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Research

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Honey Bee (Hymenoptera: Apidae) Nursing Responses to Cuticular Cues Emanating from Short-term Changes in Larval Rearing Environment

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Abstract

Honey bee larvae are dependent on the social structure of colony for their provisioning and survival. With thousands of larvae being managed collectively by groups of foragers (collecting food resources) and nurse bees (processing food and provisioning larvae), coordination of colony efforts in rearing brood depends on multiple dynamic cues of larval presence and needs. Much of these cues appear to be chemical, with larvae producing multiple pheromones, major being brood ester pheromone (BEP; nonvolatile blend of fatty acid esters) that elicits both short-term releaser effects and long-term primer effects. While BEP can affect colony food collection and processing with the signaling of larval presence, it is unclear if BEP signals individual larval needs. To understand this aspect, in a series of experiments we manipulated larval feeding environment by depriving larvae from adult bee contact for 4-h period and examined (1) nurse bee interactions with contact-deprived and nondeprived larvae and larval extracts; (2) forager bee responses to contact-deprived and nondeprived larval extracts. We also characterized BEP of contact-deprived and nondeprived larvae. We found that nurse honey bees tend to aggregate more over contact-deprived larvae when compared with nondeprived larvae, but that these effects were not found in response to whole hexane extracts. Our analytical results suggest that BEP components changed in both quantity and quality over short period of contact deprivation. These changes affected foraging behavior, but did not appear to directly affect nursing behavior, suggesting that different chemical cues are involved in regulating nursing effort to individual larvae.

Key words: honey bee, larval nutrition, nursing, gas chromatography, pheromone

Division of labor and cooperative brood care are distinctive features of eusocial insects like honey bees that provide a stable environment for rearing brood in a colony. While some members of the colony are engaged in collecting food resources, others spend their time providing for the young. This is especially critical for the honey bee larvae. Honey bee larvae are normally sessile and rely on a subset of adult workers approximately 1- to 2-wk old (termed nurse bees) for their growth and survival (Lindauer 1953). A single larva is provisioned by several nurses, all of whom patrol the brood nest, seemingly at random, examining and provisioning larvae from glandular secretions of the hypoharyngeal and mandibular glands (Haydak 1957). As no single individual bee is responsible for the entire provisioning of a specific larva, a method of recognizing larval nutritional requirement must be in place to ensure that no individual is unattended and subjected to starvation. Larvae are the only immature feeding stage and principal consumers of pollen via nurse bees (Haydak 1970, Schmickl et al. 2003). Larvae grow exponentially over 5–6 d (Thrasyvoulou and Benton 1982, Winston 1987) and even a short period of nutritional deprivation can impact their assessed value as potential queens (Sagili et al. 2018) and result in developmental changes and dwarfism in adults (Nelson and Sturtevant 1924, Jay 1964). Nurse bees rely on the incoming pollen from foragers in order to continue to provision larvae. Pollen foraging is adjusted according to amount of brood, as well as current pollen stores (Barker 1971, Al-Tikrity et al. 1972, Hellmich and Rothenbuhler 1986), suggesting a colony-level assessment of the need for protein.

Without pollen, brood rearing cannot continue for long (Haydak 1935). In colonies experimentally deprived of pollen, nurse bees adjust feeding time and frequency toward older instar larvae, eventually cannibalizing young larvae in order to utilize the nutrients toward rearing the older larvae (Schmickl and Crailsheim 2001, 2002; Schmickl et al. 2003). Larvae isolated from contact with nurse bees and therefore

deprived of their sole source of nutrition display unusual behaviors, such as crawling to the top of the cell (~12 h after removal from the colony), eventually leaving the cell (~24 h), and often dropping to the bottom of the hive where they die or are cannibalized by adults (personal observations). The behavior reminiscent of larval 'begging' behaviors is also observed in ant species *Pachychondyla caffraria* (Agbogba 1991) and *Myrmica rubra* (Creemers et al. 2003). Other social insects have been shown to respond to larval nutritional needs. For instance, larva feeding rate increased by deprivation time in *Solenopsis invicta* (Cassill and Tschinkel 1999) and *Bombus terrestris* (Pereboom et al. 2003). *Acromyrmex* spp. leaf-cutting ant larvae, isolated from food-provisioning adults for 0 to 48 h and then returned to the colony, were fed more frequently and groomed than control larvae (Lopes et al. 2005).

Honey bees utilize a vast array of chemical signals and cues to organize colony behavior (Winston 1987). Most notably, brood ester pheromone (BEP), a blend of fatty-acid esters extractable from the larval cuticle, has been shown to modulate pollen foraging, age of first foraging, and hypopharyngeal gland development in nurse honey bees (LeConte et al. 1990; Mohammedi et al. 1996; Pankiw et al. 1998; Pankiw and Page 2000, 2001; LeConte et al. 2001; Pankiw 2004). Further, it has been reported that synthetic brood pheromone can stimulate protein consumption and higher brood production in honey bee colonies (Pankiw et al. 2008; Sagili and Breece 2012). In honey bee colonies, chemical interactions happen in a chemically complex environment (Carroll and Duehl 2012). When one considers the prevalence of chemical communication within the honey bee colony, and the profound effect of larval chemical cues on colony behavior, it is not unreasonable to suggest that a chemical cue is being utilized in the signaling of larval nutritional needs, as in the case of the subsocial burrower bug *Sehirus cinctus* (Kölliker et al. 2005, Kölliker et al. 2006). It has been previously suggested that honey bee larvae produce a chemical stimulus releasing feeding behaviors too (Huang and Otis 1991).

In this study, we tested several hypotheses related to larval chemical cues (Huang and Otis 1991) by examining (1) nurse bee interactions with contact-deprived and nondeprived larvae and larval extracts and (2) forager bee responses to contact-deprived and nondeprived larval extracts. We also characterized the BEP of contact-deprived and nondeprived larvae. Finally, we observed the effects of larval chemical cues on colony-level foraging, testing the hypothesis that contact-deprived larval extracts stimulate pollen foraging beyond that of control larval extracts.

Prior studies have shown that nurse bees inspect and feed larvae that have been deprived of contact for a 4-h duration more quickly (Sagili et al. 2018). Following these results, we replicated the methods of contact deprivation, and observed whether contact deprivation of larvae resulted in any gross changes in adult mass.

Methods

Apiary Management

All experiments were performed between 2005 and 2008. Colonies were established from mixed lineage European packages purchased from a local breeder and managed for growth and parasites, and to minimize defensive phenotypes.

Experiment 1: Effects of Depriving Larvae from Adult Contact on Emergence Proportion and Weight of Bees

The queen in a honey bee colony was confined to one side of a standard deep Langstroth frame for approximately 24 h using a push cage covering the entire frame-face. The queen was then removed from the frame and confined on a different frame to prevent her from laying further eggs on the frame where she had laid eggs earlier. Four days after the eggs eclosed, half of the frame with larvae was deprived of contact with adult bees for 4 h using a 3 mm wire mesh cage pushed into the wax. The other half of the frame was not manipulated. The frame halves were photographed with a digital camera (Sony, New York, NY), larvae were counted, and the frame was returned to the colony for the larvae to develop for 2 wk. The frame was then removed from the colony and cut in half.

The two halves of the frame were stored in an incubator at 33°C, 55% RH for 2 d until bees emerged. Honey bees were emerged over 24 h, collected and freeze-killed at –20°C. We hypothesized that a short-term deprivation of larvae from adult contact would not affect emergence rates of adult bees. A subsample of 49 bees from each treatment (contact deprived and not deprived) was selected for measurement of emergence weights. The bees were immediately weighed and then placed in a drying oven at 47°C for 12 d and subsequently reweighed. We hypothesized that there would be no significant difference in wet or dry weights of newly emerged bees, regardless of treatment.

Data was checked for normality. Counts of larvae and emerged bees were analyzed by 2×2 χ^2 contingency table analysis to test for a difference in emergence by deprived versus nondeprived treatment (Sokal and Rohlf 1995). Differences in wet and dry weights by treatment were analyzed by Student's T test (Sokal and Rohlf 1995) using R v4.1.0 (R Core Team 2021)

Experiment 2: Nursing Response to Contact-deprived and Nondeprived Larvae

The experiment was conducted in an observation hive constructed of wood and plexiglass and built to hold a single pane of four Langstroth deep standard frames. Observation hives were set up several weeks prior to observation and managed to maintain approximately equal adult and larval populations and stores. Observations were repeated six times in different observation hives. For each observation experiment, side-by-side areas containing approximately 500 fourth-instar larvae on a frame were selected, and wire mesh cages of 169 cm^2 were pushed into the wax surrounding the larvae. Two mesh sizes were used, 13 mm (which did not impede adult access to larvae and served as a control) and 3 mm (which restricted adult honey bee access to larvae and served as a contact-deprived treatment). Larval areas were caged for 4 h, after which the observation hives were briefly opened, treatment areas cleared of honey bees with light puffs of smoke, and the cages removed. The treatment areas (contact-deprived and nondeprived larvae) were then recorded for 1 h with a digital camera (Sony).

Screen captures of the digital video were created using Pinnacle Studio v. 12.0 (Avid Technology, Mountain View, CA). Initial captures were taken that did not contain bees, and the number of larvae in each area were also counted. The counts of bees present over deprived and nondeprived areas were taken at 5-min intervals subsequent to the removal of cages. Counts were transformed by proportion to number of larvae present and analyzed by linear regression using the following exponential rise-to-maximum equation: $Y = a(1 - e^{-b(x)})$ where Y represents the transformed bee count, x represents time in minutes, a represents the maximum limit, and b represents the rate of increase.

Data were checked for normality. A Student's T-test was used to test the hypotheses that greater number of bees arrived at the deprived treatment areas ($a_{deprived} > a_{nondeprived}$) and also that bees arrived

more rapidly to deprived treatment areas ($b_{deprivd} > b_{nondeprived}$; Sokal and Rohlf 1995). All analyses were conducted using SPSS, 2007 version and SigmaPlot version 9.

Experiment 3: Nurse Bee Response to Hexane Extracts of Contact-deprived and Nondeprived Larvae

Larval Extract Preparation

Two side-by-side patches consisting of 200 fourth-instar larvae were caged for 4 h as described in experiment 2. Larvae from each patch were collected and soaked in approximately 20 ml 95% n-hexane (Sigma-Aldrich, St. Louis, MO) for 1 min. Extracts were filtered through a Buchner funnel (VWR, West Chester, PA) then concentrated to 200 µl under nitrogen stream and stored in sealed chromatography vials at –20°C until use. A single colony source was used for each replication, for a total of 10 separate extract sources.

Bioassay Chamber Construction and Treatment

A five-sided acrylic glass chamber was constructed to snugly fit a standard 96-well microtiter plate (VWR) with a space of 9.5 mm above the wells, approximating bee space (Supp Fig. 1 [online only]). A fresh, sterile microtiter plate was treated with 2 µl concentrated extract as follows: four-well columns of nondeprived and four-well columns of deprived larval extract separated by four columns of solvent-treated wells. Left-right orientation of treatments was randomized, but solvent treated wells always separated the treatments. Solvent was allowed to dry for a minimum of 5 min while bees were placed into the bioassay chamber.

Fifty honey bees were collected from a single colony from frames containing uncapped larvae and returned to the lab, anesthetized via chilling at –20°C to quiescence (ca. 5–10 min). Each replication used a different colony and the colonies from which the bees were obtained were not the same colonies from which the larvae were obtained for larval extracts. Anesthetized honey bees were released into the bioassay chamber, and the treated microtiter plate was fitted. The completed chamber was placed in an incubator at 55% RH and 33°C illuminated with red light. The bees were active after 5 min and a digital camera was then used to record their activity for 1 h. Twelve replications were performed with a total of 600 honey bees selected from different colonies. The number of bees present over each section was counted at 5 min intervals and the data were analyzed by χ^2 to test the hypothesis that bees are found in greater proportions over areas treated with deprived extract (Sokal and Rohlf 1995). A 0.1 bee correction was made to eliminate zero counts. All analyses and visualizations were conducted using R v4.1.0 (R Core Team 2001). Pairwise comparisons were performed with significance being determined at $P < 0.05/3$ for Bonferroni correction (Sokal and Rohlf 1995).

Experiment 4: Forager Response to Contact-deprived and Nondeprived Larval Extract

Extracts of deprived and nondeprived larvae were collected similarly as described above in experiment 3 with the exception that extracts from multiple source colonies were pooled. All pheromone-treated colonies in this experiment received aliquots from this mixture. Six replications were performed, each consisting of a newly established broodless colony that was randomly provided with three treatments: extract of 500 deprived larvae, extract of 500 nondeprived larvae, or an equal volume of solvent in a random order over the course of 3 d. Extracts or solvents were provided on glass plates (20 × 10 cm^2) and inserted in the center of the colonies after drying. Entrance counts for pollen and non-pollen foragers were taken 1 h after the treatments were introduced. All treatments and observations occurred in the morning between 8:00 am and 10:00 am.

Effects of deprived or nondeprived extract were analyzed by 3 × 2 χ^2 contingency table analysis to test the hypothesis that deprived extract released a higher proportion of pollen foragers when compared with nondeprived extract or control (Sokal and Rohlf 1995). All analyses and visualizations were conducted using R v4.1.0 (R Core Team 2001). Pairwise comparisons were performed with significance being determined at $P < 0.05/3$ for Bonferroni correction (Sokal and Rohlf 1995).

Experiment 5: Characterization of BEP of Contact-deprived and Nondeprived Larvae

This experiment was replicated 10 times, with each replication consisting of a single pool of 10 fourth-instar larvae extracted from a single frame. Each frame was removed from the hive and bees removed by brush. One half of the frame on one side was randomly selected to be deprived of adult contact (as described in experiment 2), with the other half of the frame caged, such that adult bee access was not restricted (see experiment 2). The frame was returned to the colony for 4 h after which the frame was taken to the laboratory for further processing. A total of 10 frames were taken from four colony sources (two or three frames per colony).

Larvae were extracted from frames with short pulses of water at room temperature. Pools of 10 larvae were staged by size and morphology (Dade 1977, Thrasyvoulou and Benton 1982) and weighed. Larvae were immersed in 10 ml beakers having 2 ml 95% n-hexane containing 1.0 µg < 99% purity methyl myristate (Sigma-Aldrich) as an internal standard for 1 min. Extracts were filtered through a Buchner funnel and the beaker was rinsed with a further 2 ml 95% n-hexane which was added to the extract. Extracts were then chilled at –20°C for 10 min to remove any water and water-soluble compounds. Extracts were collected into 15 ml conical vials (VWR) and evaporated at 55°C under low nitrogen stream to 1 ml for fractionation.

Extracts were fractionated using columns constructed of 4 ml glass pipettes (Fisher Scientific, Waltham, MA) plugged with a small piece of Kimwipe (Kimberly-Clark, Neenah, WI) and packed with 70-230 mesh Silica gel 60A (Sigma-Aldrich). Columns were rinsed with 10 ml each ethyl acetate, dichloromethane, and n-hexane (all <99% purity, Sigma-Aldrich). The dichloromethane fraction contained all BEP esters. Fractioned extracts were then evaporated to dryness at 40°C under nitrogen stream and reconstituted in 1 ml hexane, vortexed for 15 s at 2,000 rpm and then dried at 55°C under nitrogen and reconstituted in 0.5 ml hexane. Samples were vortexed again 15 s at 2,000 rpm and dried at 55°C and reconstituted into 0.1 ml hexane and transferred into 300 µl chromatographic vials (Alltech, Deerfield, IL). Conicals were rinsed twice with 0.1 ml hexane which was added to the sample. Samples were dried under nitrogen and reconstituted with 10 µl n-hexane containing 1.0 µg octadecane (Sigma-Aldrich) as a secondary standard.

Gas Chromatography

An HP6890 gas chromatograph (Agilent, Santa Clara, CA) with splitless programmable temperature vaporization injection and flame ionization detection was used for sample analysis. Next, 1.0 µl was injected onto an HP-88 60 m X.251 mm ID column ((88%-cyanopropyl)-methylarylpolysiloxane from Agilent, Santa Clara, CA). Inlet temperature was held at 60°C for 0.10 min and then increased to 250°C at 500 C min^{-1}. Oven temperature was held

at 50°C for 2 min, then increased at 20°C min^{-1} to 170°C, held for 3 min, increased at 30°C min^{-1} to 230°C and held at the final temperature for 6 min. Carrier gas was pure hydrogen at 2 ml min^{-1}. Individual fatty acid ester retention times were identified using > 99% purity standards (Sigma-Aldrich). To estimate quantities, the areas beneath the peaks of known amounts of each ester were calculated using GC Chemstation software version B.01.03.204 (Agilent). These data were used to generate a standard curve for each fatty acid ester expressed as a first-order regression equation. Quantified esters were corrected by proportionate error of the octadecane amount to correct for machine error and evaporative concentration, and subsequently adjusted by proportionate error of methyl myristate amount to account for methodological loss. Ester amounts were transformed as a proportion of larval weight to generate ng ester $gram^{-1}$ larvae. Transformed amounts were then summed.

Data were checked for normality. The total amount of esters (ng g^{-1}) was analyzed by ANOVA to test for differences by deprived or nondeprived treatment. Ester amounts were then transformed as a proportion of total esters. Proportions were analyzed by ANOVA to test for differences by deprived versus nondeprived treatment (Sokal and Rohlf 1995). All analyses were conducted using SPSS, 2007 version and SigmaPlot version 9.

Experiment 6: Nurse Bee Response to Contact-deprived and Nondeprived BEP Blend

Blend Formulation

Mean ester proportions as characterized in experiment 5 were used to formulate synthetic deprived and nondeprived blends of BEP (Supp Table 1 [online only]). Synthetic esters were all >99% purity and were formulated neat by volume (for liquids) or mass (for solids). The amount of total esters differed between contact-deprived and nondeprived larvae, therefore the average amount extractable from an average weight larvae was calculated for each treatment. This amount is referred to as larval equivalent (LEq) where 1 LEq for the nondeprived blend is 24.66 ng while 1 LEq for the deprived blend is 8.37 ng.

Bioassay Treatment and Analyses

The experiment was replicated 12 times. Honey bees were collected as reported in experiment 3 and a different colony source was used for each replication. A clean microtiter plate was treated similarly as above with the equivalent amount of total esters equaling 4 LEq. Four columns were treated with nondeprived BEP, four columns with deprived blend separated by four columns of solvent-treated wells. Left-right orientation of treatments was randomized, although solvent-treated wells were always in the middle. Fifty honey bees were placed into the bioassay chamber and photographs were taken of the plate every 5 min for 1 h. The number of bees over each area was then counted from the photographs.

Honey bee counts were analyzed by χ^2 to test for an effect of deprived versus nondeprived BEP extractable quantity. All analyses and visualizations were conducted using R v4.1.0 (R Core Team 2001). Pairwise comparisons were performed with significance being determined at $P < 0.05/3$ for Bonferroni correction (Sokal and Rohlf 1995).

Experiment 7: Forager Response to Contact-deprived and Nondeprived BEP Blend

Forager Response to an Equal Number of Larval Equivalents of Deprived and Nondeprived Blends

Nine replications were performed, each consisting of a single colony of approximately 4,000 honey bees. Each colony received one of three treatments on successive days, nondeprived blend, deprived blend or control. All colonies received all treatments and the order of treatment was randomized. For the bioassay, 2,000 LEq of deprived or nondeprived blend diluted in 95% n-hexane was applied to glass plates measuring 14 cm × 7 cm and allowed to dry for 5 min. Control plates consisted of an equal volume of hexane. Plates were inserted into the brood nest of the colonies and hung between the frames on wire. One hour after treatment, 5-min entrance counts were performed to record number of pollen and non-pollen foragers. All counts were performed between 10:00 and 11:00 am. The increase in absolute brood pheromone dose over experiment 4 was intended to provide for a roughly equivalent proportion of brood pheromone LEq per adult bee in these more developed colonies.

Forager Response to an Equal Amount of Total BEP Esters of Deprived and Nondeprived Blends

This experiment was set up similar to the previous with the exception that plates were treated with 2,000 LEq of nondeprived blend, 5,892 LEq of deprived, and an equal volume of hexane for controls.

For experiment 7, counts of pollen to non-pollen foragers were analyzed by 3 × 2 χ^2 contingency table analysis for effect of deprived versus nondeprived derived BEP on the pollen to non-pollen forager ratio. All analyses and visualizations were conducted using R v4.1.0 (R Core Team 2001). Pairwise comparisons were performed with significance being determined at $P < 0.05/3$ for Bonferroni correction (Sokal and Rohlf 1995).

Results

Experiment 1: Effect of Deprivation of Adult Contact on Emergence Proportion and Weight

Adult emergence proportion was not significantly affected by contact-deprived versus nondeprived larval treatment ($\chi^2 = 1.299$, df = 2, $P = 0.254$). Larval deprivation did not significantly affect honey bee emergence wet weight ($Z = -0.911$, df = 98, $P = 0.362$) with a mean emergence wet weight of 98.31 ± 1.35 mg for contact deprived and 100. 82 ± 1.120 mg for nondeprived. Further, there was no significant difference in dry weights between treatments ($Z = -1.639$, df = 98, $P = 0.101$) with a mean emergence dry weight of 15.90 ± 0.16 mg for deprived and 16.26 ± 0.15 mg for nondeprived.

Experiment 2: Nursing Response to Contact-deprived and Nondeprived Larvae

The nonlinear regression model described the nondeprived data set with an $R^2 = 0.679$ ($F_{1,76} = 159.2$, $P < 0.0001$) and the deprived data set with an $R^2 = 0.605$ ($F_{1,76} = 116.2$, $P < 0.0001$). The *a* maximum limit parameters were significantly different among the treatments with a mean of 158.91 ± 5.37 adult honey bees per 500 larvae for nondeprived and 173.54 ± 6.19 adults per 500 larvae for deprived ($Z = -2.513$, df = 10, $P = 0.031$; Fig. 1). The *b* rate of increase parameters were not significantly different among the treatments with a mean of 0.118 ± 0.019 for nondeprived and 0.092 ± 0.014 for deprived ($Z = 1.534$, df = 10, $P = 0.156$; Fig. 1).

Experiment 3: Nurse Bee Response to Hexane Extracts of Contact-deprived and Nondeprived Larvae

Counts over each of the wells did not differ among the time points for deprived ($\chi^2 = 7.38$, df = 12, $P = 0.83$), nondeprived ($\chi^2 = 14.1$, df = 12, $P = 0.29$), or solvent control ($\chi^2 = 4.56$, df = 12, $P = 0.97$) and so time points were combined to analyze treatment

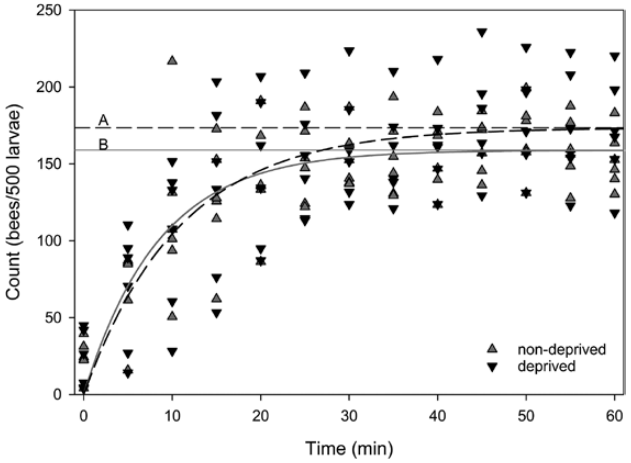

Fig. 1. Nonlinear regression of honey bee counts over deprived and nondeprived treatment areas. Counts over nondeprived areas are indicated with a gray, up-facing triangle and deprived by a black, down-facing triangle. The solid line represents the regression of nondeprived (R^2 = 0.679) and the dotted line, deprived (R^2 = 0.605). Regression followed the equation: $\text{Count} = a(1 - e^{-b(\text{time})})$ where 'a' represents the maximum limit and 'b' represents the rate of increase. Reference lines drawn from the maximal limit are denoted with labels 'A' and 'B' which denote a statistically significant difference at the $P < 0.05$ level. $N = 6$ replications representing six different colonies.

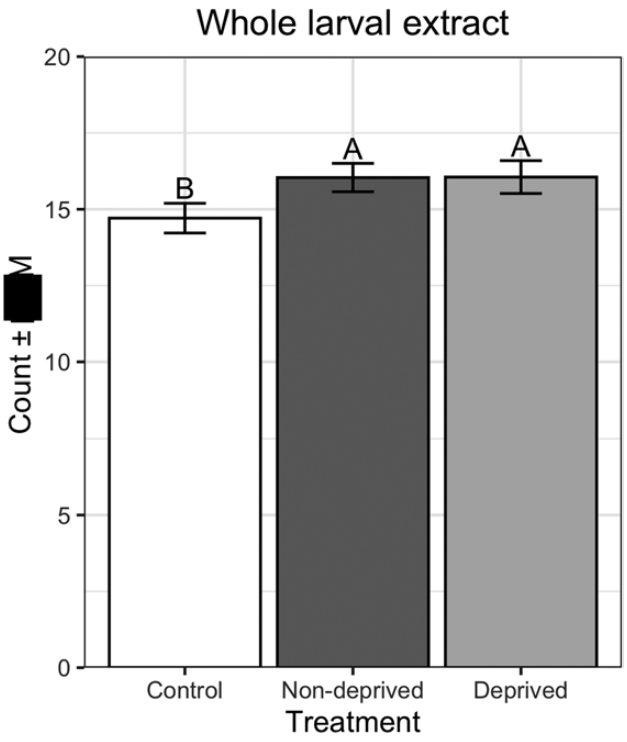

Fig. 2. Counts of nurse honey bees over bioassay chamber wells treated with no-pheromone control, nondeprived, or deprived whole larval hexane extract. Capital letters denote statistically significant ($P < 0.05/3$) subgroups as determined by χ^2 tests followed with pairwise comparisons and Bonferroni corrections. SEM indicates the standard errors for means.

effects. Treatment differences in nurse presence were significant (χ^2 = 208.790, df = 2, $P < 0.001$, Fig. 2), with counts being higher over deprived extracts than either other treatment and nurse counts being higher over nondeprived extracts compared to control. Nurse bees differentially aggregated over different wells of the bioassay chamber (χ^2 = 9.95, df = 12, P = 0.007), with counts over deprived extract treated wells being higher (16.05 ± 0.04) than those over solvent treated wells (14.71 ± 0.04, χ^2 = 7.66, df = 1, P = 0.006) but not nondeprived treated wells (16.04 ± 0.04, χ^2 = 0.001, df = 1, P = 0.96). Counts were similarly higher over nondeprived treated wells as compared to control (χ^2 = 7.49, df = 1, P = 0.006). No feeding or inspection behaviors were observed, and nurses generally aggregated over a particular portion of the plate, moving slightly but not attempting to seek egress from the bioassay chamber nor significantly probing edges (until the lights were turned on). Bees were observed antennating each other and the plate surface, but no behaviors consistent with normal nursing behavior were observed.

Experiment 4: Forager Response to Deprived and Nondeprived Larval Extract

Pollen to non-pollen forager proportions were significantly different among all extract treatments (χ^2 = 358.098, df = 2, $P < 0.0001$; Fig. 3). The highest proportion of returning pollen foragers found when colonies were treated with deprived larval extract (pollen:non-pollen forager ratio = 1.23 ± 0.10) relative to either nondeprived extract (0.80 ± 0.02, χ^2 = 22.803, df = 1, $P < 0.0001$) or control (0.17 ± 0.02, χ^2 = 350.241, df = 1, $P < 0.0001$). Proportions of returning pollen foragers were higher in nondeprived extract treated colonies were significantly higher than control as well (χ^2 = 188.283, df = 1, $P < 0.0001$).

Experiment 5: Characterization of BEP of Contact-deprived and Nondeprived Larvae

Weight of larvae selected did not differ significantly between treatments ($F_{1,19}$ = 0.818, P = 0.378) with a mean larval weight of 0.107 ± 0.005 g for nondeprived and 0.112 ± 0.002 g for deprived larvae. However, as weights ranged from 0.085 g to 0.140 g, all ester amounts were transformed into nanograms ester g^{-1} larvae. Transformed amounts of each ester were summed to give a total BEP esters g^{-1} larvae. Significantly greater amounts of BEP were extractable from nondeprived larvae with 246.6 ± 36.5 ng for nondeprived and 83.7 ± 17.0 ng for deprived larvae (ANOVA, $F_{1,19}$ = 16.382, P = 0.001). The proportions of methyl stearate (ANOVA, $F_{1,19}$ = 5.629, P = 0.029) and ethyl oleate (ANOVA, $F_{1,19}$ = 4.769, P = 0.042) differed significantly between deprived and nondeprived larvae, while all others were not significantly different (Fig. 4).

Experiment 6: Nurse Response to Contact-deprived and Nondeprived BEP Blend

Nurse bees did not aggregate preferentially over areas treated with contact-deprived or nondeprived BEP blends (χ^2 = 1.698, df = 1, P = 0.193, Fig. 5), however nurse counts were significantly higher over wells treated with either contact-deprived (18.11 ± 0.41, χ^2 = 92.2, df = 1, $P < 0.0001$) or nondeprived BEP (18.076 ± 0.40, χ^2 = 118.745, df = 1, $P < 0.0001$) blend when compared with solvent control (13.19 ± 0.32).

Experiment 7: Forager Response to Contact-deprived and Nondeprived BEP Blend

For forager response to equal LEq of contact-deprived and nondeprived BEP blend proportions of pollen to non-pollen foragers were significantly different between treatments (χ^2 = 33.890, df = 2, $P < 0.0001$, Fig. 6a). Pairwise comparisons showed all treatments to be significantly different from each other (deprived/nondeprived

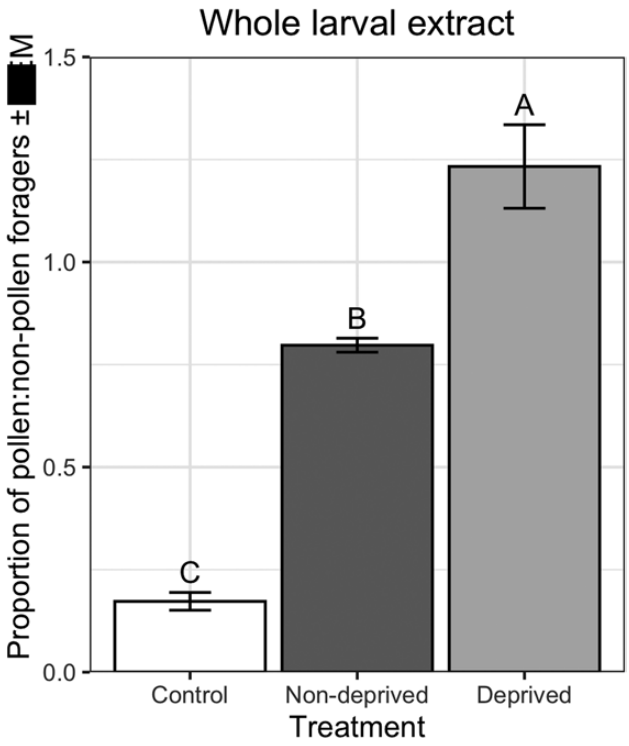

Fig. 3. Proportion of pollen to non-pollen honey bee forager returning to colony entrance over a 5-min observation period 1 h following treatment with a no-pheromone control, deprived, or nondeprived whole larva hexane extract. Capital letters denote statistically significant ($P < 0.05/3$) subgroups as determined by pairwise comparisons with Bonferroni corrections. SEM indicates the standard errors for means. $N = 12$ replications representing 12 different colonies.

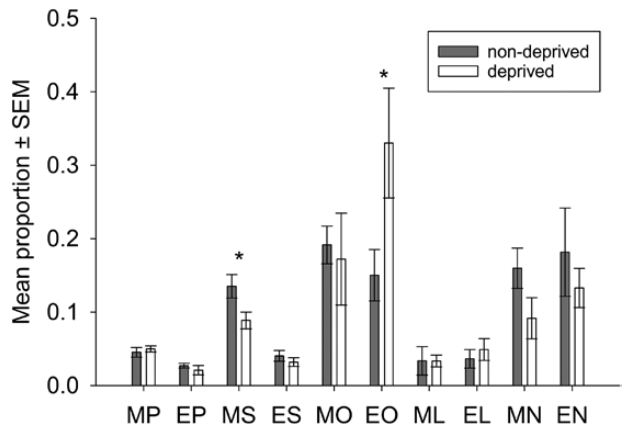

Fig. 4. Proportion of fatty acid esters of brood ester pheromone identified from the larval cuticle of nondeprived or deprived larvae. Bars are labeled with the acronyms for the (M)ethyl and (E)thyl esters of (P)almitic, (S)tearic, (O)leic, (L)inoleic, and (L)inolenic acids. Asterisks denote statistically different ($P < 0.05$) proportions among nondeprived and deprived larval extracts as determined by ANOVA to test for these differences. SEM indicates the standard errors for means. $N = 10$ replications representing four different colonies.

$\chi^2 = 33.883$, df = 1, $P < 0.0001$; control/nondeprived $\chi^2 = 8.620$, df = 1, $P = 0.003$; control/deprived $\chi^2 = 8.915$, df = 1, $P = 0.003$), with the highest proportion of pollen foragers counted after treatment with the nondeprived BEP blend (pollen: non-pollen forager ratio = 0.98 ± 0.15) and the lowest from the deprived BEP blend

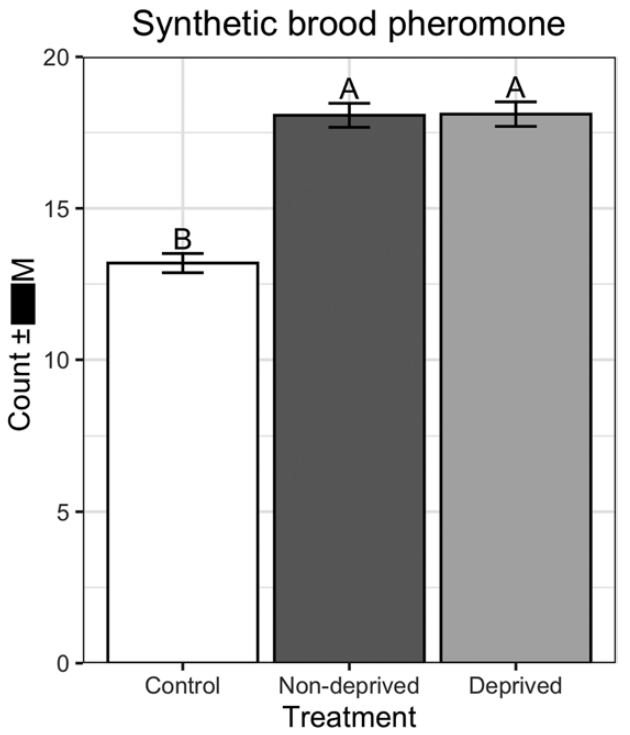

Fig. 5. Honey bee counts over wells treated with no-pheromone control, nondeprived, or deprived synthetic brood ester pheromone as formulated in Supp Table 1 (online only). Capital letters denote statistically significant ($P < 0.05/3$) subgroups as determined by χ^2 tests followed with pairwise comparisons and Bonferroni corrections. SEM indicates the standard errors for means. $N = 12$ replications representing 12 colonies.

0.67 ± 0.11), with control (hexane) treated colonies intermediate (0.78 ± 0.15, Fig. 6a).

For forager response to equal total esters of contact-deprived and nondeprived BEP blend proportions of pollen to non-pollen foragers were significantly different by treatment ($\chi^2 = 24.719$, df = 2, $P < 0.0001$, Fig. 6b). Pairwise comparisons that the nondeprived BEP blend (pollen: non-pollen forager ratio = 0.57 ± 0.09) elicited higher proportions of pollen foraging than either the deprived blend (0.51 ± 0.12, $\chi^2 = 9.014$, df = 1, $P = 0.003$) or solvent control (0.41 ± 0.07, $\chi^2 = 24.137$, df = 1, $P < 0.0001$) with the deprived blend not significantly differing from the control after correcting for multiple comparisons ($\chi^2 = 4.444$, df = 1, $P = 0.035$, Fig. 6b).

Discussion

In this study, we demonstrate that honey bee nurses respond in greater numbers to larvae that have been deprived of contact for a 4-h period (Fig. 1). The adult emergence weight data from our study suggests that the 4-h contact deprivation does not result in larval mortality or other gross long-term detrimental effects, but it is possible that this contact deprivation may impact other physiological parameters that were not examined in this study. It appears that the nurse bees are attracted to the larvae via a cue or signal, arrested by the same cue/signal, or simply remain over deprived larvae incidentally because of a higher likelihood to perform feeding behaviors (Sagili et al. 2018).

Our analytical results suggested that brood ester pheromone components changed both in quantity and quality over the short period of deprivation (4 h) of contact, with deprived larvae producing

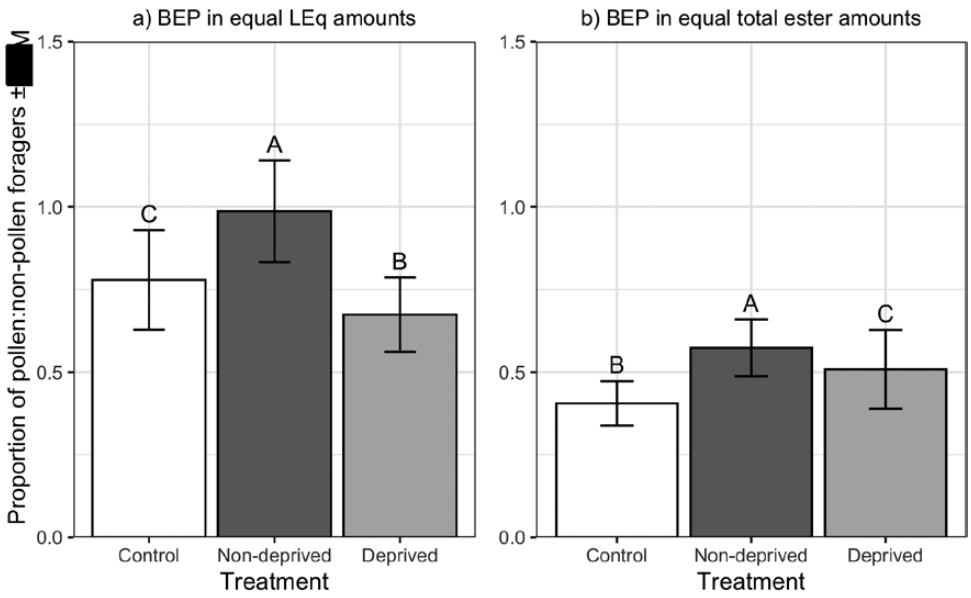

Fig. 6. Proportion of pollen to non-pollen honey bee forager returning to colony entrance over a 5-min observation period 1 h following treatment with a no-pheromone control, deprived, or nondeprived synthetic brood ester pheromone formulated according to SuppTable 1 (online only) in (a) equal LEq amounts or (b) in equal total BEP ester amounts. Capital letters denote statistically significant ($P < 0.05/3$) subgroups separately for each figure part as determined by χ^2 tests followed with pairwise comparisons and Bonferroni corrections. SEM indicates the standard errors for means. $N = 9$ replications representing nine colonies.

about a third of the total ester content as that of nondeprived larvae. BEP extracted from contact-deprived larvae had lower proportions of methyl stearate and higher proportions of ethyl oleate than nondeprived larvae (Fig. 4). Apart from its aggregate effects as a component of brood ester pheromone, ethyl oleate has been implicated as a factor in worker inhibition of behavioral maturation (Leoncini et al. 2004). If relative dosing of the various BEP components is important for the regulation of the worker switch to foraging, it may be supposed that amplifying this component may promote the retention of nurses' concomitant with the needs of the (deprived) larvae, although given that we isolated only a third of the amount of total brood pheromone from deprived larvae this cannot be considered an absolute amplification.

Capitalizing on a measurable colony response and our detection of a qualitative and quantitative difference in BEP from contact-deprived or nondeprived larvae, we designed an in vitro bioassay to model honey bee responses to potential chemical cues of contact deprivation. We found that nurse honey bees tend to aggregate more over whole hexane brood extracts rather than a solvent control, but did not preferentially aggregate over extracts of deprived or nondeprived larvae (Fig. 2). Similar trends were observed when exposed to synthetic brood ester pheromone blends alone suggesting that brood ester pheromone neither attracts nor arrests workers in the absence of further cues (Fig. 5). These findings suggest that honey bee workers are not using non-polar chemical cues or variation in BEP blend in regulating attraction or aggregation toward larvae. Given these results and the observations that nurses spend more time feeding and inspecting deprived larvae (Sagili et al. 2018), the likeliest conclusion of our observations is that any difference in the number of nurses present over deprived larvae is the result of the conduct of nursing behaviors than a direct result of any chemical cue studied here. However, earlier findings by Huang and Otis (1991) suggest that deprived larvae are subject to increased inspections even when food is experimentally deposited within their cells. It is possible that chemical cues not isolated by our non-polar extraction specifying relatively nonvolatile compounds may be involved in regulating these behaviors.

As BEP does not act exclusively to release nursing behaviors in nearby individuals, but rather serves as a social regulator for workers throughout the colony (Pankiw 2004), we also tested colony-level foraging responses to contact-deprived and nondeprived larval chemical cues. Given the assumption that deprived larvae are nutritionally stressed, it can be reasoned that colony nutritional demand would be increased, and that stimuli tracking pollen requirement would be correspondingly increased, thus increasing pollen foraging by the adult worker honey bees. Our results support this assumption, as the contact-deprived larval extracts resulted in more pollen foraging than nondeprived and solvent control extracts (Fig. 3). The results from BEP blends alone though indicated an opposite response, whether it was equal LEq (Fig. 6a) or equal total esters (Fig. 6b). These results suggest that the blend differences in BEP elicit different responses. What is unclear is whether these differences are meaningful. It has been previously shown that BEP blends differ due to population source and that these differences in blends elicit differential foraging responses (Metz et al. 2010). While in our case, source and response populations were the same, there remains the need for a carefully targeted study examining foraging effects of blend differences to separate signal from noise. As with the nurses, it appears that chemical cues from larvae elicit foraging behaviors consistent with the signaling of larval nutritional need, but that these cues are not likely pertaining to BEP alone. It could be BEP in addition to other cues. Further, it should be noted that the assay involving equal amount of total BEP esters was performed for foragers (experiment 7) only, and not for nurse bees (experiment 6).

Collectively, the results of our experiments suggest that adult bees closely monitor larval requirements and that a suite of nursing and foraging activities are modulated in response to changes in larval state. Evidence increasingly indicates that larvae are an integrative node in the honey bee information system that is vital to social organization. As BEP does not appear to be the candidate serving as

the cue for larval nutritional need in our study, further research is needed to understand this aspect. More recently, E-beta-ocimene, termed as volatile brood pheromone (vBP), has been elucidated as an important component of brood signaling, particularly for younger brood (Maisonnasse et al. 2009). vBP has been implicated in many of the same behavioral and physiological functions as BEP, impacting foraging response (Ma et al. 2016), worker physiology (Maisonnasse et al. 2009), and developmental ontogeny (Traynor et al. 2017). Finally, vBP has been directly implicated in signaling larval nutritional state (He et al. 2016). While highly volatile, vBP is also likely non-polar enough to be found in hexane extracts and may form a component of the responses observed in this study.

BEP and vBP each play an important and integrative role in regulating honey bee nursing and foraging responses. While vBP is the strongest candidate for a true larval 'begging' signal (He et al. 2016), BEP and vBP appear to work differentially, sometimes synergistically and sometimes antagonistically, to regulate colony responses. This in effect tunes workers to act in the interest of larvae of different ages or nutritional states. How these social regulators interact, and whether or not they reflect an honest signal of need remains an interesting research question, especially in the context of the potential gains from being preferentially fed to the likelihood of being selected for queen rearing (Sagili et al. 2018).

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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Author Contributions

B.N.M.: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Validation; Visualization; Writing—original draft; Writing—review and editing. P.C.: Validation; Visualization; Writing—original draft; Writing—review and editing. R.R.S.: Methodology; Project administration; Validation; Visualization; Writing—original draft; Writing—review and editing.

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