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

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In eusocial insects, daughters rear the offspring of the queen to adulthood. In the honeybee, *Apis mellifera*, nurses rear young and old larvae, which emit divergent pheromones. These larval pheromones in turn affect nurse bee behaviour and physiology. To determine whether larvae and their associated pheromones have long-term physiological and behavioural effects (a priming influence) on nurse behaviour, we examined the effects on young workers of exposure to young larvae, old larvae or the young larval pheromone e-beta ocimene (eβ) relative to a broodless control population. We also tested whether exposing nurses to the volatile eβ pheromone of young larvae was sufficient by itself to produce physiological changes similar to those caused by exposure to live larvae. 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, while priming with eβ 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.

Honeybee 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 honeybee 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 honeybees 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 honeybees in a double-repressor feedback system (Amdam et al., 2006; Amdam, Norberg, et al., 2003; Page & Amdam, 2007). JH haemolymph 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,

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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 honeybee 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 (Maisonnasse, Lenoir, Beslay, Crauser, & Le Conte, 2010; Maisonnasse 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 (eβ) 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 nonvolatile ethyl and methyl fatty acid esters, collectively known as brood ester pheromone (BEP), along with much smaller amounts of eβ (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, 1989; Huang, Otis, & Teal, 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 that 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. 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 exposing nurses to the volatile eβ pheromone of young larvae was sufficient by itself to produce physiological and endocrine changes similar to those caused by exposure to live larvae.

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 affecting the 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 (four 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 of 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 (eβ); or (4) a no brood control (NB). At the time of insertion, YL were early second-instar larvae (4 days post egg laying) and OL were early fifth-instar larvae (7 days post egg laying). Since eβ and NB treatments do not 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 honeybee larvae. Brood/empty combs were replaced daily with appropriately aged larvae or controls. At the time of removal, YL were predominantly late second instars or early third instars, which emit volatile e-beta ocimene (eβ) as their pheromone (Maisonnasse et al., 2009, 2010). OL were predominantly late fifth instars with a few cells already capped. OL primarily emit nonvolatile BEP (Trouiller, 1993; Trouiller et al., 1991). Two hours after brood comb replacements, all four nucs received an eβ 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; eβ received 10 000 larval equivalents of ocimene (0.01 g mixture of ocimene isomers including eβ; Sigma–Aldrich, St Louis, MO, U.S.A.) dissolved in 1 ml of paraffin oil. Daily brood and pheromone