

Colony-level chemical profiles do not provide reliable information about colony size in the honey bee

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Abstract. 1. Chemical communication facilitates colony function across social insects, providing workers with information about individual and colony state. Although workers use chemical cues to detect developmental transitions in individuals, it is unknown whether workers can also use colony-level chemical profiles to detect the developmental state of their colony. Indeed, it is largely unknown how colony-level chemical profiles change as colonies grow and develop.

2. Reproductive onset is a major developmental transition and, in the honey bee, *Apis mellifera*, colonies must surpass a threshold colony size before workers will invest in reproduction. Given the ubiquity of chemical communication, the present study investigated whether colony-level chemical profiles change with colony size.

3. Chemical compounds deposited by workers of three colony sizes (5000, 10 000, 15 000 workers) collected over a 4-day time-series (0, 12, 24, 48, 72, and 96 h), as well as worker cuticular lipids, were sampled.

4. In total, 26 compounds deposited on nest surfaces and 20 compounds in worker cuticular lipids were identified; it took up to 24 h for sampled nest surfaces to reach saturation in the number and amount of deposited compounds.

5. Among these compounds, no qualitative or quantitative indicators of colony size were found, suggesting that deposited chemical compounds are not semiochemicals in this context. Volatile pheromones have also been shown previously to not play a role in signaling colony size. Therefore, honey bee workers are unlikely to use deposited chemical cues to detect colony size, and must rely instead on other modalities, such as physical cues of worker density.

Key words. Superorganism, social physiology, colony size, developmental transitions, communication.

Introduction

Chemical communication facilitates colony function across the social insects (Alaux *et al.*, 2010). Akin to hormones within multicellular organisms, chemical communication forms part of the colony-level social physiology that allows groups of workers to coordinate activities within the superorganism (Johnson & Linksvayer, 2010). Temporal and spatial variation in chemical

cues provide workers with information, at both the individual and colony level.

An individual's chemical profile is not static and can change with age, task repertoire or reproductive state. In *Apis mellifera* workers, chemical profiles change as workers progress through their age-based division of labour (Kather *et al.*, 2011; Vernier *et al.*, 2019). In *A. mellifera* queens, the chemical composition of the mandibular gland changes as they transition from virgin, to mated, to laying (Kocher *et al.*, 2008). In the termite *Zootermopsis nevadensis*, four polyunsaturated alkenes are only present in reproductives that are actively producing gametes (Liebig *et al.*, 2009) and, in *Reticulitermes flavipes*, heneicosane

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is produced only by reproductive queens and kings (Funaro *et al.*, 2018). *Lasius niger* queens that are subjected to an immune challenge reduce their production of queen pheromone, 3-methylhentriacontane (Holman *et al.*, 2010). In *Camponotus floridanus*, eggs laid by high versus low fertility queens have different chemical profiles, and workers detect and respond to these differences (Endler *et al.*, 2006). These examples showcase how individual chemical profiles change with developmental state, as well as how these changes relay information to nestmates.

Chemical profiles can also change at the colony-level, and workers use this information to alter their behaviour. Nestmate recognition is the quintessential example of how workers use a colony-level odour to change their behaviour towards conspecifics (van Zweden & d'Ettorre, 2010). This colony-level bouquet, however, is not static. It changes with environmental factors, such as nest substrate (Breed *et al.*, 1988), colony diet (Liang & Silverman, 2000) or colony composition (van Zweden *et al.*, 2010). In *Camponotus fellah*, workers mark nest areas with chemical 'road signs' that help spatially organize workers within the dark nest (Heyman *et al.*, 2017). The developmental state of a colony can also be reflected in its chemical profile. In *Bombus terrestris*, the quantity and composition of lipids in the wax changes with a colony's developmental state and, if workers are exposed to wax from mature colonies, they increase their ovary development (Rottler-Hoermann *et al.*, 2016).

Some social insects, such as honey bees, function as superorganisms: a level of biological organisation above multicellular organisms but below societies, where groups of individuals form a cooperative unit to propagate their genes (Seeley, 1989; Smith & Szathmáry, 1995). This reproductive cooperation, however, also requires coordination. Just like individual cells within a multicellular organism must detect the organism's state to coordinate developmental transitions, so too must the individual workers detect their colony's state (Hölldobler & Wilson, 2009; Kennedy *et al.*, 2017). Although we know that honey bee workers use chemical profiles at the individual-level to detect individual-level developmental transitions (e.g. a queen's pheromonal bouquet to detect her mating status) (Kocher *et al.*, 2008, 2009), it is unknown whether workers also use colony-level chemical profiles to detect colony-level developmental transitions.

The first reproductive transition for a honey bee colony is when workers begin to build drone comb, the large-celled comb used for rearing male reproductives (Boes, 2010). Workers build comb collectively, although they only begin building drone comb after the colony has surpassed a threshold number of workers (approximately 5000 workers) (Smith *et al.*, 2014, 2016). Therefore, workers must somehow detect their colony's size to determine whether the colony is above or below the reproductive threshold.

How, then, do workers detect their colony's size? Living in a dark nest of parallel combs, workers only have access to local information, which they must use to assess their colony's overall size. Experimentally increasing worker density induces workers to build more drone comb than an unmanipulated control, although increasing amounts of volatile pheromones (of an unspecified blend) were reported to have no effect (Smith *et al.*, 2017). That study, however, did not investigate the potential

impact of chemical compounds that accumulate on surfaces. Honey bees deposit various exocrine secretions in the nest, which can have multiple functions (Cassier & Lensky, 1997). For example, 'footprint pheromone' helps guide foragers to the hive entrance (Butler *et al.*, 1969). Even if these chemical cues are inadvertently deposited, they form part of the chemical information present in the colony, and this chemical profile could become a reliable cue of colony size.

Does the chemical profile of a colony change with the colony's size? Chemical profiles could change in qualitative and/or quantitative ways. There could be (i) specific compounds associated with colony size; (ii) a change in compound composition (absolute or relative changes) or (iii) both. Specific compounds, for example, may be detected only in large or small colonies, or may only be detectable by our methods once sufficient individuals are present. Changes in compound composition could arise from variable degradation rates, thereby exaggerating differences between large and small colonies. Regardless of how these changes may, or may not, arise, the focus here is to first determine whether chemical profiles change with colony size and, if so, how this occurs. Subsequent experiments would be needed to test whether workers use these differences to detect their colony's size. The first step, however, is to determine whether there are reliable colony-size differences. We expect these changes to be consistent across colonies, because the transition from a small non-reproductive colony to a large reproductive colony is a developmental transition that all colonies must undergo.

Similar to how the chemical profile of an individual changes as it becomes capable of reproduction, the present study investigates whether the chemical profile of the colony changes as the colony becomes capable of reproduction. To test whether surface chemical profiles change with colony size, we used small glass plates to sample the chemical compounds deposited by workers in colonies of three sizes (5000, 10 000, or 15 000 workers). These samples were collected over a 4-day time-series (0, 12, 24, 48, 72, and 96 h) to account for potential variation in the rate of compound deposition. We also collected cuticular lipid samples from workers in the three colony sizes, aiming to determine whether there were colony-size differences in the cuticular profiles.

We found no difference in the chemical profiles collected from colonies of different sizes, nor any difference in the cuticular lipid profiles of workers from different sized colonies. The time-series data showed that it took up to 24 h for the chemical compounds to saturate on the glass, although this process was no faster in larger colonies than in smaller colonies. Therefore, we conclude that chemical compounds deposited by workers are not a reliable cue of colony size.

Materials and methods

Overview

The present study aimed to determine whether the surface chemical profile of a honey bee colony changes with colony size. We sampled worker-deposited compounds using glass plates left in colonies over a 4-day time-series (0, 12, 24, 48, 72, and 96 h),

as well as extracted cuticular lipid profiles from adult workers. We used three colony sizes (5000, 10 000 and 15 000 workers) to represent early colony development, when colonies transition to making reproductive investments.

Field methods

This research was performed at the Liddell Field Station of Cornell University, in Ithaca, New York (42°27.6'N, 76°26.7'W), using colonies of honey bees headed by naturally mated queens (*Apis mellifera linguistica*).

On 20 August 2016, we set up nine study colonies (three colonies in each of the three colony-size treatment groups: 5000, 10 000 or 15 000 workers). Each colony possessed a queen, a known number of workers, and a single frame of brood. Worker number was determined by weight (5000 workers, 0.65 kg; 10 000 workers, 1.30 kg; 15 000 workers, 1.95 kg; based on 7700 bees in 1 kg (Mitchell, 1970; Otis, 1982)). Each colony was housed in a single hive box with space for 10 deep Langstroth frames (a frame is a wooden structure used to hold beeswax comb in a moveable-frame hive). Each colony had identical nest contents: two frames of honey, one frame of worker brood, five frames of empty drawn comb, and two frames without comb. The brood frame for each colony contained uncapped brood (eggs and larvae) but no emerging adults, to maintain the colony size differences between the colonies. None of the colonies possessed any drone comb. After set-up, colonies were given 2 days to adjust. Similar methods were used in previous studies (Smith *et al.*, 2014, 2017), so we can assume that workers are capable of detecting their colony's size in such an experimental set-up.

At 19.00 h on 22 August 2016, we placed five glass plates into each colony, placed on top of the centre frame (the frame containing worker brood). Each glass plate measured 5 × 5 cm. The size and position of the glass plates were chosen so that workers had unimpeded access to walk atop and interact with the plate. After 12, 24, 48, 72, and 96 h, we removed a single glass plate from each colony and immediately washed the plate with 1 ml of hexane (SupraSolv; Merck, Darmstadt, Germany). When opening the colony to remove the glass plate, no smoke was used on the bees to avoid contaminating the glass plate. We did not induce the colonies to build comb because doing so would require feeding sugar syrup, which would have risked contaminating the glass plates. Therefore, the colonies did not build comb during the experiment but, from previous work, we know that colonies will build drone comb in August (Smith *et al.*, 2017) and that the 15 000-worker colonies would be of a sufficient size to invest in drone comb, whereas the 5000-worker colonies would not (Smith *et al.*, 2014, 2016).

On 27 August 2016, we collected cuticle samples from the workers. From each colony, we sampled three workers randomly from inside the colony, just below the lid, on top of the centre frame. Workers carrying pollen loads were not selected to avoid contamination. Each worker was placed into a vial containing 1 ml of hexane for 1 min (SupraSolv; Merck) and then removed with forceps. After each bee, the forceps were washed twice with acetone and twice with hexane. Gloves were changed between each colony.

On the same day, we also assessed the size of the colonies using the Liebefeld method, examining each side of every frame in the hive and estimating the fraction of the frame covered by adult bees (Imdorf *et al.*, 1987; Delaplane *et al.*, 2013). As expected, all colonies lost workers as a result of foraging mortality, although the differences between the colony size treatment groups remained (5000-worker colonies: 1700 ± 150 workers; 10 000-worker colonies: 3670 ± 840 workers; 15 000-worker colonies: 6470 ± 1000 workers). Assuming a linear rate of forager loss per day, on the first experimental day (2 days after installment), the colony sizes in the three treatment group were: 4060, 8200 and 12560 workers, respectively. Therefore, given previous work (Smith *et al.*, 2014, 2016), the colonies that began the experiment with 5000 workers were consistently below the reproductive threshold, whereas colonies that began the experiment with 15 000 workers were consistently above this threshold. The colonies with 10 000 workers began the experiment above the reproductive threshold (8200 workers) and ended below the reproductive threshold (3670 ± 840 workers). For the statistical analyses (see below), we account for these differences by conducting pairwise comparisons between the colony-size treatment groups.

Chemical analysis

Glass samples and cuticular extracts were analysed by gas chromatography-flame ionisation detection (GC-FID) on a GC-2014 instrument (Shimadzu Corp., Kyoto, Japan). One microlitre of each extract was injected splitless (inlet temperature 275 °C) and analysed on an HP-5MS column (Agilent Technologies Inc., Santa Clara, California): the oven was held at 50 °C for 1 min, followed by a programmed temperature increase of 10 °C min⁻¹ to a final temperature of 310 °C, which was held for 5 min. Peak areas were determined by integration in LabSolutions (Shimadzu Corp.). The resultant peaks were aligned across samples in R, version 3.3.3 (R Core Team, 2014) using the GCalignR package (Ottensmann *et al.*, 2018). Absolute abundances of compounds in the extracts were estimated by comparison of integrated peak areas with an external standard curve generated via a dilution series of *n*-undecane (analytical standard, Merck, Germany). The calibration curve, $\log[A] = 0.9621 \log[B] - 15.162$ ($r^2 = 0.999$, $P < 0.001$) generated from the external standard was used to estimate analyte concentration $[A]$ (ng μl⁻¹) based on respective peak areas $[B]$ (Shi *et al.*, 2015). For chemical identification of compounds, a subset of samples that together accounted for all peaks of interest were re-injected under identical GC conditions on an 7890B GC oven (Agilent Technologies Inc.) linked to a 5977A mass selective detector (Agilent Technologies Inc.). The tentative identities of compounds were determined by co-injection of a linear alkane series and comparison of retention indices with published standards (e.g. methyl branched hydrocarbons in Carlson *et al.*, 1998), as well as by comparisons of mass spectra and retention indices with those of reference library entries (NIST14). Relative abundance values calculated for all peaks were transformed using the Aitchison's formula, $Z_{ij} = \ln[Y_{ij}/g(Y_j)]$, where Z_{ij} is the standardized peak area i for individual sample j , Y_{ij} is the peak

area i for individual sample j and $g(Y_j)$ is the geometric mean of all peaks for individual sample j (Aitchison, 1982).

Statistical analysis

Statistical analyses were performed in R (R Core Team, 2014), with the packages: ggfortify, ggplot2, vegan and rstanarm (Tang *et al.*, 2016; Wickham, 2016; Goodrich *et al.*, 2018; Oksanen *et al.*, 2018).

For the time-series, we collected a glass plate sample at each time point (0–96 h) from each of the nine colonies (54 glass plate samples total). The time-series allowed us to determine how long it took for the glass plates to reach their maximum-level chemical profile (i.e. saturation). To determine whether the chemical profiles on the glass plates, once saturated, changed with colony size, we pooled the saturated samples (48–96 h) and compared across the three colony sizes (27 saturated glass plate samples total). The cuticular lipid profiles extracted from adult workers were collected at the end of the experiment, with three extracts collected from each of the nine colonies (27 cuticular lipid profiles total).

We used linear mixed-effects models to test for differences in the total number of compounds and the abundance of compounds detected on the glass plates. Null models (without explanatory variables) were compared with a model containing the treatment group, time spent in the colony, and their interaction, as explanatory variables, with colony ID as the random factor. Residuals were checked for normality using $Q-Q$ plots. The best-fit model was determined by using likelihood ratio tests and Akaike information criteria (AIC) comparison (Lewis *et al.*, 2011).

Principal component analysis (PCA) was used to plot the chemical profiles across the time series (0, 12, 24, 48, 72, and 96 h) and treatment group (5000, 10 000 and 15 000 workers). To test for statistical differences between the time periods or treatment groups, we used the adonis function to perform permutational multivariate analysis of variance using distance matrices (PERMANOVA), with 999 permutations, and pairwise comparisons. Data were log transformed and so, to avoid undefined values, we assigned a peak area value of 0.00001 to compounds that were undetected. All statistical tests were performed on both the absolute and relative chemical profiles.

To meet PCA assumptions, we reduced the number of compounds to not exceed the number of samples collected. For the glass samples, we originally found 54 compounds, of which 11 were clearly contaminants (e.g. siloxanes from the column). We then removed the 17 least-represented compounds across samples for a final set of 26 compounds, meeting the PCA assumptions. Each of the 26 compounds appeared in more than 10% of the glass samples. For the cuticle extracts, we originally found 69 compounds, of which seven were contaminants. We then removed the 42 least-represented compounds for a final set of 20 compounds to meet PCA assumptions. This number of compounds ($n = 20$) slightly exceeds the number of compounds identified in previously published honey bee profiles ($n = 16$) (Kather *et al.*, 2011) and is smaller than the total number of samples collected ($n = 27$). Each of these 20 compounds appeared in

more than 45% of the cuticle extracts collected. The results were no different when we repeated the same statistical analyses on the full array of chemical compounds instead of the reduced set.

Finally, we tested each of the individual chemical compounds detected on the glass plates, to determine whether they changed with colony size, using a Bayesian generalised linear model (stan_glm function). Accordingly, we pooled the saturated samples (48–96 h) and compared each individual compound across the three colony sizes (27 total samples per compound). Our priors for both the slope and intercept were sampled from a normal distribution with mean = 0 and SD = 1. We then tested whether the 95% confidence interval for each compound deviated from this prior distribution. Whether we used colony size from the beginning or the end of the experiment did not change the results obtained.

Results

Glass plates

The rationale behind using glass plates was to determine whether workers deposit chemical compounds that could be used as a cue of colony size. We collected the glass plates after 0, 12, 24, 48, 72, and 96 h to determine how the compounds on the glass changed with time, and whether the accumulation of compounds varied with colony size.

We identified 26 compounds deposited on the glass plates. This set of compounds was comprised primarily of linear and branched alkanes, alkenes, aldehydes, and alcohols. Two fatty acids, a monoterpene (cineole) and an aromatic methyl ester (methyl salicylate) were also identified from the glass samples (Table 1).

As expected, glass plates that were left in the colony for more time accumulated a greater number of compounds (Fig. 1), and a greater abundance of those compounds (Fig. 2), than glass plates that were left in the colony for less time. This temporal change was significant in linear mixed-effects models for both the number of compounds (LMER: AIC with time = 315.15; AIC without time = 319.49; $P = 0.012$), and compound abundance (LMER: AIC with time = 172.39; AIC without time = 179.72; $P = 0.002$). Including the size of the colony, however, had no effect for either the number of compounds (LMER: AIC with colony size = 321.14; AIC without colony size = 319.49; $P = 0.309$) or abundance of compounds (LMER: AIC with colony size = 179.50; AIC without colony size = 179.72; $P = 0.122$). Therefore, although the amount of time that a glass plate remained in a colony did influence the chemical profile, the size of the colony had no effect.

We then performed PCA to visualise the change in chemical profiles over time (Fig. 3). As expected, the chemical profile from glass plates that were not placed into a colony (0 h) formed a distinct cluster separate from the samples collected after only 12 h in the colony. A pairwise PERMANOVA analysis showed that the samples collected after 0 h were significantly different from all other samples (0 h versus 12, 24, 48, 72, and 96 h: $P = 0.015$). It did, however, take time for the chemical compounds to be deposited onto the glass plates; samples collected at 12 h were significantly different from the samples

Table 1. Compounds identified from the glass samples and cuticle extracts.

Compound	Present in glass samples	Present in cuticle extracts
Cineole	Yes	No
Nonanal	Yes	No
Methyl salicylate	Yes	No
Decanal	Yes	Yes
Nonanoic acid	Yes	No
Decanoic acid	Yes	No
Farnesol	No	Yes
C19:1 (Nonadecene)	No	Yes
C19 (Nonadecane)	No	Yes
Octadecenol	No	Yes
Octadecanol	No	Yes
C21 (Heneicosane)	Yes	Yes
C22 (Docosane)	Yes	Yes
Eicosenol	Yes	No
Heneicosanal	Yes	No
Docosanal	Yes	No
Docosenol	Yes	No
C25:1 (Pentacosene)	Yes	Yes
11/13-Methylpentacosane	Yes	Yes
C26 (Hexacosane)	Yes	Yes
C27:1 (Heptacosene)	Yes	Yes
Heptacosane	Yes	Yes
11/13-Methylheptacosane	Yes	No
Octacosane	Yes	Yes
11/13/15-Methylnonacosane	Yes	Yes
Triacontane	Yes	Yes
C31:2 (Hentriacontadiene)	Yes	No
Hentriacontane	Yes	Yes
13/15/17-Methylhentriacontane	Yes	Yes
C33:2 (Tritriacontadiene)	Yes	Yes
C35:1 (Pentatriacontene)	Yes	Yes

Tentative chemical identification is based on retention indices and mass spectra. [Colour table can be viewed at [wileyonlinelibrary.com](#)].

collected at 72 and 96 h ($P = 0.015$). Samples collected at 24 h were no different from samples collected after 48, 72 or 96 h ($P > 0.05$).

A PERMANOVA analysis confirmed that the amount of time significantly improved the model ($P = 0.001$), whereas colony size did not ($P = 0.727$), nor did an interaction between time and colony size ($P = 0.775$). Therefore, the size of the colony did not significantly influence the rate at which chemical compounds were deposited onto the glass. These results are for the absolute chemical composition data; the results were identical when the relative composition data were used: PERMANOVA for relative composition: time improves the model ($P = 0.001$); colony size does not ($P = 0.735$); interaction of time and colony size does not ($P = 0.595$).

We concluded that the amount of compounds deposited onto the glass plates reached a plateau at 24 h, although there was no effect of colony size on the speed at which they were deposited. Knowing that it took 24 h for the glass plates to saturate, we then pooled the samples collected at 48, 72, and 96 h to determine whether there was an effect of colony size once the amounts of deposited compounds had reached a maximum.

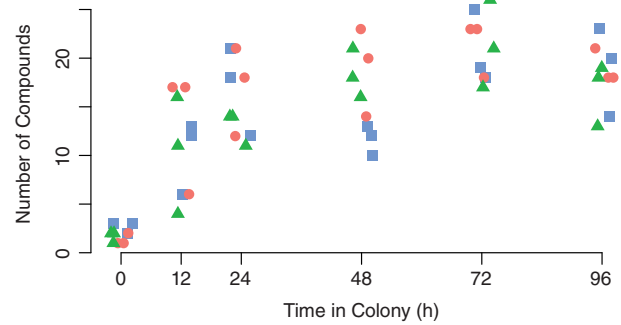


Fig. 1. The number of compounds deposited on the glass plates increased with time (noise added to the x-axis to prevent points from overlapping). There was no significant effect of colony size on the number of compounds deposited. Colonies with 5000 workers: green triangles; 10000 workers: red circles; 15000 workers: blue squares. [Colour figure can be viewed at [wileyonlinelibrary.com](#)].

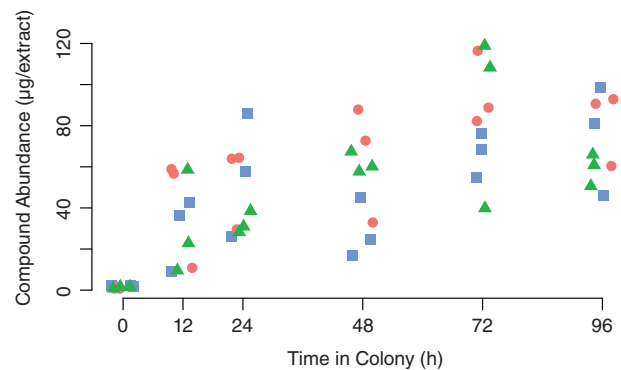


Fig. 2. The total abundance of compounds deposited on the glass plates increased with time (noise added to the x-axis to prevent points from overlapping). There was no significant effect of colony size on the abundance of compounds deposited. Colonies with 5000 workers: green triangles; 10000 workers: red circles; 15000 workers: blue squares. [Colour figure can be viewed at [wileyonlinelibrary.com](#)].

A PCA on the maximum-level chemical profiles (48–96 h) showed strong overlap between colonies of different sizes (Fig. 4). Adding colony size to a PERMANOVA analysis did not significantly improve the model ($P = 0.128$), nor were any of the pairwise PERMANOVA comparisons significant (colony size: 5000 vs. 10000 workers; 10000 vs. 15000; 5000 vs. 15000; $P > 0.05$). As before, these results are for absolute chemical composition; the results were identical for relative composition: PERMANOVA for relative composition: colony size does not improve the model ($P = 0.178$); pairwise comparisons were not significant (5000 vs. 10000 workers; 10000 vs. 15000; 5000 vs. 15000; $P > 0.05$).

Finally, we tested whether any of the individual compounds deposited on the glass changed in abundance with colony size (pooled samples from 48, 72, and 96 h). Using a Bayesian generalised linear model, we did not find any significant relationship between colony size and the abundance of a given compound (95% confidence interval = 0.00–0.00). This result held regardless of whether we used the initial colony size or

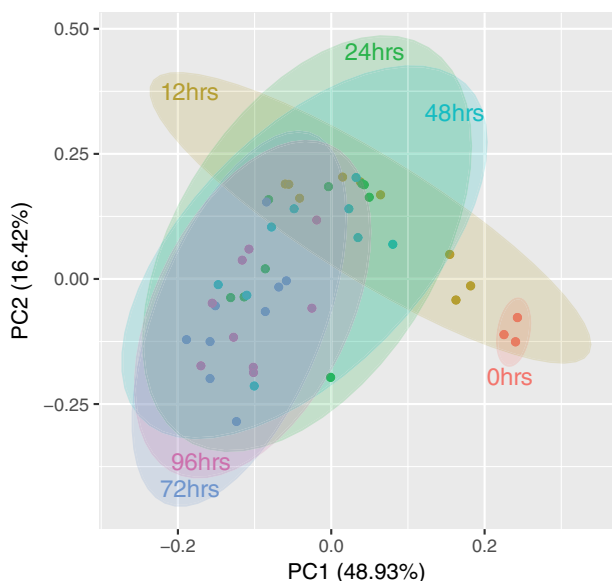


Fig. 3. The first two principal components (PC) from the principal component analysis of the compounds deposited onto the glass. Samples collected at 0 h ($n = 9$, overlapping points) were significantly different from samples collected at all other time points (12, 24, 48, 72, and 96 h; $n = 9$ per time point). Samples collected after 12 h were significantly different from samples collected at 72 and 96 h. By 24 h, the chemical profiles were no different from the glass plates left in the colonies for a longer time (48, 72, and 96 h). There was no significant effect of colony size, nor was there a significant interaction between time and colony size. [Colour figure can be viewed at wileyonlinelibrary.com].

the colony size at the end of the experiment. Therefore, given the samples collected, none of the individual compounds varied with colony size.

Cuticle

Glass plates are an effective way to sample the chemical compounds deposited by workers within the colony. However, it is possible that chemical compounds detected upon contact could still be different in colonies of different sizes, just that the workers do not deposit these compounds onto the surface of the nest. Therefore, at the end of the experiment (27 August 2016), we extracted cuticular lipids from workers in each colony. These data were then subjected to PCA, to determine if the cuticular compounds were different between workers sampled from colonies of different sizes.

We identified 20 compounds in the cuticle extracts. This set of compounds was made up almost entirely of alkanes and alkenes, although two alcohols and an aldehyde were also identified (Table 1).

A PCA plot showed strong overlap between the cuticular extracts of workers from colonies that began the experiment with 5000, 10 000 and 15 000 workers (Fig. 5). There was no significant difference between the different colony sizes (PERMANOVA, $P = 0.108$), nor were any pairwise PERMANOVA comparisons significant (colony size: 5000 vs. 10 000 workers;

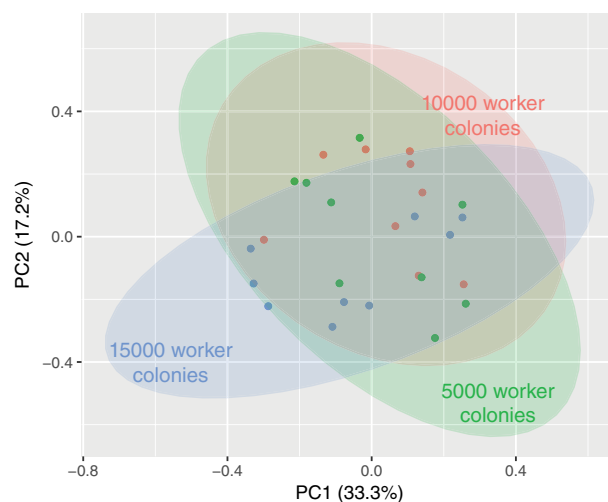


Fig. 4. The first two principal components (PC) from the principal component analysis of the chemical profiles after they reached their maximum abundance (48–96 h). There was no significant difference in the chemical profiles from colonies with 5000, 10 000 or 15 000 workers, even when only considering the samples collected after the overall abundance had plateaued. [Colour figure can be viewed at wileyonlinelibrary.com].

10 000 vs. 15 000; 5000 vs. 15 000; $P > 0.05$). Note, however, that, because the cuticular extracts were taken at the end of the experiment, the colony sizes had decreased, though the differences between the colony sizes remained (see Materials and methods). The results presented here are for the absolute chemical composition data; the results were identical for relative composition: PERMANOVA for relative composition: colony size does not improve the model ($P = 0.469$); pairwise comparisons were not significant (5000 vs. 10 000 workers; 10 000 vs. 15 000; 5000 vs. 15 000; $P > 0.05$).

Discussion

We found no evidence for a surface deposited or worker-cuticle related chemical cue of colony size. The compounds deposited on the glass plates were not different for colonies of different sizes (Fig. 4), nor were there any differences in the cuticular extracts of workers from these colonies (Fig. 5). We do show that the interior of the nest (as inferred from deposition rates on glass plates) accumulates these compounds quickly, reaching a plateau within 24 h (Fig. 3), although the rate of accumulation was no different for colonies of different sizes. These results, together with findings from Smith *et al.* (2017), provide further evidence that it is unlikely for honey bee workers to use colony-level chemical signals to communicate colony size.

Although social anatomy describes the parts of the superorganism (e.g. castes of workers), social physiology characterizes the way in which communication occurs between those parts (Johnson & Linksvayer, 2010). This pathway can be direct, from one worker to another, such as when a forager advertises a food site with a waggle dance. Communication can also be broadcast widely, to all members of the colony, such as when a

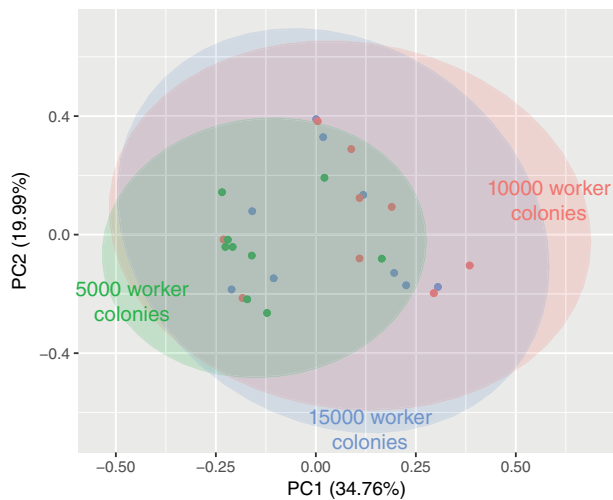


Fig. 5. The first two principal components (PC) from the principal component analysis of the cuticular extracts. There was no significant difference between the chemical profiles of workers sampled from colonies of different sizes. [Colour figure can be viewed at wileyonlinelibrary.com].

worker releases alarm pheromone during a defensive response (reviewed in Nouvian *et al.*, 2016). For widely broadcast information, chemical cues are typical, such as social pheromones. Our evidence, however, shows that colony-level chemical communication is an unlikely pathway for relaying information about a colony's size, or for coordinating early developmental transitions.

Honey bees are highly mobile within their nest, and this shared environment is a good platform for information flow within the colony (Seeley, 1998). Of the potential modalities for communication (e.g. chemical, physical, or visual), the modality that will be used depends on the reliability of information within that modality. Chemical cues appeared to represent a plausible mechanism for workers to detect colony size because deposition of compounds could be performed passively and could scale with worker number. Indeed, a combination of queen tarsal and mandibular gland pheromones have been shown to inhibit workers from building queen cups, the first step in the female reproductive pathway (Lensky & Slabezki, 1981). Examining chemical profiles at the colony level, however, we found that the surface chemical profile of a colony containing fewer than 5000 workers was no different from a colony containing up to 15000 workers. Previous work ruled out volatile pheromones as a cue of colony size (Smith *et al.*, 2017), and so chemical compounds, regardless of volatility, are unlikely cues for workers to detect the size of their colony, at least at the colony sizes tested. Our experiments did not explicitly test for a potential role of queen pheromones, so we cannot exclude this possibility. We do, however, show that the colony-level chemical profile, which encompasses all colony members, is not a reliable indicator of colony size in the honey bee.

Although it is possible that our experimental design did not give the workers sufficient time (7 days) to detect their colony's size, we do know from previous work that workers will detect

their colony's size, and also make reproductive investments based on that size, in as little as 8 days (Smith *et al.*, 2017). Furthermore, workers detect colony size in contexts beyond reproductive investments. For example, foraging strategy is influenced by colony size (Eckert *et al.*, 1994; Beekman *et al.*, 2004; Donaldson-Matasci *et al.*, 2013). Foragers from large colonies return with larger nectar loads than do foragers from small colonies (Eckert *et al.*, 1994); foragers from small colonies collect pollen more often than do foragers from large colonies (Beekman *et al.*, 2004). Therefore, colony size, whether directly or indirectly assessed, influences worker behaviour in multiple contexts, and so we expect workers to be able to quickly assess and adjust their behaviour based on the size of their colony. Of course, the mechanism by which they detect their colony's size is unknown, and could vary depending on context.

The rationale for using a time-series was to see how chemical profiles might change over time with colony size, especially the rate of saturation. The size of a colony, however, also changes during the time-series (see methods). The colonies that started the experiment with 5000 workers were consistently below the reproductive threshold; colonies with 15000 workers were consistently above the reproductive threshold. Colonies with 10000 workers began the experiment above the threshold, and finished below the threshold, so they represent colonies roughly at the reproductive threshold. A sample collected after 96 h from a 10000-worker colony therefore would have been deposited by workers that detected their colony's size as above the reproductive threshold for part of the experiment, and below the reproductive threshold for part of the experiment. Despite this caveat, our pairwise comparisons did not find differences even between the 5000- and 15000-worker colonies, which were reliably below and above the reproductive threshold throughout the experiment.

It is also possible that each colony has its own unique chemical cue of colony size. We favour the idea that something as important as coordinating developmental transitions would be shared across colonies, akin to reproductive hormones in humans, such as the kisspeptin/GPR54 system (Trevisan *et al.*, 2018). There is, however, the possibility that each colony has a unique colony-level trigger that changes as the colony grows. To test this hypothesis, one would have to sample the chemical profile of a colony throughout its growth and development, ideally from its founding through reproduction, while also accounting for environmental influences, such as identifying nectar. Based on our analysis, however, we did not identify a universal honey bee chemical cue for colony size, at least not in the glass samples or worker cuticle extracts we collected.

Workers, then, presumably rely on other modalities for detecting the size of their colony, such as physical stimuli, like worker density (Smith *et al.*, 2017). Indeed, physical cues have been shown to drive developmental transitions, for example, the desert locust, where touching the hind femur induces a behavioural switch from the solitary to the gregarious form (Simpson *et al.*, 2001). Whether a similar physical cue is operating at the colony-level in honey bees awaits further investigation. The results here do not discount the many ways in which workers do use chemical cues as part of the social physiology of the superorganism (Alaux *et al.*, 2010). Our study, however, did

not find colony-level chemical cues to be a reliable indicator of colony size in the honey bee.

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MLS, CJK, KB, and AK conceived the ideas and designed methodology. MLS collected the data. MLS, CJK and KB analysed the data. MLS led the writing of the manuscript. All authors contributed critically to the drafts and gave their final approval to the manuscript submitted for publication.

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