et al. 2007). In the case of American Foulbrood (AFB) infections, larvae only become infected when spores of Pl reach the gut lumen and germinate. Adult bees function as vectors, carrying spores but never becoming infected with vegetative forms themselves (Genersch 2010). Nonetheless, when nurses cannibalize infected larvae, they may come into contact with vegetative forms of AFB. Nurse bees that ‘offer’ contaminated food (unprocessed pollen or nectar) to queens when infections are present in a colony could theoretically promote the activation of social immunization by the TgIP mechanism proposed by Salmela et al. (2015), in a ‘feeding with intent’ scenario.

Alternatively, workers could also boost the immune system of queens by transferring immune elicitors by feeding queens with glandular secretions. This process would involve the epithelial resorption of the respective immune elicitors in the workers’ lumen to the haemocoel, their subsequent secretory passage through hypopharyngeal glands (HPG) into the royal jelly and their resorption into the queen’s haemocoel when feeding. This seems an intricate and overcomplicated route... workers, contributing to the activation of an immune response. If such a transfer could occur, it would not occur in the case of Ringer-injected workers where the queens’ immune system was shown to be only partially activated, and this mechanism could account for the sole effect of the altered CHC profiles.

Despite these uncertainties, the study findings clearly suggest that the immune status of queens can be modulated according to the health of the individuals within the colony. Because our previous research has shown that honeybee queens that have been exposed to an immune challenge can influence the immune competence of subsequently produced progeny (Hernández López et al. 2014), the findings of the current study point to the existence of a mechanism of social immunity by which queens regulate the immunity of their offspring upon detecting chemical cues emitted by diseased nestmates. It is undoubtedly beneficial for the colonies if queens can detect infections, without necessarily becoming infected themselves, and pass resistance on to their progeny. Our experiments have shown that queen immune activation occurred, not in response to direct exposure to a pathogenic agent, but was associated with the detection of specific (altered) chemical cues emitted by diseased nestmates. The instances of detection of diseased nestmates are postulated to occur with higher frequency than instances of direct infection of queens in a colony because the latter only occurs when an infection load has overwhelmed the colony’s disease control mechanisms (Cremer et al. 2007). Additionally, whether nurse bees actively ‘offer’ a contaminated diet to queens in such situations also contributes to the enhancement and support of social immunization.

If offspring are primed after the queen has detected diseased individuals due to changes in their CHC profile, this priming mechanism is not mediated by the maternal transfer of bacterial fragments. Instead, epigenetic factors such as post-translational histone modifications or DNA methylation may be involved. These modifications have been recently demonstrated to have an impact on honeybee behaviour and development (Lyko et al. 2010; Dickmann et al. 2013; Wojciechowski et al. 2014). Differences in queen and worker behaviour and reproduction are related to the differences inherent...
in the DNA methylation patterns of hundreds of genes in the brain, which in turn are determined by ambient stimuli (e.g. different supplementation regimes of royal jelly in the larval diet during development) (Lyko et al. 2010). As insects have been used as models to study the epigenetic basis of infections, an increasing number of epigenetic modifications resulting from host-pathogen interactions have been reported (Mondor et al. 2004; Poulin & Thomas 2007; Gómez-Díaz et al. 2012; Mukherjee et al. 2012, 2015). Mukherjee et al. (2012) reported for the first time that pathogenic bacteria affect the regulation of histone acetyltransferases (HATs) and histone deacetylases (HDACs) in insects, resulting in manipulation of the host immunity and development. They concluded that the acetylation/deacetylation of histones causes transcriptional reprogramming in response to wounding or infection and affects the expression of antimicrobial peptides. In the light of our results, we hypothesize that when the queen detects diseased nestmates using her antennae, the balance in the enzymatic activity of HATs and HDACs (or other epigenetic factors) could be disrupted and the expression of antimicrobial peptides could be promoted. If these changes take place in reproductive cells, they could be inherited.

This study had certain limitations in that it did not address the expression of genes that are associated with the cellular responses, which depend upon more complex processes such as the differentiation of prohemocytes into various types of mature hemocytes, cell adhesion, degranulation, encapsulation and opsonization. Due to the complexity of these processes, the selection of key genes that would reveal the status of the cellular response in queens presents significant challenges. In the future, however, we propose to search for specific CHCs or specific combinations thereof that can contribute to immune activation in queens and conduct whole transcriptome analyses in queens to identify the full repertoire of regulated genes. If a parallel study of epigenetic modifications that might take place in queens (and larvae) exposed to healthy or unhealthy nestmates was conducted, we could study whether the observed immune activation in queens contributes to heritable differences.

Conclusions

Chemical cues such as pheromones and cuticular hydrocarbons function as signals for social insects, helping them to modulate tasks among individuals with regard to colony requirements. These cues also allow healthy individuals to recognize diseased nestmates and elicit hygienic or aggressive behaviours in the former towards the latter. In this study, we evaluated correlations between immune responses in queens and chemical cues emitted by immune-challenged nestmates. Our study provides evidence that specific (altered) chemical cues emitted by diseased nestmates can be detected by queens upon physical contact with the latter or CHC extracts of the latter and, consequently, an immune activation response in queens occurs. These findings indicate that chemical cues are not only used to regulate the partitioning of tasks among workers or discriminate between intraspecific and intercolony individuals as previously believed, but also act as immunologic cues that allow queens to detect the presence of pathogens in the colony and respond accordingly. The contribution of CHCs to immunity has not been previously described. Future research will enable us to clarify whether the immune activation in queens subsequently accounts for heritable changes in offspring. The study expands our knowledge of chemical communication in insects and sheds light on a potential new mechanism for social immunity.

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HYDROCARBONS AS IMMUNE ELICITORS IN HONEYBEES

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Data accessibility
qPCR raw data available at doi:10.5061/dryad.sg46n.

J.H.L. contributed to experimental design. J.H.L. performed queen experiments, genetic analysis and statistical analysis and drafted the manuscript. W.S. performed chemical analysis. J.H.L., W.S., K.C. and U.R.G. performed interpretation of data. J.H.L., W.S., K.C. and U.R.G. made corrections and approved the final version of the manuscript.

Supporting information
Additional supporting information may be found in the online version of this article.
Table S1 List of identified cuticular hydrocarbons in worker bees (% ± standard deviation) and statistical significances.

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