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

Tarli E. Conroy[a,*], Luke Holman[a,*]

[a]School of BioSciences, University of Melbourne, Royal Parade, Parkville, VIC 3010, Australia

ARTICLE INFO

Keywords:
Cuticular Hydrocarbons
Evolutionary immunology
Social immunity
Hygienic behaviour
Lipopolysaccharide

ABSTRACT

Animals living in large societies are especially vulnerable to pathogens, as their close proximity facilitates the spread of infections. 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. Here, we perform experiments with immune-challenged honey bee workers (*Apis mellifera*). We find that workers treated with an inert immune challenge (LPS) that mimics infection with Gram-negative bacteria quickly exit the hive, either voluntarily or by being dragged out by other workers; bees exiting the hive subsequently died. In a second experiment, we find that healthy workers treated with surface chemicals from LPS-treated bees are evicted from the hive more often than controls, indicating that immune-challenged bees produce chemical cues that cause 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 was immune challenged, relative to procedural controls. Our findings suggest that immune-challenged bees altruistically banish themselves, and that workers evict sick individuals (which are identified via olfactory cues), putatively because of (kin) selection to limit the spread of pathogens within colonies.

INTRODUCTION

Colonial animals face a heightened risk of infectious disease, which can spread rapidly when conspecifics come into contact often (Naug and Camazine, 2002; Pie et al., 2004). One might therefore predict more social species to evolve stronger immune defences, in response to the elevated opportunities for disease transmission. However, eusocial Hymenoptera appear to buck this trend. Honey bees and ants have fewer immune defence genes than solitary insects such as *Drosophila*, putatively because they lost immune genes following the evolution of eusociality (Evans et al., 2006; Gadau et al., 2012). Additionally, an interspecific comparative analysis found a negative correlation between colony size and physiological immune responses (López-Uribe et al., 2016), suggesting that communal living might instead select for *weaker* immune defences.

In light of these genetic and physiological findings, as well as observations of their behaviour, researchers hypothesise that eusocial insects combat pathogens using 'social immunity', which reduces pathogen exposure and selects for reduced investment in physiological immune defences (Cremer et al., 2007). 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 (Cremer et al., 2007). For example, many eusocial insects collectively remove waste from their colonies (Bot et al., 2001), or coat the interior of the nest with antimicrobial agents collected from plants or produced in their own bodies (Christe et al., 2003; Simone-Finstrom and Spivak, 2010). Many species have compartmentalised nests that help to contain the spread of pathogens; for example, leaf cutter ants have a separate garbage dump, and workers from the dump tend not to venture into the rest of the colony (Hart and Ratnieks, 2001). Some bees deal with infestations of parasitic mites by identifying and removing infected larvae and pupae (Spivak, 1996), or by 'mummifying' foreign objects or even live intruders with wax and propolis (Greco et al., 2010).

Studies have reported that honeybees respond behaviourally to sick individuals, in contexts that suggest social immunity. Baracchi et al. (2012) 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 (Baracchi et al., 2012). 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 (van Zweden and d'Ettorre, 2010), the queen (Holman, 2018), and workers of differ ages and task specialties (Greene and Gordon, 2003). Secondly, Richard et al. (2008) 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 (Hamilton, 1964) 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 (typically

*Corresponding author
luke.holman@unimelb.edu.au (L. Holman)
ORCID(s): 0000-0002-7268-2173 (L. Holman)

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 – the course of action that maximises the worker's inclusive fitness may be to leave the colony permanently. Consistent with this hypothesis, Rueppell et al. (2010) 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 that this behaviour represented 'altruistic suicide' by workers perceiving themselves to be close to death and/or infectious, which has evolved due to indirect fitness benefits to the individual's healthy relatives. Similarly, Heinze and Walter (2010) found that moribund ants affected by a fungal infection left their nests and remained outside until death, potentially because of kin selection to prevent the infection of nestmates. 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 (Kazlauskas et al., 2016).

In light of this research, we hypothesise that honeybees (and perhaps other eusocial insects) use a multi-pronged approach to combat infection that involves both collective behaviour (e.g. the quarantining of sick individuals by the society) as well as individual responses (altruistic self-quarantine). We here investigate these ideas using behavioural experiments on social immunity in the European honeybee, *Apis mellifera*. Our experiments utilise 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 (Imler et al., 2000; Aubert and Richard, 2008; Kazlauskas et al., 2016). In Experiment 1, we treated worker bees with LPS or various procedural controls, re-introduced them to their natal hive, and then estimated the rates at which bees from each treatment were ejected from the hive or left voluntarily. In Experiment 2, we transferred the surface chemicals of immune-challenged bees to healthy bees, and tested whether the healthy bees were ejected from their colony 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. Kazlauskas et al., 2016), 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 controls, giving a total of four treatments in Experiment 1 and two in Experiments 2-3. First, Experiment 1 included an 'Intact control', which did not involve a treatment solution (see below). Secondly, Experiment 1 used heat-treated LPS in Ringers, created by placing a random sample of the LPS aliquots into a heat block at 100°C for 30 minutes. Heat-treated LPS is commonly used as a control in immunological studies of mammals or mammalian cells, even though many of these studies subsequently find that heat treatment has no effect on the immune-stimulating effect of LPS (Singh-Jasuja et al., 2000; Panjwani et al., 2002; Millar et al., 2003; Motojima et al., 2010; Coveney et al., 2015). We nevertheless included heat-treated LPS in Experiment 1 to test whether it might be a useful control in experiments on insects.

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 incapable of flight or walking, but were 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 to prevent cross-contamination). 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 which treatment they belonged to; 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), so that the average order in which bees were processed

was the same for all treatments (preventing confounds due to the time spent under anaesthesia, etc.).

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 they had been collected from. The hive entrance was then recorded for up to two hours (sometimes cut short due to rain or technical issues) 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 multinomial 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 with no apparent involvement from 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 dropped). Four bees emerged from the hive and then re-entered (1 intact control, 1 Ringers, 2 heat-treated LPS); the significance of this behaviour is not clear, so we excluded these four observations when analysing the data. 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).

Experiment 2: The role of cuticular odours

To improve the per-treatment sample size, and because of the uncertain immunogenicity of 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 (Holman et al., 2010); indeed, Richard et al. (2008) 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 (e.g. if 71 control bees and 66 LPS bees survived, we placed a random 66 individuals per treatment into each vial). We then submerged each bee in 500μL hexane (HPLC grade; Sigma ref. 34859) per bee, and then gently shook the vials by hand for 30 seconds to facilitate dissolution of all hexane-soluble epicuticular chemicals. We then pooled an equal volume of each extract into a single vial.

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 colour with treatment. Because hexane is toxic, we applied the extracted CHCs to the bees indirectly following Smith et al. (2012), by pipetting 20μl of the appropriate hexane solution onto the surface of deionised water in a 10mL glass beaker. 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 marked, 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 over 26th May - 28th June 2019.

Experiment 3: 'Social distancing' following immune challenge

Like Experiment 2, this experiment used 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

The response variable for Experiments 1 and 2 was categorical with three possibilities: each individual bee either left the hive voluntarily, was forced out, or stayed inside for the duration of the observation period. We therefore analysed these experiments with a Bayesian multinomial logistic model (an

extension of binomial logistic models to >2 outcomes), implemented in the R package `brms` via the `categorical` family (Bürkner, 2017). For Experiments 1 and 2, we ran three candidate models: one containing treatment, hive, and the treatment × hive interaction, one lacking the interaction, and one that lacked the interaction plus the treatment effect. All three models also included the observation time (to the nearest minute) as a covariate. We compared the fit of these three models using posterior model probabilities (PMP; estimated using bridge sampling via the `post_prob` function with a flat prior). For all experiments, the 'treatment × hive' model was significantly outperformed by the 'treatment + hive' model, and so the latter was used when estimating the treatment effect sizes. 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 standard frequentist approaches; McElreath, 2020). Experiment 3 had a binary response variable and was therefore modelled using the binomial family in `brms`. Our statistical approach was the same as before, except that each model also included the random factor 'pair ID', to account for repeated measurements of each pair of bees, and it was not necessary to include the observation time covariate.

For models of Experiments 1 and 2 we ran four chains per model (5000 iterations per chain with 2500 each discarded as burn-in), and confirmed model convergence and fit via $\hat{R}$ statistics and posterior predictive plots. Experiment 3 models required additional iterations (20,000 with 10,000 burn-in) to ensure adequate effective sampling. To make inferences from the best-fitting model, we calculated the posterior difference in means between pairs of treatments for each outcome, for planned contrasts relevant to our biological hypotheses (e.g. for Experiment 1 we tested whether bees were forced out more frequently in the LPS treatments, relative to each of the controls). We use log odds ratios throughout as a standardised measure of effect size.

Data and code availability

All the raw data and R code used in this paper is available at https://lukeholman.github.io/social_immunity/.

Results

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

Figure 1A shows the estimated percentage 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 six planned contrasts between treatment groups. Complete statistical results for Experiment 1 are given in Tables S1-2. The 'treatment + hive' model (PMP: 0.35) was about half as probable as the 'hive' model (PMP: 0.65), with the 'treatment × hive' model being much less probable than either (PMP < 0.001).

Despite the inconclusive PMP results, parameter estimates from the 'treatment + hive' model indicated that the treatment groups differed significantly in the rate at which they left the hive. 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 0.9979 (for both LPS and heat-treated LPS, by coincidence; Table S2), which can be interpreted similarly to a one-tailed p-value of 0.0021. Hereafter, we write PP to represent 1 minus this posterior probability, for easier comparison to the familiar *p*-value.

More bees from the two LPS treatments remained inside relative to the Ringers control, but not significantly so (Tables S1-2; PP = 0.10 and PP = 0.084). Additionally, Ringers-treated bees were non-significantly less likely to remain inside the hive than bees in the intact control (PP = 0.12). Bees in the LPS and heat-treated LPS treatments left the hive at similar rates (PP = 0.43). Bees from LPS and heat-treated LPS treatments were also more likely to leave the hive voluntarily, in some cases significantly so, relative to the intact control (Figure 1B; Table S2). Heat-treated LPS also had a marginally significant positive effect on the proportion of bees that were forced out, relative to the the Ringers control (PP = 0.055), though the corresponding LPS versus Ringers comparison did not show evidence of a difference (PP = 0.24).

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

Figure 2A shows the estimated percentage 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 of the LPS treatment relative to the Ringers control. Complete statistical results for Experiment 2 are given in Tables S3-4. The 'treatment + hive' model (PMP: 0.47) was approximately equally likely as the 'hive' model (PMP: 0.53), while the 'treatment × hive' model was improbable (PMP: 0.003).

Figures 2A-B 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 (Tables S3-4; PP = 0.005). Correspondingly, fewer bees from the LPS treatment remained inside the hive (PP = 0.005). Interestingly, there was no difference in the rate at which the two treatment groups left the hive *voluntarily* (PP = 0.29).

We also used the model to test whether the odour treatment altered the proportion of bees that were forced out the hive, among the subset of bees that did not remain inside. 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 individuals coated with odours from LPS-treated bees were more likely to be forced out (as opposed to leaving voluntarily), relative to controls.

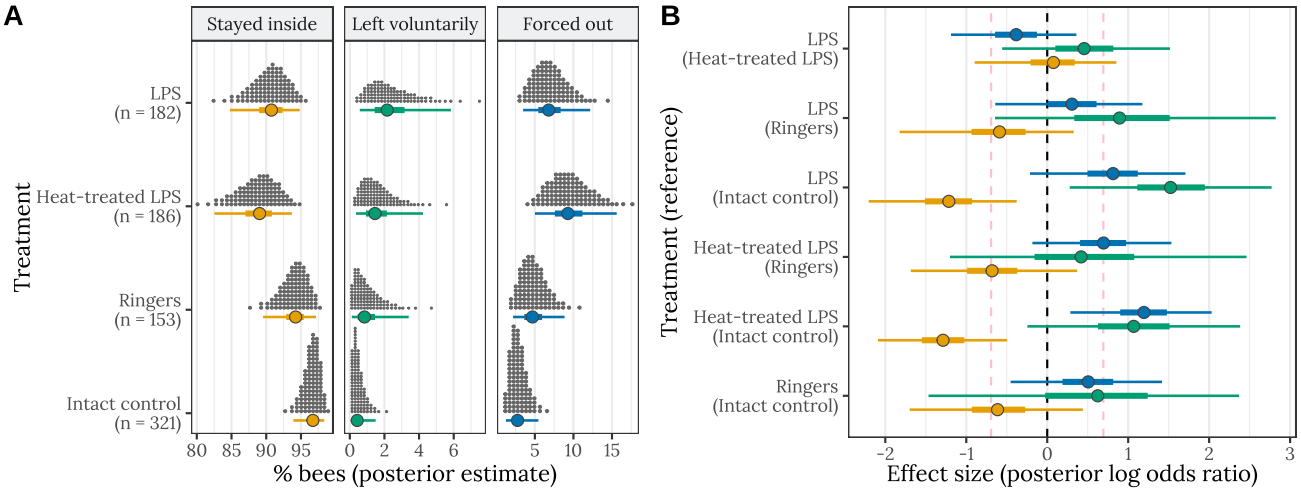

Figure 1: Results of Experiment 1. Panel A shows the posterior estimate of the mean % bees staying inside the hive (left), leaving voluntarily (middle), or being forced out (right), for each of the four treatments. The quantile dot plot shows 100 approximately equally likely estimates of the true % bees, 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.

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 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), though not strongly so, and the effect size was moderate (Figure 3C; log odds ratio: -0.37, 95% CIs: -0.76 to 0.02).

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 (non-significantly) to the Ringers control. There was no difference in which the rate at which the Ringers and intact controls bees left the hive. Most of the bees that left the hive in Experiment 1 (especially in the LPS treatments) were forced out by other colony members, though many appeared to leave voluntarily, by walking out and then dropping to the ground. Very few left by flying, unlike in an earlier study that reported 'altruistic self-removal' by honeybees with experimentally impaired health; this difference may reflect the different methodology employed (Rueppell et al. 2010 challenged bees with CO_2 or hydroxyurea, rather than LPS). These bees were frequently found dead on subsequent days, and many were predated by Vespid wasps within moments of dropping to the ground, illustrating that the hive-exiting behaviour is unlikely to represent an attempt to temporarily self-quarantine. From Experiment 1, we conclude that wounded and/or immune-stimulated bees tend to leave the hive, sometimes without assistance, and sometimes by being pulled out by other workers. Furthermore, our results provide some evidence that LPS and heat-treated LPS have similar effects on the immune response in honeybees, contrary to our expectations, but consistent with a number of other experiments finding that heat treatment does not reduce the immune-stimulating effects of LPS (see Methods).

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, in light of evidence that insects develop a distinct chemical profile in a matter of hours following an immune challenge (Richard et al., 2008; Holman et al., 2010). 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 bees that received Ringers. Interestingly, there was no treatment effect on the rate at which bees left the hive voluntarily. We hypothesise that the bees treated with extracts from LPS-treated individuals were preferentially forced out because they were perceived to be immune-challenged by other individuals, but they did not leave voluntarily at elevated rates because their average health was the same as bees in the control group.

In light of past findings that immune-challenged ants

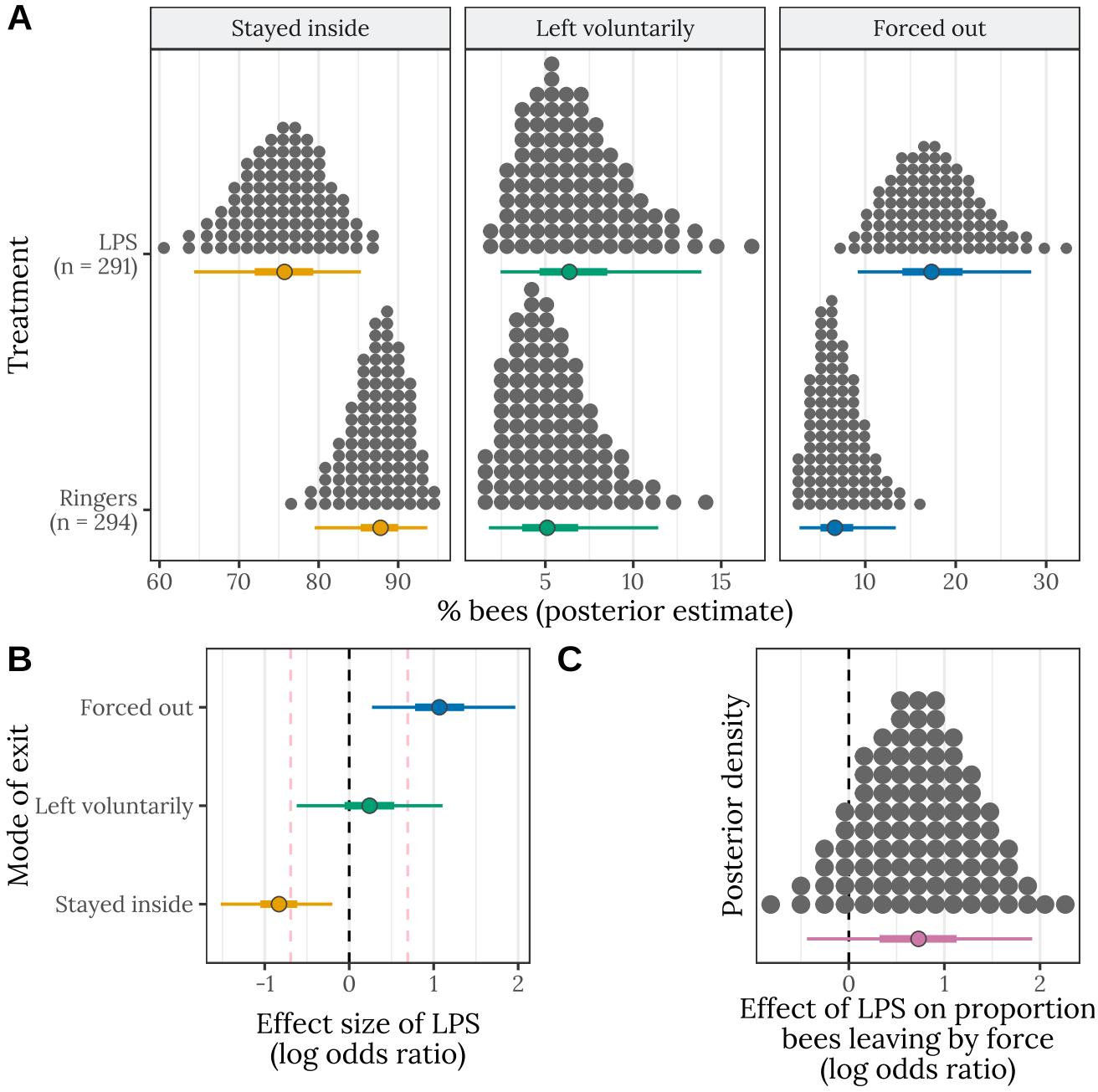

Figure 2: Results of Experiment 2. Panel A shows the same information as Figure 1A. Panel B gives the posterior estimates of the effect size (log odds ratio) of the LPS treatment as a log odds ratio, for each of the three possible outcomes; the details are the same as in Figure 1B. Panel C shows the 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.

(Bos et al., 2012) and bees (Richard et al., 2008; Kazlauskas et al., 2016) 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 mild but statistically significant decline in the proportion of time spent in contact, suggesting a behavioural in the immune-challenged 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 (Richard et al., 2008); 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 (Kazlauskas et al., 2016), though there may also be direct fitness benefits (or non-adaptive explanations) of sickness behaviour, especially given that it occurs in non-eusocial animals (e.g. Sullivan et al., 2016).

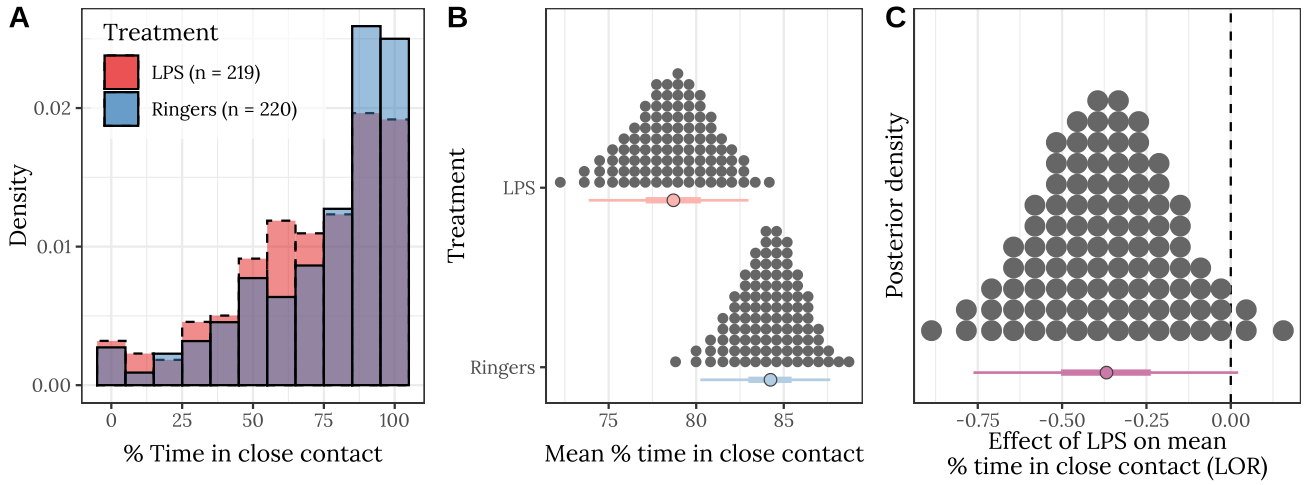

Figure 3: Results of Experiment 3. Panel A shows the frequency distribution of the % time in close contact, for pairs of bees from the LPS treatment and the Ringers control. Panel B shows the posterior estimates of the mean % time spent in close contact; the details of the quantile dot plot and error 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.

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. van Zweden and d'Ettorre, 2010). 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 6 hours, and there were concommitant changes in the expression of genes involved in CHC biosynthesis (Richard et al., 2012). Another reason to suspect CHCs is that the insect innate immune response involves changes in the expression of genes in the IMD pathway, which has pleiotropic effects on lipid metabolism/homeostasis (e.g. Kamareddine et al., 2018), providing a plausible mechanistic link between the CHC profile and immune status. However, no study has yet manipulated the CHC profile without confounds – our study and that of Richard et al. (2008) both involved solvent washes rather than CHCs specifically – so the involvement of CHCs remains to be demonstrated.

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'; Gould and Lewontin, 1979). 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 (Forni et al., 2014); 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 (Kamareddine et al., 2018); the key feature distinguishing these two hypotheses is the presence of a net inclusive fitness benefit to workers that signal for themselves to be removed. 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*; Nielsen and Holman, 2012). 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.

Acknowledgements

We are very grateful to Jean-Pierre Scheerlinck, Heidi Wong, and Daisy Kocher for their assistance with the experiments. This work benefited from an Australian Research Council Discovery Project grant to LH (DP170100772).

References

Aubert, A. and F.-J. Richard (2008). Social management of LPS-induced inflammation in *Formica polyctena* ants. *Brain, Behavior, and Immunity 22*(6), 833–837.

Baracchi, D., A. Fadda, and S. Turillazzi (2012). Evidence for antiseptic behaviour towards sick adult bees in honey bee colonies. *Journal of Insect Physiology 58*(12), 1589–1596.

Bos, N., T. Lefèvre, A. Jensen, and P. d'Ettorre (2012). Sick ants become unsociable. *Journal of Evolutionary Biology 25*(2), 342–351.

Bot, A. N., C. R. Currie, A. G. Hart, and J. J. Boomsma (2001). Waste management in leaf-cutting ants. *Ethology Ecology & Evolution 13*(3), 225–237.

Bürkner, P.-C. (2017). Advanced Bayesian multilevel modeling with the R package `brms`. *arXiv preprint:1705.11123*.

Christe, P., A. Oppliger, F. Bancalà, G. Castella, and M. Chapuisat (2003). Evidence for collective medication in ants. *Ecology Letters 6*(1), 19–22.

Coveney, A. P., W. Wang, J. Kelly, J. H. Liu, S. Blankson, Q. Di Wu, H. P. Redmond, and J. H. Wang (2015). Myeloid-related protein 8 induces self-tolerance and cross-tolerance to bacterial infection via TLR4-and TLR2-mediated signal pathways. *Scientific Reports 5*(1), 1–14.

Cremer, S., S. A. Armitage, and P. Schmid-Hempel (2007). Social immunity. *Current biology 17*(16), R693–R702.

Evans, J., K. Aronstein, Y. P. Chen, C. Hetru, J.-L. Imler, H. Jiang, M. Kanost, G. Thompson, Z. Zou, and D. Hultmark (2006). Immune pathways and defence mechanisms in honey bees apis mellifera. *Insect Molecular Biology 15*(5), 645–656.

Forni, D., R. Cagliani, C. Tresoldi, U. Pozzoli, L. De Gioia, G. Filippi, S. Riva, G. Menozzi, M. Colleoni, M. Biasin, et al. (2014). An evolutionary analysis of antigen processing and presentation across different timescales reveals pervasive selection. *PLoS Genetics 10*(3), e1004189.

Gadau, J., M. Helmkampf, S. Nygaard, J. Roux, D. F. Simola, C. R. Smith, G. Suen, Y. Wurm, and C. D. Smith (2012). The genomic impact of 100 million years of social evolution in seven ant species. *Trends in Genetics 28*(1), 14–21.

Gould, S. J. and R. C. Lewontin (1979). The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. *Proceedings of the royal society of London. Series B. Biological Sciences 205*(1161), 581–598.

Greco, M. K., D. Hoffmann, A. Dollin, M. Duncan, R. Spooner-Hart, and P. Neumann (2010). The alternative pharaoh approach: stingless bees mummify beetle parasites alive. *Naturwissenschaften 97*(3), 319–323.

Greene, M. J. and D. M. Gordon (2003). Cuticular hydrocarbons inform task decisions. *Nature 423*(6935), 32–32.

Hamilton, W. D. (1964). The genetical evolution of social behaviour. II. *Journal of Theoretical Biology 7*(1), 17–52.

Hart, A. G. and F. L. Ratnieks (2001). Task partitioning, division of labour and nest compartmentalisation collectively isolate hazardous waste in the leafcutting ant *Atta cephalotes*. *Behavioral Ecology and Sociobiology 49*(5), 387–392.

Heinze, J. and B. Walter (2010). Moribund ants leave their nests to die in social isolation. *Current Biology 20*(3), 249–252.

Holman, L. (2018). Queen pheromones and reproductive division of labor: a meta-analysis. *Behavioral Ecology 29*(6), 1199–1209.

Holman, L., C. G. Jørgensen, J. Nielsen, and P. d'Ettorre (2010). Identification of an ant queen pheromone regulating worker sterility. *Proceedings of the Royal Society B: Biological Sciences 277*(1701), 3793–3800.

Imler, J.-L., S. Tauszig, E. Jouanguy, C. Forestier, and J. A. Hoffmann (2000). LPS-induced immune response in *Drosophila*. *Journal of Endotoxin Research 6*(6), 459–462.

Kamareddine, L., W. P. Robins, C. D. Berkey, J. J. Mekalanos, and P. I. Watnick (2018). The *Drosophila* immune deficiency pathway modulates enteroendocrine function and host metabolism. *Cell metabolism 28*(3), 449–462.

Kazlauskas, N., M. Klappenbach, A. M. Depino, and F. F. Locatelli (2016). Sickness behavior in honey bees. *Frontiers in Physiology 7*, 261.

López-Uribe, M. M., W. B. Sconiers, S. D. Frank, R. R. Dunn, and D. R. Tarpy (2016). Reduced cellular immune response in social insect lineages. *Biology Letters 12*(3), 20150984.

McElreath, R. (2020). *Statistical rethinking: A Bayesian course with examples in R and Stan*. CRC press.

Millar, D. G., K. M. Garza, B. Odermatt, A. R. Elford, N. Ono, Z. Li, and P. S. Ohashi (2003). Hsp70 promotes antigen-presenting cell function and converts T-cell tolerance to autoimmunity *in vivo*. *Nature Medicine 9*(12), 1469–1476.

Motojima, M., T. Matsusaka, V. Kon, and I. Ichikawa (2010). Fibrinogen that appears in Bowman's space of proteinuric kidneys *in vivo* activates podocyte Toll-like receptors 2 and 4 *in vitro*. *Nephron Experimental Nephrology 114*(2), e39–e47.

Naug, D. and S. Camazine (2002). The role of colony organization on pathogen transmission in social insects. *Journal of theoretical Biology 215*(4), 427–439.

Nielsen, M. L. and L. Holman (2012). Terminal investment in multiple sexual signals: immune-challenged males produce more attractive pheromones. *Functional Ecology 26*(1), 20–28.

Panjwani, N. N., L. Popova, and P. K. Srivastava (2002). Heat shock proteins gp96 and hsp70 activate the release of nitric oxide by APCs. *Journal of Immunology 168*(6), 2997–3003.

Pie, M. R., R. B. Rosengaus, and J. F. Traniello (2004). Nest architecture, activity pattern, worker density and the dynamics of disease transmission in social insects. *Journal of Theoretical Biology 226*(1), 45–51.

Richard, F. J., A. Aubert, and C. M. Grozinger (2008). Modulation of social interactions by immune stimulation in honey bee, *Apis mellifera*, workers. *BMC Biology 6*(1), 50.

Richard, F.-J., H. L. Holt, and C. M. Grozinger (2012). Effects of immunostimulation on social behavior, chemical communication and genome-wide gene expression in honey bee workers (apis mellifera). *BMC Genomics 13*(1), 558.

Rueppell, O., M. K. Hayworth, and N. Ross (2010). Altruistic self-removal of health-compromised honey bee workers from their hive. *Journal of Evolutionary Biology 23*(7), 1538–1546.

Simone-Finstrom, M. and M. Spivak (2010). Propolis and bee health: the natural history and significance of resin use by honey bees. *Apidologie 41*(3), 295–311.

Singh-Jasuja, H., H. U. Scherer, N. Hilf, D. Arnold-Schild, H.-G. Rammensee, R. E. Toes, and H. Schild (2000). The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. *European Journal of Immunology 30*(8), 2211–2215.

Smith, A. A., B. Hölldobler, and J. Liebig (2012). Queen-specific signals and worker punishment in the ant *Aphaenogaster cockerelli*: the role of the Dufour's gland. *Animal Behaviour 83*(3), 587–593.

Spivak, M. (1996). Honey bee hygienic behavior and defense against *Varroa jacobsoni*. *Apidologie 27*(4), 245–260.

Sullivan, K., E. Fairn, and S. A. Adamo (2016). Sickness behaviour in the cricket *Gryllus texensis*: comparison with animals across phyla. *Behavioural Processes 128*, 134–143.

van Zweden, J. S. and P. d'Ettorre (2010). Nestmate recognition in social insects and the role of hydrocarbons. In G. J. Blomquist and A.-G. Bagnères (Eds.), *Insect Hydrocarbons: Biology, Biochemistry and Chemical Ecology*. Cambridge: Cambridge University Press.

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	-14.87	9.43	-35.35	2.07	0.0446	*
Treatment: Ringers	0.66	0.97	-1.39	2.43	0.2321	
Treatment: Heat-treated LPS	1.30	0.61	0.13	2.53	0.0153	*
Treatment: LPS	1.68	0.60	0.53	2.90	0.0020	**
hive1	-1.51	2.62	-6.60	3.58	0.2808	
hive2	3.13	1.48	0.84	6.62	0.0012	**
hive3	-1.52	1.60	-4.78	1.56	0.1718	
Observation duration (minutes)	0.09	0.09	-0.07	0.28	0.1477	
% bees forced out						
Intercept	-7.40	6.71	-20.82	5.74	0.1341	
Treatment: Ringers	0.55	0.45	-0.34	1.44	0.1143	
Treatment: Heat-treated LPS	1.30	0.41	0.52	2.10	0.0004	***
Treatment: LPS	0.97	0.42	0.16	1.80	0.0113	*
hive1	-0.39	2.52	-5.31	4.67	0.4367	
hive2	-0.12	0.65	-1.39	1.16	0.4306	
hive3	-0.76	1.52	-3.83	2.20	0.3094	
Observation duration (minutes)	0.04	0.07	-0.10	0.18	0.2885	

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	0.98	8.09	-17.01	17.18		
	Log odds ratio	0.05	0.43	-0.90	0.85	0.4255	
LPS (Ringers)	Difference in % bees staying inside	-9.54	9.87	-33.80	5.35		
	Log odds ratio	-0.62	0.54	-1.82	0.33	0.1047	
LPS (Intact control)	Difference in % bees staying inside	-17.27	10.86	-42.03	-1.89		
	Log odds ratio	-1.23	0.46	-2.21	-0.38	0.0021	**
Heat-treated LPS (Ringers)	Difference in % bees staying inside	-10.52	9.66	-31.87	5.76		
	Log odds ratio	-0.68	0.51	-1.69	0.37	0.0840	~
Heat-treated LPS (Intact control)	Difference in % bees staying inside	-18.25	10.72	-40.41	-2.13		
	Log odds ratio	-1.29	0.40	-2.09	-0.49	0.0021	**
Ringers (Intact control)	Difference in % bees staying inside	-7.73	8.93	-29.75	4.96		
	Log odds ratio	-0.61	0.54	-1.70	0.44	0.1158	
% bees leaving voluntarily							
LPS (Heat-treated LPS)	Difference in % bees leaving voluntarily	3.64	6.63	-5.35	22.30		
	Log odds ratio	0.46	0.53	-0.56	1.52	0.1919	
LPS (Ringers)	Difference in % bees leaving voluntarily	6.27	10.01	-5.24	35.00		
	Log odds ratio	0.95	0.90	-0.64	2.82	0.1396	
LPS (Intact control)	Difference in % bees leaving voluntarily	9.51	11.02	0.13	40.61		
	Log odds ratio	1.53	0.63	0.28	2.77	0.0091	**
Heat-treated LPS (Ringers)	Difference in % bees leaving voluntarily	2.64	8.40	-11.88	25.56		
	Log odds ratio	0.49	0.94	-1.20	2.46	0.3086	
Heat-treated LPS (Intact control)	Difference in % bees leaving voluntarily	5.87	8.33	-0.48	30.99		
	Log odds ratio	1.07	0.66	-0.25	2.38	0.0546	~
Ringers (Intact control)	Difference in % bees leaving voluntarily	3.23	7.79	-6.36	26.72		
	Log odds ratio	0.58	0.98	-1.47	2.37	0.2559	
% bees forced out							
LPS (Heat-treated LPS)	Difference in % bees forced out	-4.62	6.30	-20.93	4.80		
	Log odds ratio	-0.39	0.39	-1.19	0.36	0.1601	
LPS (Ringers)	Difference in % bees forced out	3.27	6.31	-7.38	19.17		
	Log odds ratio	0.30	0.46	-0.64	1.18	0.2446	
LPS (Intact control)	Difference in % bees forced out	7.76	8.27	-0.83	28.69		
	Log odds ratio	0.80	0.48	-0.21	1.71	0.0535	~
Heat-treated LPS (Ringers)	Difference in % bees forced out	7.88	7.87	-1.34	27.16		
	Log odds ratio	0.69	0.44	-0.18	1.53	0.0556	~
Heat-treated LPS (Intact control)	Difference in % bees forced out	12.38	10.49	0.32	36.95		
	Log odds ratio	1.18	0.44	0.29	2.03	0.0078	**
Ringers (Intact control)	Difference in % bees forced out	3.23	7.79	-6.36	26.72		
	Log odds ratio	0.58	0.98	-1.47	2.37	0.2559	

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	-6.37	6.67	-19.69	6.68	0.1683	
Treatment: LPS	0.38	0.44	-0.48	1.25	0.1974	
hive1	0.11	1.52	-2.87	3.08	0.4657	
hive2	-0.18	0.68	-1.51	1.16	0.3902	
hive3	0.08	2.53	-4.84	5.11	0.4913	
Observation duration (minutes)	0.03	0.07	-0.10	0.16	0.3366	
% bees forced out						
Intercept	-5.18	6.71	-18.32	7.89	0.2217	
Treatment: LPS	1.10	0.43	0.29	1.99	0.0039	**
hive1	-0.03	1.54	-3.06	2.97	0.4900	
hive2	-0.85	0.71	-2.27	0.49	0.1120	
hive3	0.05	2.55	-4.89	5.09	0.4928	
Observation duration (minutes)	0.01	0.07	-0.12	0.15	0.4140	

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	-11.95	4.94	-22.21	-2.75		
Log odds ratio	-0.84	0.33	-1.52	-0.20	0.0048	**
% bees leaving voluntarily						
Absolute difference in % bees leaving voluntarily	1.34	2.62	-3.72	7.09		
Log odds ratio	0.24	0.44	-0.62	1.11	0.2881	
% bees forced out						
Absolute difference in % bees forced out	10.61	4.63	2.54	20.46		
Log odds ratio	1.08	0.43	0.27	1.97	0.0047	**

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	1.68	0.14	1.40	1.96	0.0000	***
Treatment: LPS	-0.37	0.20	-0.76	0.02	0.0328	*
hive1	-0.20	0.17	-0.54	0.14	0.1300	
hive2	0.14	0.16	-0.16	0.46	0.1800	
hive3	-0.23	0.18	-0.58	0.12	0.1021	

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	5.54	3.01	-0.33	11.50		
Log odds ratio	-0.37	0.20	-0.76	0.02	0.0328	*