Social immunity and chemical communication in the honeybee: immune-challenged bees enter enforced or self-imposed exile

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ABSTRACT

Animals living in large colonies are especially vulnerable to infectious pathogens, and may therefore have evolved additional defences. Eusocial insects supplement their physiological immune systems with 'social immunity', a set of adaptations that impedes the entrance, establishment, and spread of pathogens in the colony. We here find that honey bee workers (*Apis mellifera*) that had been experimentally immune-challenged with lipopolysaccharide (LPS) often exited the hive and subsequently died; some of these workers were dragged out by other individuals, while others appeared to leave voluntarily. In a second experiment, we found that healthy workers treated with surface chemicals from LPS-treated bees were evicted from the hive more often than controls, indicating that immune-challenged bees produce chemical cues or signals that elicit their eviction. Thirdly, we observed pairs of bees in the lab, and found that pairs spent more time apart when one member of the pair had received LPS, relative to controls. Our findings suggest that immune-challenged bees altruistically banish themselves, and that workers evict sick individuals which they identify using olfactory cues, putatively because of (kin) selection to limit the spread of pathogens within colonies.

BACKGROUND

Colonial animals face a heightened risk of infectious disease, which can spread rapidly when conspecifics are in frequent contact (1; 2). One might therefore predict social species to have evolved stronger immune systems. This prediction has received only partial support in the eusocial Hymenoptera, and indeed some evidence suggests that these highly colonial species have comparatively weak immune systems. Interspecific comparative analyses have found both positive and negative correlations between colony size and physiological immune responses (3; 4; 5), suggesting that advanced sociality does not necessarily create selection for stronger immunity, and might even select for weaker immunity.

Inspired by the lack of a clear strengthening of the immune system among eusocial taxa, as well as observations of their behaviour, researchers have proposed that eusocial insects combat pathogens using 'social immunity', which provides a complementary defence system that reduces pathogen exposure and thereby weakens selection for stronger individual immune defences (6). Social immunity is defined as the set of behavioural, physiological, and organisational adaptations that impede the entrance, establishment, and spread of pathogens in the colony (6). For example, many eusocial insects collectively remove waste from the nest (7), or coat its interior with antimicrobial substances collected from plants or produced in their own bodies (8; 9). Many species have compartmentalised nests that help to contain

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the spread of pathogens; for example, leaf cutter ant nests have a separate garbage dump, and workers from the dump tend not to venture into the rest of the colony (10). Some bees deal with infestations of parasitic mites by identifying and removing infected larvae and pupae (11), or by 'mummifying' foreign objects or even live intruders with wax and propolis (12).

Studies have reported that honeybees respond behaviourally to sick individuals, in contexts that suggest social immunity. Baracchi *et al.* (13) introduced newly-emerged worker bees infected with deformed wing virus (DWV) to the hive alongside healthy controls, and observed that the infected bees were frequently ejected from the hive by other workers while controls were not. Moreover, DWV-infected bees produced a different blend of cuticular hydrocarbons, as measured by gas chromatography (13). Cuticular hydrocarbons (CHCs) are a layer of waxy chemicals on the body surface that prevents desiccation and has many important functions in chemical communication, e.g. in the identification of nestmates (14), the queen (15), and workers of differ ages and task specialties (16). Secondly, Richard *et al.* (17) found that applying chemical cues extracted from the body surface of experimentally immune-challenged bees to healthy bees caused the latter to elicit more antennation and allogrooming from nestmates. Together, these results suggest that workers can detect sick nestmates (possibly via chemical cues such as CHCs), and that they sometimes respond behaviourally by investigating, avoiding, and/or ejecting sick individuals.

Furthermore, kin selection theory (18) predicts that some social insects might have evolved 'altruistic' responses to sickness. In advanced eusocial species like honeybees, workers rarely breed, and instead reproduce their alleles indirectly by providing assistance to relatives such as their mother queen. Thus, as soon as the worker's presence flips from having a beneficial to a detrimental effect on the fitness of its relatives – such as when the worker picks up an infectious pathogen – leaving the colony permanently may become the course of action that maximises the worker's inclusive fitness. Consistent with this idea, Rueppell *et al.* (19) observed that worker bees exposed to harmful doses of CO_2 or hydroxyurea flew out of the colony and did not return; the authors hypothesised this behaviour to be 'altruistic suicide' that evolved via kin selection to prevent harm to related nestmates. Similarly, Heinze and Walter (20) found that moribund ants affected by a fungal infection left their nests and remained outside until death. Another study reported that immune-challenged bees showed reduced movement, and also reduced social interactions, which was hypothesised to be an adaptation that limits disease transmission (21).

In light of this research, we hypothesise that *Apis mellifera* honeybees (and perhaps other eusocial insects) use a multi-pronged approach to social immunity that involves both collective (e.g. eviction of sick individuals by the society) as well as individual behaviours (such as altruistic self-isolation). We here investigate these ideas using behavioural experiments on social immunity involving bacterial lipopolysaccharides (LPS), which are non-living cell wall components found in Gram-negative bacteria. LPS elicits a strong response from the innate immune system in many organisms, including honey bees (21; 22; 23). In Experiment 1, we treated worker bees with LPS or vari-

ous procedural controls, re-introduced them to their natal hive, and then estimated the rates at which bees remained inside, left voluntarily, or were ejected by other workers. In Experiment 2, we transferred surface chemicals from immune-challenged bees to healthy bees, and tested whether the healthy bees left the hive more often than controls. In Experiment 3, we observed pairs of bees in which one member was immune-challenged, to test whether they became more or less gregarious relative to controls.

Methods

Experimental animals

We utilised five honeybee colonies, each housed in a Langstroth hive on the Parkville campus of The University of Melbourne. Most of the work was carried out in in April – June 2019, except one replicate of Experiment 1 conducted in December 2019. The workers used in our experiments were collected by opening up a hive and selecting a centrally-located frame containing developing brood, then brushing a haphazardly-selected sample of workers clinging to the frame into a plastic container for transport to the lab. By selecting within-nest bees that did not fly in response to the disturbance of opening the hive, we aimed to preferentially collect younger bees that have not yet begun performing outside-nest tasks such as guarding and foraging. Therefore, our default expectation is that most of these bees would remain inside when returned to the hive.

Experimental immune challenge and controls

Following similar experiments (e.g. 21), we diluted LPS (from serotype 055:B5 *E. coli*; Sigma-Aldrich) to 0.5mg/mL in a sterile physiological saline (Ringer's solution, prepared from autoclaved, double-distilled water), and then stored it in aliquots at −18°C prior to the experiments. For all three experiments, we included Ringer's solution with no added LPS (henceforth 'Ringers') as a control; aliquots of Ringers were prepared and frozen at the same time as the LPS-containing aliquots, from the same batch of Ringer's solution. For Experiment 1 only, we included two additional treatments, such that there were four treatments in Experiment 1 and two in Experiments 2-3.

The two treatments unique to Experiment 1 were an 'Intact control' that did not involve any treatment solution (see below), and a heat-treated LPS treatment. We prepared the heat-treated LPS by placing a random sample of the LPS-in-Ringers aliquots into a heat block at 100°C for 30 minutes, following advice from an immunologist colleague who recommended the use of heat-treated LPS as a control. Heat-treated LPS is commonly compared to standard LPS in immunological studies of mammals or their cells, apparently in the belief that LPS is chemically altered by heating in a way that reduces its immune-stimulating effect. However, LPS is highly heat-stable (24), and these experiments have repeatedly found no evidence that heat treatment reduces the immunogenicity of LPS (e.g. 25; 26; 27; 28; 29), such that heat-treated LPS may not be a useful control. We nevertheless included heat-treated LPS in Experiment 1 following

the advice received, but one should bear in mind that heat treatment likely did not prevent LPS from stimulating the immune system.

To administer these three solutions (Ringers, heat-treated LPS in Ringers, and LPS in Ringers), bees were anaesthetised in small groups (30-40 individuals) by placing them in a −18°C freezer inside a plastic container until they were immobile but still moving their appendages (typically *c.* 6 minutes). We then kept the containers of bees over ice, and monitored them to maintain this state of light anaesthesia. Using a stereomicroscope and an entomological pin (0.25mm; sterilised in ethanol and a candle flame between uses), we then randomly selected a bee, dipped the pin into one of the treatment solutions, and then inserted the pin through the pleural membrane between the fourth and fifth tergal segments to a distance of roughly 1mm (using a different pin for each treatment solution). Bees in Experiment 1's 'Intact control' control were handled similarly (i.e. anaesthetised and manipulated under the microscope), but were not punctured with a pin. After treatment, bees were marked on the thorax with a dot of coloured paint to identify their treatment group; we used a different pairing of colours and treatments for each experimental replicate to prevent confounding. For all experiments, we applied the treatments on a rotation (e.g. control-LPS-control-LPS for Experiment 2), preventing confounds due to the time spent under anaesthesia or order of processing.

Experiment 1: Do immune-challenged bees leave the hive?

Experiment 1 utilised four treatments: the intact control, and the treatments in which bees were punctured with Ringers, heat-treated LPS, or LPS. After applying these treatments as described above, bees were housed in their treatment groups, in the dark at 25°C, for 18±1 hours. We then removed any bees that had died or showed impaired mobility, and reintroduced the remainder to the hive, by opening the hive and returning them to the central frame where they had been collected. The hive entrance was then recorded for up to two hours by an observer who stood by the hive (mean observation time: 97.5 minutes). We also video recorded the hive entrance to double-check each observation (done blind by a second observer).

We recorded a categorical response variable with three possible outcomes: bees either stayed inside the hive for the duration of the observation period, left the hive voluntarily, or were forced out. We recorded a bee as leaving voluntarily when it walked or flew out of the hive without interacting with any other workers. We recorded the 'forced out' outcome when the focal bee was pulled out of the hive by one or more other workers using their mandibles. Only bees that left the landing board at the hive entrance were classified as having exited the hive. Only two bees left the hive by flying away: the rest did so by dropping to the ground, or being dragged and dropped. Four bees emerged from the hive and then re-entered (1× intact control, 1× Ringers, 2× heat-treated LPS); the meaning of this behaviour is not clear, so these four observations were excluded from analysis. Experiment 1 was replicated over four hives: three in Autumn (30/04, 3/05 and 9/05 in 2019), and one in Spring/Summer (19/11/2019; added to increase the sample size).

Experiment 2: The role of cuticular odours

To improve the per-treatment sample size, and because of uncertainty regarding the heat-treated LPS treatment, Experiment 2 involved just two treatments: Ringers and LPS. We first collected a sample of *c.* 200 workers from inside a hive, anaesthetised them, and punctured them with either Ringers or LPS in Ringers as in Experiment 1 (except that we did not apply a paint mark). We then housed these bees at 25°C in the dark for 24 hours in their treatment groups, to give enough time for large changes in the cuticular odour profile to occur following puncturing with LPS. Earlier experiments have shown that 24h is enough time for LPS-treated insects to develop a substantially different chemical profile relative to Ringers-treated controls (30); indeed, Richard *et al.* (17) observed changes after only 4h.

Next, we freeze-killed the surviving bees and placed an equal number from each treatment into two 20mL glass vials. We then added 500μL hexane (HPLC grade; Sigma ref. 34859) per bee, and then gently shook the vials by hand for 60 seconds to facilitate dissolution of hexane-soluble epicuticular chemicals, then pipetted the extract into aliquots in 2mL glass vials.

On the same day that we prepared the chemical extracts, we collected a further 200 bees from the same hive, which were cold-immobilised and then marked with one of two paint colours. As before, we used different treatment-colour pairings for each hive to avoid confounding. Because hexane is toxic, we applied the extracted CHCs to the bees indirectly by pipetting 20μL of the appropriate hexane solution onto the surface of deionised water in a 10mL glass beaker (31). After waiting a few seconds for the hexane to evaporate, we then dipped an anaesthetised, paint-marked bee through the surface of the water, and swirled it in the water's surface to allow it to pick up hydrophobic solutes (e.g. cuticular hydrocarbons) that were floating on the water's surface. The odour-coated bees were then reintroduced to their hive, and their subsequent emergence was recorded over the next 1-2 hours (mean 97.5 minutes), as in Experiment 1. Experiment 2 was replicated across four hives between 26th May and 28th June 2019.

Experiment 3: 'Social distancing' following immune challenge

Like Experiment 2, this experiment had two treatments: Ringers and LPS. We collected approximately 250 bees from a brood frame as before, paired them at random, and then randomly assigned each pair to one of the two treatments. One of the bees in each pair was punctured with either Ringers or LPS, while the other was left intact. Each pair was placed into a 22mL glass test tube stoppered with cotton wool. All of the test tubes were put into a ZebraTower video recording cabinet (Viewpoint, France), then recorded under infrared illumination (invisible to bees). We then analysed the videos using 'scan sampling'; specifically, we examined video stills separated by 120s intervals, and recorded whether or not each pair of bees was in close contact (defined as within 1.5cm of each other) in each video still (i.e. the response variable was binary). The observation period lasted 3.5 hours, and began 30 minutes after closing the video recording cabinet to allow time for the bees to settle following the disturbance. Videos were transcribed blind with

respect to treatment and hive. Experiment 3 was replicated across four hives in the same time period as for Experiment 2.

Statistical analysis

Our main statistical analyses for Experiments 1 and 2 use an unfamiliar type of model, which we nevertheless believe is the right choice given the data. Following reviewer feedback we first present an analysis that uses a more standard statistical approach. Specifically, we re-coded the response variable to have only two categories, namely 'Left the hive' or 'Stayed inside', which facilitated analysis using a binomial generalised linear model (GLM) with treatment and hive as fixed effects.

The main analysis used a Bayesian multinomial logistic model, which is an extension of binomial models to >2 outcomes, implemented in the R package `brms` (32). As for the simpler model, we included treatment and hive as fixed effects. We specified a prior distribution of $N(\mu = 0, \sigma^2 = 3)$ for all fixed effect estimates, in order to 'regularise' the parameter estimates; this helps to prevent overfitting, and is conservative compared to using a flatter prior or frequentist approaches (33). Experiment 3 had a binary response variable and was therefore modelled using the binomial family in `brms`. The model included the same two fixed effects as before (treatment and hive), and we additionally included the random factor 'pair ID', to account for repeated measurements of each pair of bees. We ran four chains for the Bayesian models of Experiments 1 and 2 (5000 iterations per chain; first 2500 discarded as burn-in), and confirmed model convergence and fit via $\hat{R}$ statistics and posterior predictive plots. The model for Experiment 3 required additional iterations (20,000 per chain, with 10,000 burn-in) to ensure adequate effective sampling. To make inferences from each model we calculated the posterior estimate of the difference in means between each pair of treatments, using log odds ratios as a standardised measure of effect size (effectively running 'planned contrasts').

Results

Experiment 1: Do immune-challenged bees leave the hive?

In the preliminary GLM, we found a significant effect of treatment (Likelihood ratio test; $p = 0.012$) and hive ($p < 0.0001$) on the proportion of bees leaving the hive. More bees left in the two LPS treatments relative to the intact control ($p = 0.022$ and $p = 0.032$), though there was no significant difference between the intact and Ringers controls ($p = 0.25$).

Figure 1A uses the results of the the Bayesian multinomial model to display the posterior estimates of the percentages of bees from each treatment group that stayed inside the hive, left voluntarily, or were forced out, while Figure 1B shows the standardised effect size for planned contrasts between treatment groups for each of the three response

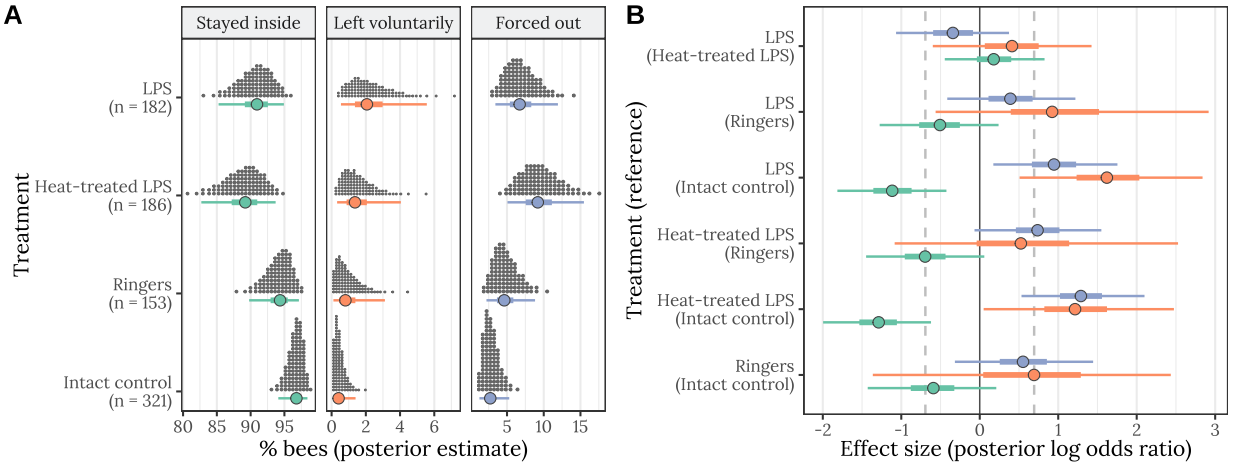

Figure 1: Results of Experiment 1 (n = 842 bees). Panel A shows the posterior estimate of the mean % bees staying inside the hive (left), leaving voluntarily (middle), or being forced out (right), for each of the four treatments. The quantile dot plot shows 100 approximately equally likely estimates of the true % bees, and the horizontal bars show the median and the 50% and 95% credible intervals of the posterior distribution. Panel B gives the posterior estimates of the effect size of each treatment, relative to one of the other treatments (the name of which appears in parentheses), and expressed as a log odds ratio (LOR). Positive LOR indicates that the % bees showing this particular outcome is higher in the treatment than the control; for example, more bees left voluntarily (green) or were forced out (orange) in the LPS treatment than in the intact control. The dashed lines mark $LOR = 0$, indicating no effect, and $LOR = \pm log(2)$, i.e. the point at which the odds are twice as high in one treatment as the other.

categories. Table S1 shows the model's parameter estimates, and Table S2 shows the results of planned contrasts comparing each pair of treatments (as in Figure 1B).

Bees treated with LPS or heat-treated LPS were significantly less likely to stay inside the hive compared to the intact control: the posterior probability that the true effect size is negative was over 99.99% for both LPS and heat-treated LPS (Table S2); this percentage can be interpreted similarly to a one-tailed p-value of 0.0001. Hereafter, we write PP to represent 1 minus this posterior probability, for easier comparison with the familiar p-value.

Furthermore, bees that received LPS or heat-treated LPS were less likely to stay inside the hive than those that received the Ringers control; this difference was significant at the conventional threshold for the heat-treated LPS group (PP = 0.038) but not the LPS group (PP = 0.089). Bees were also somewhat more likely to leave the hive if treated with Ringers, relative to the intact control (PP = 0.072). There was no difference between the LPS and heat-treated LPS (PP = 0.29). Some of the estimated effect sizes (Figure 1B, Table S2) were quite large (e.g. Log odds ratio: 1.00 for the 'Left voluntarily' outcome when comparing the LPS and Ringers treatments), indicating that the LPS treatments doubled or tripled the rates at which bees left the hive by either mode of exit.

The results were similar when considering the numbers of bees that were forced out, or left voluntarily (Figure 1, Table S2), though the differences were more apparent for the 'Forced out' outcome, perhaps because this outcome occurred more commonly (5-10% of bees) than the 'Left voluntarily' outcome (1-3%), increasing our precision to measure the treatment effects. In particular we note differences between the intact control and the two LPS treatments,

and weaker differences (significant, in the case of the 'Forced out' outcome) with the Ringers control.

Experiment 2: Role of cuticular odours in behavioural responses to immune challenge

In the preliminary GLM analysis, we found a significant effect of both treatment and hive (Likelihood ratio tests; $p < 0.0001$) on the proportion of bees leaving the hive. More bees left in the group perfumed with odours from LPS-treated bees relative to the control.

Figure 2A uses the results of the the Bayesian multinomial model to display the posterior estimates of the percentages of bees from each treatment group that stayed inside the hive, left voluntarily, or were forced out, while Figure 2B shows the standardised effect size of the LPS treatment, for each of the three response categories. Table S3 shows the model's parameter estimates, and Table S4 shows the results of planned contrasts comparing each pair of treatments (as in Figure 2B).

Figures 2A-B and Tables S3-4 illustrate that bees coated with chemical extracts from LPS-treated bees were forced out of the hive significantly more often than were those treated with chemical extracts from Ringers-treated bees (PP = 0.0048). The estimated effect sizes (Figure 2B, Table S4) were quite large (e.g. Log odds ratio: 1.08 for the 'Forced out' outcome), indicating that perfuming with odours from LPS-treated bees almost tripled the rate at which bees were forced out relative to the Ringers control. Interestingly, there was no difference in the rate at which the two treatment groups left the hive *voluntarily* (PP = 0.29), and we were able to ascertain that the true effect size lies close to zero with moderate certainty.

We also tested whether the odour treatment altered the proportion of bees that were forced out, among the subset that left the hive. Figure 2C plots the posterior estimate of the effect size of the LPS treatment on this proportion, expressed as a log odds ratio. The posterior median log odds ratio was 0.73, with 95% credible intervals of -0.23 to 1.71 (PP = 0.11). The result is not statistically significant, though it provides some evidence that the proportion of hive-leavers that were forced out was higher in the LPS treatment than the control.

Experiment 3: 'Social distancing' following immune challenge

Figures 3A-C illustrate that pairs of bees in which one individual had been treated with LPS spent less time in close contact than pairs in which one individual had received Ringers. Figure 3A shows histograms of the raw data, illustrating that bees in the control group more often spent >90% of the 3.5 hour observation in close contact, while LPS treatment bees were over-represented among pairs that spent most of their time apart. Tables S5-6 give the associated statistical results; the difference in mean % time in close contact (Figure 3B) was statistically significant (PP = 0.033), and the effect size was moderate (Figure 3C; log odds ratio: -0.37).

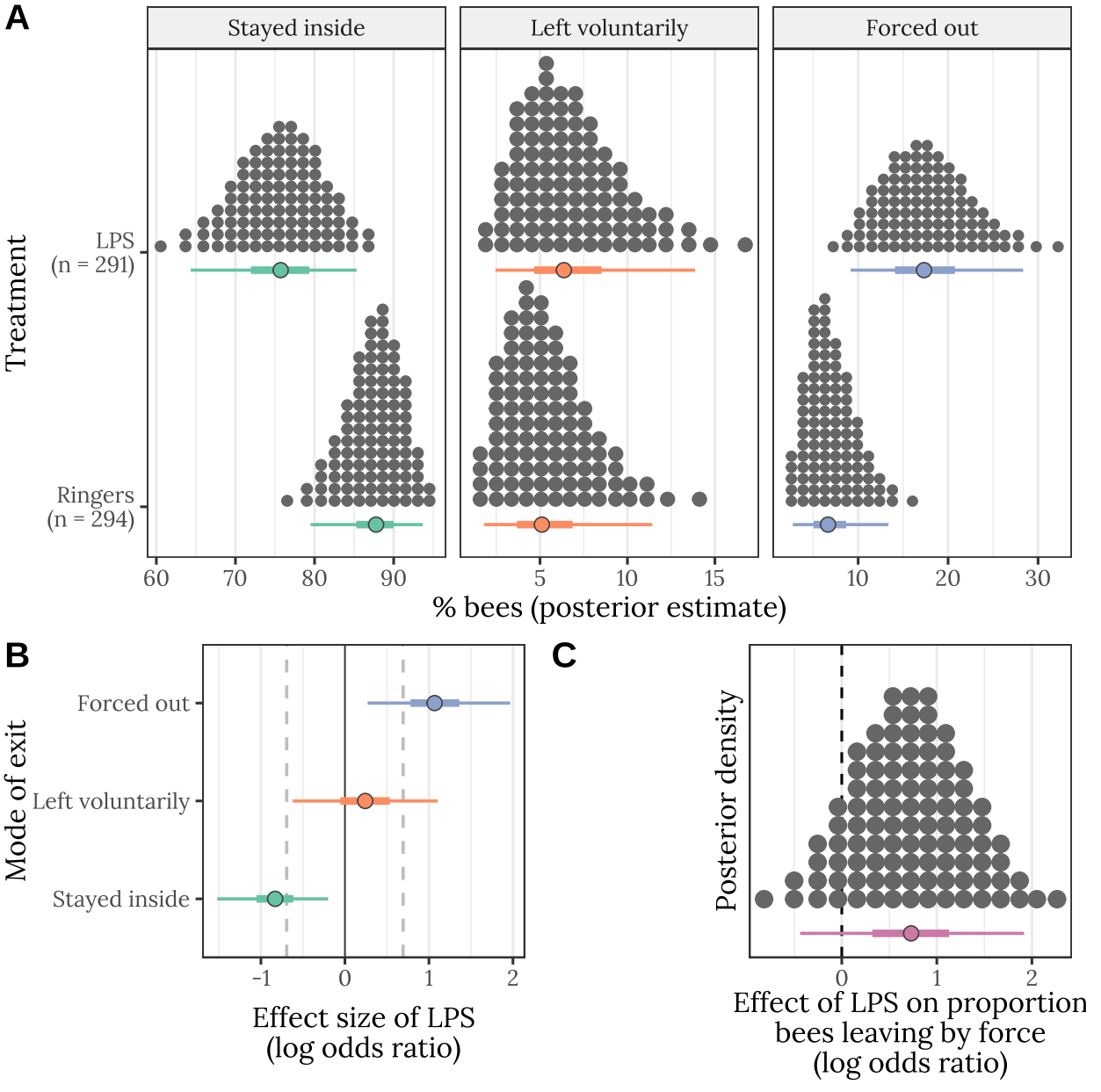

Figure 2: Results of Experiment 2 (n = 585 bees). Panel A shows the same information as Figure 1A. Panel B gives the posterior estimates of the effect size (log odds ratio) of the LPS treatment as a log odds ratio, for each of the three possible outcomes; the details are the same as in Figure 1B. Panel C shows the posterior estimate of the effect of the LPS treatment on the proportion of bees observed leaving the hive by force, as opposed to leaving voluntarily.

Discussion

Experiment 1 revealed that bees pierced with standard or heat-treated LPS were more likely to leave the hive compared to the intact control and (to a lesser extent) the Ringers control. There was no difference in the rate at which the Ringers and intact controls bees left the hive, and no difference between standard and heat-treated LPS. Most of the bees that left the hive in Experiment 1 (especially in the LPS treatments) were forced out by other colony members, though

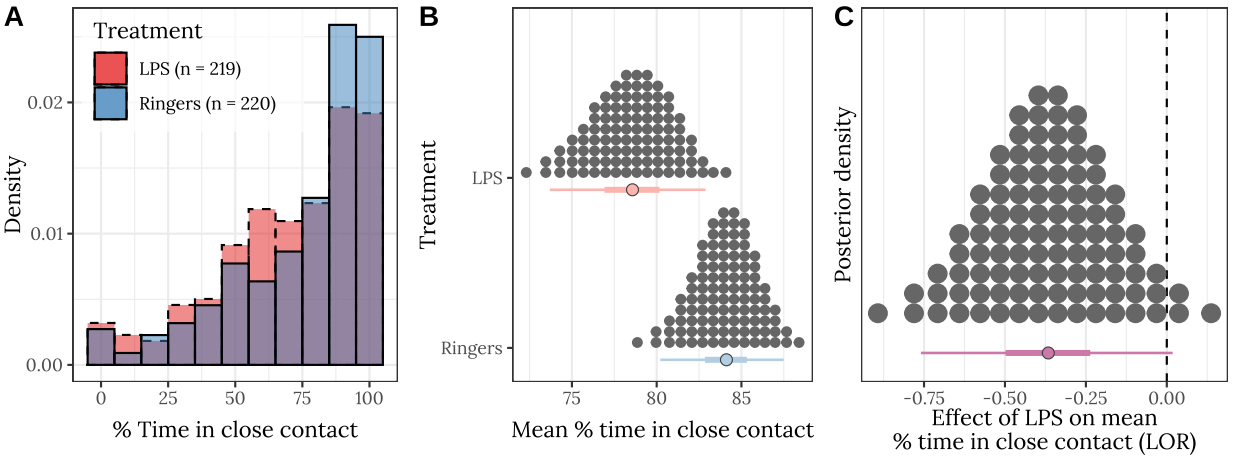

Figure 3: Results of Experiment 3 (n = 439 bees). Panel A shows the frequency distribution of the % time in close contact, for pairs of bees from the LPS treatment and the Ringers control. Panel B shows the posterior estimates of the mean % time spent in close contact; the details of the quantile dot plot and error bars are the same as described for Figure 1. Panel C shows the effect size (LOR; log odds ratio) associated with the difference in means in Panel B.

many appeared to leave voluntarily, by walking out and then dropping to the ground without any apparent involvement from other workers. Very few left by flying, unlike in an earlier study (19) that reported "altruistic self-removal" by honeybees with experimentally-impaired health; this discrepancy may reflect different methodologies (the other study challenged bees with CO_2 or hydroxyurea, rather than LPS). These bees were frequently found dead on the same or subsequent days, and many were predated by Vespid wasps within seconds of dropping to the ground, illustrating that the hive-exiting behaviour is unlikely to represent efforts to temporarily self-quarantine. From Experiment 1, we conclude that bees that are wounded (as in the Ringers control) and/or immune-stimulated tend to leave the hive, both with and without the apparent involvement of other workers.

The removal of immune-stimulated bees by their nestmates implies that the former produce signals or cues which allow them to be identified for removal from the colony. We hypothesised that at least some of these signals/cues would be olfactory, since Hymenoptera rapidly develop a distinct chemical profile after an immune challenge (17; 30). We tested this hypothesis in Experiment 2, and found that bees coated with hexane-soluble chemicals extracted from the body surface of immune-challenged bees were ejected from the colony threefold more often than controls treated with chemicals from Ringers-treated bees. Interestingly, there was no treatment effect on the rate at which bees left the hive voluntarily. We hypothesise that this result stems from the mismatch created in Experiment 2 between the personal information held by the experimental individuals (which correctly perceived themselves to be healthy), and the chemical cues on their body surface (which came from immune-challenged individuals), causing the bees to be forced out by their nestmates, but not to leave voluntarily.

In light of past findings that immune-challenged ants (34) and bees (17; 21) engage in fewer social interactions,

Experiment 3 tested whether pairs of bees in which one member had received an immune challenge spent less time in close contact than control pairs. We recorded a statistically significant decline in the proportion of time spent in contact, suggesting a behavioural response to immune challenge in the treated individual, the healthy individual paired with them, or both. A previous study recorded that bees directed more aggression and grooming behaviours towards immune-challenged bees (17); behavioural effects like this could underpin our results. Another study recorded that LPS-treated bees showed reduced locomotion and antennated other individuals less often, which might also explain our results. Such 'sickness behaviour' might have been shaped by kin selection to limit disease transmission (21), though there may also be direct fitness benefits (or non-adaptive explanations) for sickness behaviour, especially given that it occurs in non-eusocial animals (e.g. 35).

The chemical cues that distinguish healthy and immune-challenged individuals remain to be determined. Cuticular hydrocarbons (CHCs) are one likely possibility, given that honeybees utilise CHCs for chemical recognition in several other contexts (e.g. 14). Furthermore, the CHC profile changes rapidly following an immune challenge, in both eusocial and non-eusocial insects. For example, honeybee workers injected with Gram-negative bacteria began to produce relatively more unsaturated and shorter-chained hydrocarbons within 6h, and there were concommitant changes in the expression of genes involved in CHC biosynthesis (36). Another reason to suspect CHCs is that the insect innate immune response involves changes in the expression of genes that have pleiotropic effects on lipid metabolism/homeostasis, such as those in the IMD pathway (e.g. 37), such that there are a plausible mechanistic links between the CHC profile and immune status. However, no study has yet manipulated the CHC profile without confounds – our study and earlier, similar experiments (17) involved solvent washes rather than treatment with CHCs only – so the involvement of CHCs remains to be demonstrated conclusively.

Another outstanding question is whether the changes in the external chemical cues of immune-challenged bees represent an adaptation, or simply a non-adaptive consequence of other processes (i.e. a 'spandrel'; 38). Under the adaptive hypothesis, sick bees that purposefully signal their illness would be the superorganismal equivalent of infected vertebrate cells, which use MHC class I proteins to 'present' antigens to cytotoxic T cells, which then destroy the infected cell. Presumably, the antigen-presenting system evolved adaptively (39); it could be framed as a kin-selected adaptation because the self-sacrificing cells confer a benefit to other cells in the body that carry the same alleles. Under the non-adaptive model, immune-challenged bees might produce modified chemical cues for reasons other than eliciting their own removal, e.g. because of pleiotropic links between immunity and metabolism (37); the key feature distinguishing these two hypotheses is the presence of a net inclusive fitness benefit to workers that solicit their own removal. In support of the non-adaptive hypothesis, immune challenge has also been found to affect the CHC profile of non-social insects that appear to have no need for social immunity (e.g. the beetle *Tenebrio molitor*; 40). To begin establishing whether chemical signalling of immune status has evolved adaptively, one could test whether social

insects undergo uniquely strong chemical or behavioural changes following an immune challenge, relative to non-social insects, in a formal phylogenetic study.

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Data and code availability

Raw data and documented R code and output is available at lukeholman.github.io/social_immunity.

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Online Supplementary Material

Social immunity and chemical communication in the honeybee: immune-challenged bees enter enforced or self-imposed exile, by Tarli Conroy and Luke Holman.

The figures and tables in this document, along with the with the R code used to generate them, can also be viewed online at https://lukeholman.github.io/social_immunity/

Table S1: Table summarising the posterior estimates of each fixed effect in the best-fitting model of Experiment 1. This was a multinomial model with three possible outcomes (stay inside, leave voluntarily, be forced out), and so there are two parameter estimates for the intercept and for each predictor in the model. 'Treatment' is a fixed factor with four levels, and the effects shown here are expressed relative to the 'Intact control' group. 'Hive' was also a fixed factor with four levels; unlike for treatment, we modelled hive using deviation coding, such that the intercept term represents the mean across all hives (in the intact control treatment), and the three hive terms represent the deviation from this mean for three of the four hives. Lastly, observation duration was a continuous predictor expressed to the nearest minute. The p column gives the posterior probability that the true effect size is opposite in sign to what is reported in the Estimate column, similarly to a p-value.

Parameter	Estimate	Est. Error	Lower 95% CI	Upper 95% CI	PP	
% bees leaving voluntarily						
Intercept	-5.43	0.71	-6.97	-4.19	0.0000	***
Treatment: Ringers	0.67	0.95	-1.33	2.45	0.2282	
Treatment: Heat-treated LPS	1.29	0.62	0.12	2.55	0.0162	*
Treatment: LPS	1.68	0.60	0.55	2.88	0.0021	**
hive1	-3.22	1.48	-6.64	-0.94	0.0003	***
hive2	1.90	0.58	0.91	3.17	0.0000	***
hive3	-0.08	0.64	-1.28	1.27	0.4311	
% bees forced out						
Intercept	-3.66	0.36	-4.40	-3.01	0.0000	***
Treatment: Ringers	0.56	0.45	-0.31	1.46	0.1062	
Treatment: Heat-treated LPS	1.31	0.40	0.55	2.12	0.0004	***
Treatment: LPS	0.97	0.41	0.19	1.77	0.0068	**
hive1	-1.76	0.58	-3.07	-0.79	0.0000	***
hive2	-0.42	0.36	-1.15	0.31	0.1194	
hive3	0.07	0.28	-0.47	0.65	0.4161	

Table S2: This table gives statistics associated with each of the contrasts plotted in Figure 1B. Each pair of rows gives the absolute effect size (i.e. the difference in % bees) and standardised effect size (as log odds ratio; LOR) for the focal treatment, relative to the treatment shown in parentheses, for one of the three possible outcomes (stayed inside, left voluntarily, or forced out). A LOR of $|log(x)|$ indicates that the outcome is x times more frequent in one treatment compared to the other, e.g. $log(2) = 0.69$ and $log(0.5) = -0.69$ correspond to a two-fold difference in frequency. The PP column gives the posterior probability that the true effect size has the same sign as is shown in the Estimate column; this metric has a similar interpretation to a one-tailed p value in frequentist statistics.

Comparison	Metric	Estimate	Est.Error	Lower 95% CI	Upper 95% CI	PP	
% bees staying inside							
LPS (Heat-treated LPS)	Difference in % bees staying inside	1.57	2.85	-3.82	7.33		
	Log odds ratio	0.18	0.33	-0.45	0.83	0.2911	
LPS (Ringers)	Difference in % bees staying inside	-3.31	2.59	-8.60	1.62		
	Log odds ratio	-0.51	0.39	-1.28	0.24	0.0895	~
LPS (Intact control)	Difference in % bees staying inside	-5.83	2.24	-10.66	-1.93		
	Log odds ratio	-1.11	0.36	-1.81	-0.42	8e-04	***
Heat-treated LPS (Ringers)	Difference in % bees staying inside	-4.88	2.79	-10.53	0.44		
	Log odds ratio	-0.70	0.39	-1.45	0.06	0.0367	*
Heat-treated LPS (Intact control)	Difference in % bees staying inside	-7.40	2.49	-12.75	-3.03		
	Log odds ratio	-1.30	0.35	-2.00	-0.62	1e-04	***
Ringers (Intact control)	Difference in % bees staying inside	-2.52	1.91	-6.72	0.75		
	Log odds ratio	-0.60	0.41	-1.43	0.21	0.0721	~
% bees leaving voluntarily							
LPS (Heat-treated LPS)	Difference in % bees leaving voluntarily	0.79	1.13	-1.21	3.46		
	Log odds ratio	0.41	0.51	-0.60	1.42	0.2121	
LPS (Ringers)	Difference in % bees leaving voluntarily	1.36	1.38	-1.10	4.47		
	Log odds ratio	0.99	0.87	-0.56	2.92	0.1183	
LPS (Intact control)	Difference in % bees leaving voluntarily	1.97	1.22	0.30	4.95		
	Log odds ratio	1.64	0.60	0.51	2.84	0.0025	**
Heat-treated LPS (Ringers)	Difference in % bees leaving voluntarily	0.57	1.19	-1.83	3.16		
	Log odds ratio	0.58	0.91	-1.08	2.53	0.2668	
Heat-treated LPS (Intact control)	Difference in % bees leaving voluntarily	1.18	0.89	0.03	3.43		
	Log odds ratio	1.23	0.62	0.05	2.47	0.0213	*
Ringers (Intact control)	Difference in % bees leaving voluntarily	0.61	0.92	-0.65	2.95		
	Log odds ratio	0.65	0.95	-1.36	2.43	0.2363	
% bees forced out							
LPS (Heat-treated LPS)	Difference in % bees forced out	-2.36	2.60	-7.79	2.48		
	Log odds ratio	-0.34	0.37	-1.07	0.37	0.1843	
LPS (Ringers)	Difference in % bees forced out	1.95	2.18	-2.21	6.35		
	Log odds ratio	0.39	0.42	-0.41	1.22	0.1741	
LPS (Intact control)	Difference in % bees forced out	3.86	1.93	0.61	8.12		
	Log odds ratio	0.95	0.41	0.17	1.75	0.0078	**
Heat-treated LPS (Ringers)	Difference in % bees forced out	4.31	2.54	-0.38	9.60		
	Log odds ratio	0.74	0.41	-0.07	1.55	0.0374	*
Heat-treated LPS (Intact control)	Difference in % bees forced out	6.22	2.36	2.18	11.43		
	Log odds ratio	1.29	0.40	0.53	2.10	5e-04	***
Ringers (Intact control)	Difference in % bees forced out	0.61	0.92	-0.65	2.95		
	Log odds ratio	0.65	0.95	-1.36	2.43	0.2363	

Table S3: Table summarising the posterior estimates of each fixed effect in the best-fitting model of Experiment 2. This was a multinomial model with three possible outcomes (stay inside, leave voluntarily, be forced out), and so there are two parameter estimates for the intercept and for each predictor in the model. 'Treatment' is a fixed factor with two levels, and the effect of LPS shown here is expressed relative to the 'Ringers' treatment. 'Hive' was a fixed factor with four levels; unlike for treatment, we modelled hive using deviation coding, such that the intercept term represents the mean across all hives (in the Ringers treatment), and the three hive terms represent the deviation from this mean for three of the four hives. Lastly, observation duration was a continuous predictor expressed to the nearest minute. The PP column gives the posterior probability that the true effect size is opposite in sign to what is reported in the Estimate column, similarly to a *p*-value.

Parameter	Estimate	Est. Error	Lower 95% CI	Upper 95% CI	PP	
% bees leaving voluntarily						
Intercept	-3.51	0.36	-4.26	-2.86	0.0000	***
Treatment: LPS	0.37	0.44	-0.48	1.26	0.1962	
hive1	0.75	0.34	0.08	1.41	0.0146	*
hive2	-0.39	0.45	-1.32	0.41	0.1867	
hive3	-0.99	0.49	-2.05	-0.11	0.0130	*
% bees forced out						
Intercept	-3.75	0.38	-4.56	-3.05	0.0000	***
Treatment: LPS	1.10	0.43	0.29	1.98	0.0035	**
hive1	0.29	0.35	-0.42	0.97	0.1970	
hive2	-0.96	0.48	-2.00	-0.10	0.0127	*
hive3	-0.48	0.38	-1.25	0.23	0.0967	~

Table S4: This table gives statistics associated with each of the contrasts plotted in Figure 2B. Each pair of rows gives the absolute (i.e. the difference in % bees) and standardised effect size (as log odds ratio; LOR) for the LPS treatment, relative to the Ringers control, for one of the three possible outcomes (stayed inside, left voluntarily, or forced out). A LOR of $|log(x)|$ indicates that the outcome is x times more frequent in one treatment compared to the other, e.g. $log(2) = 0.69$ indicates a two-fold difference in frequency. The PP column gives the posterior probability that the true effect size has the same sign as is shown in the Estimate column; this metric has a similar interpretation to a one-tailed p value in frequentist statistics.

Metric	Estimate	Est.Error	Lower 95% CI	Upper 95% CI	PP	
% bees staying inside						
Absolute difference in % bees staying inside	-11.90	4.91	-22.04	-2.86		
Log odds ratio	-0.83	0.33	-1.49	-0.21	0.0053	**
% bees leaving voluntarily						
Absolute difference in % bees leaving voluntarily	1.32	2.60	-3.69	7.12		
Log odds ratio	0.24	0.44	-0.62	1.13	0.2944	
% bees forced out						
Absolute difference in % bees forced out	10.58	4.59	2.57	20.64		
Log odds ratio	1.08	0.43	0.27	1.95	0.0039	**

Table S5: Table summarising the posterior estimates of each fixed effect in the best-fitting model of Experiment 3 that contained the treatment effect. This was a binomial model where the response variable was 0 for observations in which bees were not in close contact, and 1 when they were. 'Treatment' is a fixed factor with two levels, and the effect of LPS shown here is expressed relative to the 'Ringers' treatment. 'Hive' was a fixed factor with four levels; unlike for treatment, we modelled hive using deviation coding, such that the intercept term represents the mean across all hives (in the Ringers treatment), and the three hive terms represent the deviation from this mean for three of the four hives. The model also included one random effect, 'pair ID', which grouped observations made on each pair of bees, preventing pseudoreplication. The PP column gives the posterior probability that the true effect size is opposite in sign to what is reported in the Estimate column, similarly to a p-value.

Parameter	Estimate	Est. Error	Lower 95% CI	Upper 95% CI	PP	
Intercept	1.67	0.14	1.40	1.95	0.0000	***
Treatment: LPS	-0.37	0.20	-0.76	0.02	0.0316	*
hive1	-0.21	0.17	-0.55	0.13	0.1110	
hive2	0.15	0.16	-0.17	0.48	0.1836	
hive3	-0.23	0.18	-0.58	0.12	0.0924	~

Table S6: Pairs in which one bee had received LPS were observed in close contact less frequently than pairs in which one bee had received Ringers. The PP column gives the posterior probability that the true effect size is opposite in sign to what is reported in the Estimate column, similarly to a p-value.

Metric	Estimate	Est.Error	Lower 95% CI	Upper 95% CI	PP	
Absolute difference in % time in close contact	5.55	3.0	-0.27	11.52		
Log odds ratio	-0.37	0.2	-0.76	0.02	0.0316	*