Young and old honey bee (*Apis mellifera*) larvae differentially prime
the developmental maturation of their caregivers

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Abstract:

In eusocial insects daughters rear the offspring of the queen to adulthood. In the honey bee, *Apis mellifera*, nurses rear young and old larvae, which emit divergent pheromones. These larval pheromones in turn impact nurse bee behaviour and physiology. To determine whether larvae and their associated pheromones have long-term physiological and behavioral effects (a priming influence) on nurse behaviour, we examined the impacts on young workers of exposure to young larvae, old larvae, or the young larval pheromone e-beta ocimene (e β) relative to a broodless control population. We also tested whether exposing nurses to the volatile e β pheromone of young larvae was sufficient by itself to produce physiological changes similar to those caused by exposure to live larvae. In order to disentangle the releaser and primer effects of brood pheromones on age of first foraging and collection bias, bees were primed for only the first 10 days of adult life.

The early environment of maturing caregivers was found to alter the circulating titres of vitellogenin and juvenile hormone in nurses. Live brood treatments suppressed ovary activation. The brood environments also significantly reduced the age of first foraging (AFF), while priming with e β increased the proportion of pollen foragers. Collectively, these results support the view that the reproductive regulatory network is sensitive to colony conditions, and this network is used to mediate the foraging division of labour.

Honey bee workers exhibit a division of labour characterized by temporal polyethism (Calderone & Page, 1988; Seeley, 1982, 1995; Wilson, 1971) where young adult bees, known as nurses, care for and feed immature brood inside the hive, while older workers forage for resources outside the hive (Winston, 1987). This behavioural transition is a key component of colony plasticity in responding adaptively to fluctuating internal and external environmental conditions (Robinson, 1992). Substantial physiological and behavioural changes occur during the shift from nurse to forager (Nelson, Ihle, Fondrk, Page, & Amdam, 2007; Robinson, 1992). Workers pass through this key transition at different rates, with some individuals showing precocious behavioural development and foraging bias for protein rich pollen, while others mature more slowly and preferentially collect nectar (Amdam, Csondes, Fondrk, & Page, 2006; Calderone & Page, 1988; Page & Amdam, 2007; Ribbands, 1952; Siegel, Fondrk, Amdam, & Page, 2013).

The transition to foraging is regulated by both intrinsic and extrinsic factors. Among the primary endogenous influences are juvenile hormone (JH) and vitellogenin (VG). JH mediates honey bee development, maturation and social behaviour (Amdam & Omholt, 2003; Rachinsky & Hartfelder, 1990; Sullivan, Jassim, Fahrbach, & Robinson, 2000; Wirtz & Beetsma, 1972). VG, an egg-yolk precursor produced by the fat body (Excels, 1974), is associated in honey bees with increased immunity (Amdam et al., 2004) and oxidative stress resistance (Seehuus, Norberg, Gimsa, Krekling, & Amdam, 2006). VG is also used by nurse bees to produce larval food (Amdam, Norberg, Hagen, & Omholt, 2003; Seehuus, Norberg, Krekling, Fondrk, & Amdam, 2007). JH and VG co-regulate each other in most honey bees in a double-repressor

feedback system (Amdam et al., 2006; Amdam, Norberg, et al., 2003; Page & Amdam, 2007). JH hemolymph titres and rates of biosynthesis are low in nurses and high in foragers, while VG titres are high in nurses and low in foragers (Amdam, Norberg, et al., 2003; Ihle, Page, Frederick, Fondrk, & Amdam, 2010). An early foraging onset can be induced by either inhibition of vitellogenin mRNA (*vg*) production through RNAi (Ihle et al., 2010) or artificial increase of JH titre through injection or topical application (Robinson, 1992; Robinson & Vargo, 1997). However, removal of the corpora allata, the glands that produce JH (Tobe & Stay, 1985), delays but does not eliminate the transition to foraging (Sullivan et al., 2000), suggesting that factors outside the VG/JH feedback loop are also influential.

An extrinsic factor that influences the timing of transition is the presence of immature honey bee brood (eggs, larvae and pupae) (Amdam, Rueppell, Fondrk, Page, & Nelson, 2009; Dreller, Page, & Fondrk, 1999; Pankiw & Page, 2001). Larvae, confined in a cell of the wax comb, are completely dependent on the care provided by nurses and must communicate their nutritional needs via pheromones (Le Conte, Sreng, & Poitout, 1995; Le Conte, Sreng, & Trouiller, 1994; Sagili & Pankiw, 2009). Pheromones play regulatory roles in many animal societies (Wilson, 1971) by inducing rapid but short-lived behavioural responses or by exerting long term physiological effects in the recipient that promote a delayed behavioural response (Le Conte & Hefetz, 2008; Wilson & Bossert, 1963). The signals emitted by developing larvae change as they age (Maisonasse, Lenoir, Beslay, Crauser, & Le Conte, 2010; Maisonasse et al., 2009; Trouiller, 1993; Trouiller, Arnold, Le Conte, & Masson, 1991), reflecting their changing nutritional needs (Le Conte et al., 1994). These varied pheromone blends influence

foraging behaviour and resource collection (nectar, pollen) in their caregivers to ensure appropriate nutritional resources (Traynor, 2014; Traynor, Le Conte, & Page, 2015). Young worker larvae, which require protein-rich food produced by nurse bees, emit the volatile pheromone e-beta-ocimene (e β) and stimulate more pollen foraging (Traynor et al., 2015). Compared with young larvae, old larvae destined to become workers are restrictively fed diets with quadruple the sugar content (Asencot & Lensky, 1988; Rhein, 1956; Shuel & Dixon, 1968) and half the protein content (Kunert & Crailsheim, 1987). The more mature larvae emit predominantly non-volatile ethyl and methyl fatty acid esters, collectively known as brood ester pheromone (BEP), along with much smaller amounts of e β (Trouiller, 1993; Trouiller et al., 1991). Larval development is thus orchestrated by the interplay of larval signals and nurse feeding responses.

Nurse bees produce the protein rich food fed to developing larvae from paired hypopharyngeal glands (HPG) (Snodgrass, 1925). To activate their HPG, bees must consume protein and have contact with larvae for 3 days (Huang, Otis, & Teal, 1989; Huang & Otis, 1989). The protein is then presumably used to produce VG which is converted to brood food in the HPG (Amdam, Norberg, et al., 2003; Seehuus et al., 2007). Gland activity is positively correlated with gland size (Deseyn & Billen, 2005; Knecht & Kaatz, 1990).

Pheromones from larvae stimulate increased nursing behaviour, longer larval feeding visits, increased HPG protein content, and the preparation of new cells for an expanded brood nest (Sagili, 2007; Sagili & Pankiw, 2009; Sagili, Pankiw, & Metz, 2011). These signals may also have longer term consequences on caregivers and prime their physiology, so that they

transition to foraging earlier and adjust the proportion of nectar to pollen that they collect to ensure the upcoming needs of the developing brood are met.

To determine how larvae of different ages affect the development and later behaviour of young workers, through both their different nutritional demands and pheromonal signals, we measured several physiological characteristics involved in the transition from nursing to foraging, the rate of behavioural maturation determined via the age of first foraging, and forage composition. Based on earlier model predictions (Maisonnasse et al., 2010), we hypothesized that maturing in an environment of young larvae for the first 10 days of adult life would accelerate maturation by priming individuals to collect protein rich pollen required for early larval development and thus transitioning out of the hive more rapidly to forage for critical protein resources. Old larvae, in contrast, require continual, but restricted feeding and thus should prolong the nursing phase of their caregivers. In order to disentangle the releaser and primer effects of brood pheromones on age of first foraging and collection bias, bees were primed for only the first 10 days of adult life with different brood treatments or received a control of no brood. The bees receiving different brood treatments were then combined into a common garden environment (to ensure identical releaser stimulus across treatment groups) and allowed to forage to determine the age of first foraging (AFF) and proportion of pollen collected. We also tested whether nurse exposure to the volatile e β pheromone of young larvae was sufficient by itself to produce physiological and endocrine changes similar to those caused by exposure to live larvae.

MATERIALS AND METHODS

Replicates: The experiment detailed below was replicated three times, each replicate trial commencing one day later than the previous replicate. To avoid potential effects of “nucleus” colony (nuc) position impacting results, the relative positioning of the treatment nucs was changed for each replicate.

Bees: We used a commercially available Italian stock to produce newly emerged bees (Williams et al., 2013). For 24 h, six queens were individually caged on six empty combs to oviposit. The combs of eggs were transferred into full size colonies and the larvae raised to adulthood in a brood box placed above a queen excluder, which prevented the queen from laying additional eggs. The combs of experimental larvae were removed and placed in an incubator set at 34 °C and 50% humidity the day prior to adult emergence. The following morning newly emerged (NE) bees were collected and paint marked (4 colours) on the abdomen to indicate treatment. This focal cohort of NE marked bees was added to four small nucs containing about 1.5 kg bees. Each nuc was provided with one caged, mated queen, one comb of honey, one empty comb, one comb of wax foundation and one comb containing the treatment.

Treatments: Each nuc received one of four possible brood treatments: 1) young larvae (YL); 2) old larvae (OL); 3) e-beta ocimene (e β); or a control 4) the absence of brood (NB). At the time of insertion, YL were early 2nd instar larvae (4 d post egg laying) and OL were early 5th instar larvae (7 d post egg laying). Since e β and NB treatments don't require live brood, they received

an empty comb. Appropriately aged larvae were obtained by caging queens in additional colonies on empty combs for 18 h. Each brood treatment consisted of ~ 2000 cells of honey bee larvae. Brood/empty combs were replaced daily with appropriately aged larvae or controls. At the time of removal, YL were predominantly late 2nd instar or early 3rd instar, a stage that emits volatile e-beta ocimene (e β) as their pheromone (Maisonnasse et al., 2010; Maisonnasse et al., 2009). OL were predominantly late 5th instar with a few cells already capped. OL primarily emit non-volatile BEP (Trouiller, 1993; Trouiller et al., 1991). Two hours after brood comb replacements, all four nucs received an e β pheromone/control treatment. This was contained within a glass petri dish placed below the brood nest area and covered with mesh to prevent direct contact by the bees (Maisonnasse et al., 2010). YL, OL and NB received 1 ml of paraffin oil as a control; e β received 10,000 larval equivalents of ocimene (0.01 g mixture of ocimene isomers including e β ; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 1 ml of paraffin oil. Daily brood and pheromone treatments were continued for 10 d to provide four different early priming environments, then bees were sampled as described below. All remaining bees were then combined into a common hive as described below.

Sample Collection: After being exposed to one of the four early priming environments, 7-12 bees were collected from each treatment colony for hemolymph extraction as the amount of extractable hemolymph can vary (Hartfelder et al., 2013). Bees were anesthetized by chilling. From each- bee, 1 μ l of hemolymph was siphoned into a glass capillary tube after piercing the cuticle above the second tergite. For each JH sample the 1 μ l of hemolymph from three randomly

selected individuals of the same treatment group and replicate were pooled and stored at -80°C in 1 ml 50% acetonitrile (EMD Chemicals, Gibbstown, NJ) until analysis. For VG protein samples, an additional 1 μl hemolymph per bee was extracted, evacuated into an Eppendorf tube, and stored at -80°C until analysis. Subsequently, the gut was removed and the individual abdominal cuticle sample with its adherent layer of fat body was dissected into 500 μl of TRIzol (Invitrogen, Carlsbad, CA) and stored at -80°C until *vg* mRNA expression could be analysed. For ovary and hypopharyngeal gland assessment, an additional 25 bees per group were collected and frozen at -80°C until dissection.

RNA Extraction and cDNA Synthesis of Fat Body: Fat bodies dissected from the sampled bees were homogenized in TRIzol. RNA was extracted following Invitrogen's instructions. The quality and quantity of RNA were determined via spectrophotometry (Nanovue, GE Healthcare, Piscataway, NJ). DNase (RNase-free, DNase kit, Applied Biosystems, Bedford, MA, USA) was added to the each RNA extract to remove trace DNA contaminants. One μg of such treated RNA was used for reverse transcription following an established method (Y. Wang, Brent, Fennern, & Amdam, 2012) using TaqMan® Reverse Transcription Reagents (Applied Biosystems).

Quantitative Real-time PCR Analysis: First-strand cDNA was used for real-time quantitative PCR (RT-qPCR) assays. Sixteen samples were randomly picked from each treatment group for *vg* gene (AJ517411) expression analysis (Y. Wang, Brent, et al., 2012); each replicate was represented by 4-7 samples for each treatment. The biological samples from the four treatment

groups were run together on every PCR plate, enabling *vg* expression comparisons. Each sample had three technical replicates run on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems) for measuring *vg* transcript levels in comparison with those of the reference gene *actin* by means of the Delta-Delta Ct method (Y. Wang, Brent, et al., 2012). Studies have shown that *actin* is stably expressed throughout all stages in honey bees (Lourenço, Mackert, dos Santos Cristino, & Simões, 2008; Reim, Thamm, Rolke, Blenau, & Scheiner, 2013). By monitoring negative control samples (without reverse transcriptase) and melting curve analysis, we verified that the RT-qPCR assays were not confounded by DNA contamination or primer dimers (Vandesompele, De Paepe, & Speleman, 2002).

Hemolymph VG Protein Analysis: Individual hemolymph samples for all four treatment groups were subjected to one-dimensional SDS-electrophoresis using 4-10% polyacrylamide gels. Each gel included two samples from each treatment group and three β -galactosidase standard samples (1.25, 2.5 and 3.75 μ g) of *Escherichia coli* (Sigma), randomly distributed among lanes. Each replicate was represented by 4-7 samples that were randomly distributed across gels. Sample aliquots containing 0.5 μ l hemolymph were mixed with loading buffer (Laemmli 2 $\times$, Sigma) and boiled for 2 min. Electrophoresis on 4–10% polyacrylamide gels (Bio-Rad, Hercules, CA) was performed according to published protocols (Amdam, Simões, Guidugli, Norberg, & Omholt, 2003; Seehuus et al., 2006). Electrophoresis was carried out at a constant current of 15mA for 1 h at 20°C. Gels were stained with 0.025% Coomassie blue dissolved in a solution of acetic acid (7%), methanol (40%) and purified water that was also used for gel destaining. VG was

identified by molecular mass (180-190 KD) in comparison with known protein markers (Bio-Rad).

Hemolymph JH Titre: JH titres were estimated using the gas chromatography/mass spectrometry (GC-MS) method of (Bergot, Schooley, & Dekort, 1981) as modified by (Shu, Ihl Park, Ramaswamy, & Srinivasan, 1997) and detailed in (Amdam, Page, Fondrk, & Brent, 2010). Samples were eluted through aluminium oxide columns successively with hexane, 10% ethyl ether–hexane and 30% ethyl ether–hexane. Samples were derivatized with methyl-d alcohol (Sigma-Aldrich, St Louis, MO, USA) and trifluoroacetic acid (Sigma-Aldrich) and then subjected to a second series of aluminium oxide elutions (30% ethyl ether-hexane, then 50% ethyl-acetate–hexane). Purified samples were analysed on an HP 7890A Series GC (Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m × 0.25 mm Zebron ZB-WAX column (Phenomenex, Torrence, CA, USA) and coupled to an HP 5975C inert mass selective detector (Agilent Technologies). Helium was the carrier gas. MS analysis occurred in the SIM mode, monitoring at m/z 76 and 225 to ensure specificity for the d₃-methoxyhydrin derivative of JHIII. Farnesol (Sigma-Aldrich) was used as an internal standard in each sample to ensure consistent peak calibration. Total abundance was quantified against a standard curve of derivatized JHIII (Sigma-Aldrich). The assay's detection limit is 1 pg.

Foraging Activity, Age of First Foraging, and Pollen Foraging: When the focal cohorts reached 10 d of age, and sample collection had ceased, the combs and the queen were removed from each

of the four colonies and the bees were left to form a cluster inside the empty hive. After sunset, when all foragers had returned to the hive, the entrance was screened and the four hives moved into a cool, dark location overnight. In the morning the bees from the four different hives were misted with water so they could not fly, then combined into a ventilated wooden box. They formed one large 6 kg cluster and were allowed to mix overnight to lose their individual colony odours. The following morning, when the marked cohort of bees was 12 d old, this combined colony was introduced into a full size hive, provided with a different mated queen, two combs of larvae, two combs of pupae, three empty combs, and two honey combs. The hive entrance was opened and the bees were allowed to forage.

Foraging activity was monitored daily in four 20 min intervals for 18 d and the exact start time of each interval was recorded. During monitoring periods, the hive entrance was closed with mesh screening so that returning foragers could still orient to the hive odours and land on the entrance board, but were barred from entering the hive. All marked foragers were collected into a cage and cold anesthetized. The collected bees were sorted by paint marks into treatment, then the number of pollen and non-pollen foragers for each group was tallied. Foragers with visible pollen loads on their hind legs were categorized as pollen foragers while those with no noticeable loads were considered non-pollen foragers. Since the hive was monitored daily, the adult age at capture represents a bee's age of first foraging (AFF). To add gradation to the AFF, the time collected was converted to morning (08:00-11:00), midday (11:00-14:00), or afternoon (14:00-17:00) and 0.3 or 0.6 d were added on to the AFF if the bee was captured during the midday or afternoon time window, respectively. Pollen foraging activity was determined by comparing the

proportion of pollen foragers for each treatment group. For a subset of pollen foragers, one pollen pellet was removed from the hind leg and weighed. Because bees carry a balanced pollen load (Winston, 1987), the weight of the single pellet was doubled to estimate total pollen load collected. Pollen load mass was calculated across all collected foragers to provide a better representation of pollen intake per colony. All NE marked bees were counted at the time of introduction. When > 50% of each treatment had foraged, the experiment was terminated (Pankiw & Page, 1999). A census was conducted for all marked bees that remained in the hive, and these were recorded as non-foragers. The influence of treatment on foraging activity was determined using survival analysis, with data for bees that never transitioned to foraging being censored.

Ovariole Number, Ovary Activation & HPG Development: Reproductive physiology influences individual behavioural response thresholds, the age of first foraging and foraging preference (Page, 2013; Page & Amdam, 2007; Page, Rueppell, & Amdam, 2012), so we assessed ovary condition in bees of each treatment at 10 d. Each of the three experimental replicates was represented by 10 to 15 bees for every treatment. The paired ovaries were dissected into saline (0.25 mol/l NaCl) and the ovariole number for each was determined under a microscope. Additionally, the state of oocyte development (ovary activation) within each ovary was scored using a 5 point scale (Pernal & Currie, 2000): stage 0 = no follicle development, stage 1 = slight enlargement, stage 2 = presence of distinct cells leading to swellings and constrictions, stage 3 =

egg volume exceeding that of the nutritive follicle, stage 4 = presence of fully formed eggs. The mean score of the ovary pair was used for analysis.

Nurse bees produce brood food from paired hypopharyngeal glands (Snodgrass, 1925). The activity of the gland is positively correlated with its size (Deseyn & Billen, 2005; Knecht & Kaatz, 1990). Numerous globular acini attach to the long, slender main channel of the HPG. These acini increase in diameter until six days after adult emergence, and thereafter decrease in size. The gland continues to atrophy with increased age. Bees dissected for ovary analysis also had both HPGs removed from the head capsule into saline. HPG development was scored under a microscope at 100x magnification on a scale from stage 1 to 4 based on acini size and globe density (Hess, 1942): Stage 1) atrophied; 2) slightly swollen with noticeable spacing between acini; 3) swollen with small spacing between acini, capable of producing brood food; and 4) fully developed and tightly clustered, channel obscured by acini.

Statistical Analysis: Age of first foraging was not normally distributed, therefore survival analysis was used to analyze the data. The day of initiation of foraging is equivalent to “death” and allows the use of this procedure (Amdam et al., 2009; Pankiw, 2004; Pankiw & Page, 2001). To determine the influence of treatment, replicate and potential interaction effects, a Parametric Fit analysis with Frechet distribution was performed on the whole model, and Likelihood Ratio Tests to analyse individual model effects when the whole was significant. Since there was no interaction between treatment and replicate, Log-Rank survival analysis was conducted for treatment and replicate individually. Generalized linear models (GLM) were used to analyse

treatment and replicate effects on VG and JH titres, with correction for overdispersion when necessary. These tests were followed by LSD Student T-tests post-hoc analyses only when initial results were significant (Sokal & Rohlf, 1995). Data were log transformed for vg mRNA. JH data fit a Poisson distribution. Ovary activation and HPG development were not normally distributed and were analysed with Wilcoxon rank sums. Replicate had no effect on ovary activation, so we pooled across all three replicates. To determine significant differences between treatment groups, we used the Wilcoxon method for non-parametric comparisons of each pair and significant differences ($\alpha = 0.05$) are reported. Total ovarioles, ovary activation and HPG development were compared using Spearman's rank correlations, to determine if they were co-regulated. Whether treatment, age or replicate influenced pollen collection was analysed with a nominal logistic fit and likelihood ratio test, as pollen foraging is categorical and not continuous. The proportion of total pollen foraging activity was compared to controls for each treatment using Welch's t-test, which is robust when variance is unequal across groups. Significant differences were confirmed with a 2 x 2 contingency table comparing total number of pollen foragers and non-pollen foragers for each of the four treatment groups. The impact of early priming environment on the successful transition to foraging was calculated with 2 x 4 contingency tables comparing the total number of bees that transitioned to the total number still present in the colony at census, using the online programs available at <http://www.vassarstats.net>. All other analyses were conducted using JMP Pro 11 (SAS, Cary, NC).

RESULTS

Age of First Foraging: Since genetic composition and colony environment (encompassed in replicate) and early brood environment (treatment) can influence the timing of transition from in-hive tasks to foraging, we included both factors and their interaction in our survival analysis model. Both treatment and replicate significantly influenced the AFF, but there was no interaction of these factors (Parametric Survival Fit: whole model: $\chi^2 = 70.47$, $df = 11$, $P < 0.0001$; Likelihood Ratio Test on interaction: $\chi^2 = 10.99$, $df = 6$, $P = 0.089$). AFF varied by treatment (Log-Rank: $\chi^2 = 22.85$, $df = 3$, $P < 0.0001$) with the median AFF 1.8 days later for the no brood control compared to any of the brood treatments (Table 1). While e β had an impact similar to that of live brood treatments on AFF for the first week of monitoring, the pheromone treatment was more similar to the no brood control in the later days of the experiment (Fig. 1). AFF also varied by replicate (Fig. 2, Log-Rank: $\chi^2 = 30.86$, $df = 2$, $p < 0.0001$).

Pollen Foraging Activity and Pollen Load: Pollen foraging provides the colony with rich protein resources. The proportion of workers foraging for pollen was significantly influenced by AFF ($\chi^2 = 211.88$, $df = 18$, $P < 0.001$), and treatment ($\chi^2 = 13.98$, $df = 3$, $P < 0.003$), but not replicate ($\chi^2 = 1.46$, $df = 2$, $P = 0.48$). While the total number of foraging focal bees was greatest immediately after the treatment groups were combined into a common garden experiment at 12 d of age, the proportion of pollen foragers peaked around 21 d (Fig. 3). Pollen foraging can vary greatly day to day, and there were no statistical differences across groups at any given age. Over the entire experiment, bees primed by an e β environment had 40% more pollen foragers than

control bees reared in a NB environment (Fig. 4, Welch's t-test: $t = 2.19$, $df = 1169.1$, $P = 0.029$). The YL group had almost 30% more pollen foragers than controls, but as overall pollen foraging was low among naïve bees transitioning to foraging for the first time, this was not significantly different from controls.

Transition to Foraging: Brood priming environment significantly influenced the number of bees that successfully transitioned to foraging (Table 2; $\chi^2 = 11.43$, $df = 3$, $P = 0.010$), with more bees reared in the OL environment switching tasks compared to the control and e β priming environment. The YL group did not differ from any other treatment.

Activation of the Ovaries and HPG: Ovary activation was not significantly influenced by replicate (Wilcoxon: $\chi^2 = 0.51$, $df = 2$, $P = 0.77$), so we pooled results across the three replicates. Early priming environment influenced worker reproductive physiology (Fig. 5). Overall ovary activation, measured as stage of follicle development at 10 d post-emergence, was low among all treatment groups (Mean $\pm$ SE for NB = 1.18 ± 0.13 ; OL = 0.56 ± 0.12 ; YL = 0.75 ± 0.18 ; e β = 1.17 ± 0.19), due to the presence of a caged queen in each nuc. Bees reared in the OL and YL environment had significantly fewer activated ovaries than those reared without brood or with only e β pheromone. HPG activation was significantly influenced by both replicate (Wilcoxon: $\chi^2 = 13.49$, $df = 2$, $P = 0.0012$) and early priming environment (Wilcoxon: $\chi^2 = 28.40$, $df = 3$, $P < 0.0001$). HPG development was reduced in the control bees reared without brood in all three replicates, while the three different brood treatments resulted in bees with well-developed HPGs

appropriate for producing brood food, though there were slight variations by replicate (Fig. 6). Total ovariole number and ovary activation are significantly and positively correlated across all bees (Spearman $\rho = 0.23$, $n = 136$, $P = 0.007$).

vg mRNA in Fat Body: *Vg* mRNA expression levels influence JH titres and thus impact the transition to foraging. The early brood environment experienced by the bees influenced *vg* mRNA expression regardless of replicate (Fig. 7 top, GLM: Treatment: $\chi^2 = 9.28$, $df = 3$, $p = 0.0258$; Replicate: $\chi^2 = 1.60$, $df = 2$, $P = 0.450$); *Vg* mRNA was significantly down-regulated in bees reared with old larvae compared to those raised in NB ($t_{60} = 2.71$, $P = 0.009$) or YL ($t_{60} = 2.05$, $P = 0.044$) environments.

VG Hemolymph Protein: Early environment had a significant impact on the levels of circulating VG protein in the hemolymph regardless of replicate (Fig. 7 bottom, GLM: Treatment: $\chi^2 = 10.73$, $df = 3$, $p = 0.0133$; Replicate: $\chi^2 = 0.011$, $df = 2$, $P = 0.994$). Bees reared with OL had significantly less VG than those raised in NB ($t_{54} = 2.89$, $P = 0.006$), YL ($t_{54} = 2.67$, $P = 0.010$), and eß ($t_{54} = 2.47$, $P = 0.017$) environments.

JH Titres: Early environment had a significant impact on the levels of circulating JH in the hemolymph (GLM: Treatment: $\chi^2 = 12.52$, $df = 3$, $P = 0.0058$). Both live brood environments primed workers by significantly raising JH titres relative to the broodless environments (Fig. 8;

$F_{3,41} = 6.81, P < 0.001$). There was no difference between bees exposed to NB and e β ($t_{41} = 0.82, P = 0.42$) or between OL and YL ($t_{41} = 0.68, P = 0.50$).

DISCUSSION

Although it was previously established that brood can impact the foraging behaviour of nestmates (Amdam et al., 2009; Dreller et al., 1999; Hellmich & Rothenbuhler, 1986; Pankiw, Page, & Fondrk, 1998; Tsuruda & Page, 2009), we now demonstrate that the specific age composition of brood (OL vs. YL) and the pheromones of young larvae influence the foraging response of their caretakers. Feeding and interacting with larvae primes the physiology of young adult bees, significantly impacting the reproductive physiology and VG/JH titres that regulate the transition from nursing to foraging (see Table 3).

Exposure of maturing workers to YL or OL impacted the development of the nurse-aged bees in fundamental ways and, as expected, stimulated greater HPG development than in bees reared with no brood. Despite having no access to live larvae, bees in the e β environment also had better developed HPGs compared to those reared without brood. This finding seemingly contradicts previous studies showing that HPGs of isolated young workers exposed to e β do not develop (Maisonnasse et al., 2010). Traynor et al. (2014) showed that e β stimulates HPG growth in caged bees, so long as the bees possess ovaries with a higher number of ovarioles as that trait appears to prime them toward caregiving. Current findings show that e β alone is sufficient to stimulate HPG development. This difference between studies may indicate that while the volatile

brood pheromone can stimulate HPG development, it can only do so in bees primed for caregiving or within the context of a colony, where a queen and honeycombs are also present to provide synergistic stimuli. Additional stimuli may be a prerequisite given that activation of the HPGs to feed larvae is an energetically expensive commitment, requiring nurses to consume pollen and convert it into protein rich brood food (Brouwers, 1983; Hrassnigg & Crailsheim, 1998). Even under normal colony conditions, key stimuli may be necessary to induce HPG development. Previous work showed that nurse bees only activate their HPGs after three days of rearing brood (Huang & Otis, 1989) and that direct contact with larvae is required (Huang et al., 1989). However, these earlier reports used mixed age larvae, which may not have produced e β concentrations adequate to stimulate HPG development compared to young larvae.

In previously run releaser experiments, exposure to young larvae or e β biased foraging toward pollen collection (Traynor et al., 2015). In this experiment, we found that bees exposed to e β had 40% more pollen foragers than control bees reared in a NB environment (Fig. 4), while YL group had almost 30% more pollen foragers than controls. Overall pollen foraging was low among naïve bees transitioning to foraging for the first time; the brood stimulus of only two frames of open larvae in the common garden environment may not have been great enough to induce heavy pollen foraging.

The presence of larvae typically suppresses ovary activation in workers (Jay, 1970). Overall, we saw very low levels of ovary activation in our experiment, but only bees reared in the live larvae (OL and YL) environments having significantly reduced ovary activation compared to NB controls. Similarly, although the larval pheromones e β and BEP have been

shown to suppress ovary activation in cage trials, and BEP was demonstrated to suppress ovary activation in small queenless field colonies of 1,500 bees (Arnold et al., 1994; Maisonnasse et al., 2009; Mohammadi, Paris, Crauser, & Le Conte, 1998), we found bees reared in the e β environment had ovary activation comparable to the control (NB) bees (Fig. 5). This low rate of ovary activation may have been due to the suppressive effects of the caged queen present in all nucs. Differences in ovary activation between NB and e β exposure may only become apparent in a queenless situation. Across all bees, ovariole number was positively correlated to ovary activation, as has been found repeatedly (Amdam et al., 2006; Graham et al., 2011; Makert, Paxton, & Hartfelder, 2006). A possible explanation for the increased ovarian activation seen in bees exposed only to the young larval pheromone in this experiment is that e β stimulated the nurse age bees to activate their HPG to feed larvae, but with no larvae to receive the protein-rich food, the nurse aged bees stored the excess protein into vitellogenic oocytes. Such a flexible repurposing of resources within workers has been postulated for social insects (Bier, 1954, 1958; Hölldobler & Wilson, 1990).

The test bees were the predominant nurse aged bees in the colonies, as no other bees were allowed to emerge during the 10 days of priming. The bees reared with old larvae significantly depleted their own vitellogenin stores, as evident in reduced circulating hemolymph VG protein and downregulated transcription of *vg* mRNA. Compared to young larvae, old larvae are substantially larger (Rembold & Kremer, 1980; D. Wang, 1965) and consume more food per unit of time. Because nurse bees convert VG into protein rich jelly in the HPG (Amdam, Norberg, et al., 2003), the more intensive demands of caring for OL may deplete circulating VG faster than it

can be replenished. This in turn would cause a rise in JH titres that would induce the transition to foraging at an earlier age compared to workers reared without brood, given that the JH and VG are typically in a negative feedback loop (Amdam & Omholt, 2003; Amdam *et al.*, 2009; Nelson *et al.*, 2007). Such an early transition to foraging after exposure to old larvae is demonstrated in this experiment (Fig. 1 and Table 1 & 2), where AFF differences with the YL treatment group become more evident toward the latter half of the experiment.

Our results contradict some of the model predictions by Maisonnasse *et al.* (2010), who postulated exposure to old larvae would delay the transition to foraging. Their model proposed delayed foraging of bees caring for old larvae because exposure to high levels of BEP produced by old larvae has been linked to a delay in transition (Le Conte, Mohammedi, & Robinson, 2001). However, reports of the purported influence of BEP on age of first foraging has been varied, with some experiments showing an acceleration of maturation (Pankiw, Roman, Sagili, & Zhu-Salzman, 2004) and others a delay (Alaux *et al.*, 2009; Le Conte *et al.*, 2001). Both worker age and BEP dose impact the timing of the transition to foraging (Alaux *et al.*, 2009; Sagili, 2007; Sagili & Pankiw, 2009), as does worker genotype (Page, 2013; Pankiw & Page, 1999, 2001; Tsuruda & Page, 2009), suggesting a multi-component regulatory system.

Typically the transition to foraging is preceded by extensive brood feeding, leading to VG depletion and a subsequent rise in JH (Amdam *et al.*, 2009; Smedal, Brynem, Kreibich, & Amdam, 2009). However, the nurses reared in the YL and e β environments did not experience this depletion, having elevated *vg* and VG titres compared to NB, and yet transitioned to foraging earlier than bees reared without brood. We postulate that exposure to e β lowers the individual's

response threshold to JH, so that the transition to foraging can be induced by lower JH levels. Another possibility is that young larvae and e β influence other redundant regulatory systems outside the VG/JH loop or a complimentary component of the feedback loop. The insulin signalling system is one possibility because it is sensitive to nutritional cues and is located upstream of JH production (Y. Wang, Kocher, et al., 2012), and is thus in a position to influence the transition to foraging. We know redundancy is built into the regulatory system as allatectomized bees, rendered incapable of producing JH, can still transition to foraging successfully (Sullivan et al., 2000). Genetic selection for pollen hoarding has demonstrated an uncoupling of JH and VG in low-strain bees (Ihle et al., 2010; Nelson et al., 2007), again highlighting the redundancy of the regulatory system as these bees still initiate foraging, albeit later in life.

The priming influences of OL, YL and e β all impacted endocrine physiology in different ways and decreased the age of first foraging compared to NB (Table 3). While all three brood treatments had identical ages when 25% of the focal cohort transitioned to foraging, slight differences become apparent by the time 75% have transitioned. Bees exposed to an OL stimulus showed the expected pattern of precocious foragers, exhibiting low VG and high JH titres at 10 days of age. Bees primed with YL showed a previously un-encountered pattern of high VG and high JH; the higher VG titres may have led to their slightly delayed median age of first foraging compared to OL. Bees primed with e β demonstrated a pattern of high VG and low JH typically seen in bees with a prolonged the nursing phase, and they foraged later than bees exposed to live larvae. The difference in AFF between the three treatment groups is small and only discernible in

the second half of the experiment (Fig. 1 and Table 1). This suggests that the common garden environment of the full-size colony, with a regular broodnest and egg-laying queen, may suppress endocrine differences that arise from stage-specific larval stimuli, so that only nurses exposed to NB significantly delayed their transition to foraging.

These results highlight the malleability of reproductive physiology in the division of labour, allowing a variety of early priming influences to shift endocrine titres and influence the age of first foraging. Later experience may mute the effect of an early environment, diminishing differences of early life trajectories. The direct modes of action of larvae and their pheromones on ovary activation and consequent endocrine and behavioural changes in their caregivers remain to be determined.

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Figures

Figure 1. Age of first foraging by treatment. Age of first foraging as a function of early priming environment for all bees plotted using survival analysis with age of foraging substituted for “death”. Bees that never transitioned to foraging were censored. No Brood = NB, red, Old Larvae = OL, green; Young Larvae = YL, blue; e-beta ocimene = eβ, orange. Number that foraged and censored, median AFF, 25% and 75% quartiles reported in Table 1.

Figure 2. Age of first foraging by replicate. Age of first foraging as a function of replicate for all bees plotted using survival analysis with age of foraging substituted for “death”. Bees that never transitioned to foraging were censored. Replicate 1 = red (foraged = 633, censored = 86), replicate 2 = green (foraged = 931; censored = 108); replicate 3 = blue (foraged = 942; censored = 181).

Figure 3. Foraging over Time. Mean of the three replicates of each of four treatments for each age. Top row = total foragers for given age; middle row = proportion of total foragers that collected pollen; bottom row = cumulative proportion of pollen foragers during the experiment. Treatment indicated by colour (No Brood = NB = black, Old Larvae = OL = dark grey; Young Larvae = YL = light grey; e-beta ocimene = eβ = very light grey).

Figure 4. Pollen Foraging. Mean proportion of pollen foragers + S.E. as a function of the early priming environment. No Brood = NB, Old Larvae = OL; Young Larvae = YL; e-beta ocimene = eβ. We had an *a priori* assumption that pollen foraging would differ between brood treatments and controls and thus compared each treatment to the control group using Welch's t-tests. All samples were collected on the first day of foraging (regardless of chronological age) and were pooled from three replicates per treatment. Significant differences between treatments ($\alpha < 0.05$) are indicated by different letters.

Figure 5. Ovary activation by early priming environment. Mean + S.E. ovary activation stage (0 = no follicle development, 1 = slight enlargement, 2 = distinct cells with swellings and constrictions, 3 = egg volume exceeds nutritive follicle, 4 = fully formed eggs). No Brood = NB (n = 33); Old Larvae = OL (n = 34); Young Larvae = YL (n = 34); e-beta ocimene = eβ (n = 35). Significant differences between treatments ($\alpha < 0.05$) are indicated by different letters.

Figure 6. HPG development by replicate and early priming environment. Mean + S.E HPG development stage (1 = atrophied, 2 = slightly swollen and wide spaced acini; 3) swollen and slight spaced acini, 4) fully developed and tightly clustered acini). No Brood = NB (n = 33); Old Larvae = OL (n = 34); Young Larvae = YL (n = 34); e-beta ocimene = eβ (n = 35). Significant differences ($\alpha < 0.05$) within each replicate are indicated by different letters.

Figure 7. *vg* mRNA and VG protein by treatment. Mean + S.E. *vg* mRNA titres in fat body (top) and VG protein concentration in hemolymph as a function of early priming environment. No Brood = NB ($n = 16$ *vg* & 13 VG); Old Larvae = OL ($n = 15$ *vg* & 16 VG); Young Larvae = YL ($n = 17$ *vg* & 15 VG); e-beta ocimene = eβ ($n = 16$ *vg* & 14 VG). Significant differences between treatments ($\alpha < 0.05$) are indicated by different letters.

Figure 8. Mean + S.E. juvenile hormone titers as a function of early priming

environment. No Brood = NB ($n = 12$), Old Larvae = OL ($n = 11$); Young Larvae = YL ($n = 11$); e-beta ocimene = eβ ($n = 11$). Significant differences ($\alpha < 0.05$) are indicated by different letters.

Table 1. Age of First Foraging by Treatment. Survival analysis of the transition to foraging by the focal cohort of bees with age at time of first foraging (AFF) substituted for death. The early priming environment (treatment) had a significant impact on median AFF. The pheromone treatment e β was similar to the live brood treatments in the early part of the experiment, but by the time 75% of the focal cohort had foraged the impacts of e β were almost indistinguishable from the no brood control.

Treatment	Foraged	Censored	Median AFF (95% CI)	25% foraged	75% foraged
NB	656	112	19.8 (19.0-20.3)	14.3	26.0
OL	622	67	17.3 (16.3-18.3)	13.6	23.6
YL	638	90	17.8 (16.6-18.6)	13.6	24.0
e β	590	106	18.0 (16.6-18.6)	13.6	25.6

Treatment = the 10 day priming environment the focal cohort of bees experienced. NB = no brood control; OL = old larvae; YL = young larvae; e β = e-beta ocimene pheromone. Foraged = the number of individual bees from the focal cohort that successfully transitioned to foraging. Censored = the number of individual bees from the focal cohort that never started foraging and were still present at the cessation of the experiment. Median AFF = the median age of first foraging. 95% CI = 95% confidence interval.

Table 2. 2x4 Contingency tables of transition to foraging. Some bees never transitioned from in-hive tasks such as nursing to outside foraging during the 30 days of the experiment. The early priming environments had a significant effect on the likelihood of transition to foraging.

Treatment	Foraged	Never Foraged	% Never Foraged
NB	656	112	14.5%
OL	622	67	9.7%
YL	638	90	12.4%
eβ	590	106	15.2%

$\chi^2 = 11.5$, $df = 3$, $P = 0.009$

Table 3. Significant effects compared to the control of no brood. For each characteristic measured, the three brood treatments (OL, YL, e β) were compared against the control of NB. Significant differences ($\alpha = 0.05$) are indicated by $\uparrow$ if upregulated or higher and $\downarrow$ if downregulated or lower in comparison to NB. AFF = age of first foraging; Ovy # = total ovarioles; Ovy Act = ovary activation; HPG = hypopharyngeal gland development; VG = vitellogenin; JH = juvenile hormone.

SIGNIFICANT EFFECTS COMPARED TO THE CONTROL OF NO BROOD								
	Median AFF	Proportion Foraged	Pollen Foraging	Ovy Act	HPG	vg mRNA	VG protein	JH
OL	$\downarrow$	$\uparrow$	NS	$\downarrow$	$\uparrow$	$\downarrow$	$\downarrow$	$\uparrow$
YL	$\downarrow$	NS	NS	$\downarrow$	$\uparrow$	NS	NS	$\uparrow$
e β	$\downarrow$	NS	$\uparrow$	NS	$\uparrow$	NS	NS	NS

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