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Primer effects of a brood pheromone on honeybee behavioural development

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Primer pheromones are thought to act in a variety of vertebrates and invertebrates but only a few have been chemically identified. We report that a blend of ten fatty-acid esters found on the cuticles of honeybee larvae, already known as a kairomone, releaser pheromone and primer pheromone, also act as a primer pheromone in the regulation of division of labour among adult workers. Bees in colonies receiving brood pheromone initiated foraging at significantly older ages than did bees in control colonies in five out of five trials. Laboratory and additional field tests also showed that exposure to brood pheromone significantly depressed blood titres of juvenile hormone. Brood pheromone exerted more consistent effects on age at first foraging than on juvenile hormone, suggesting that the primer effects of this pheromone may occur via other, unknown, mechanisms besides juvenile hormone. These results bring the number of social factors known to influence honeybee division of labour to three: worker–worker interactions, queen mandibular pheromone and brood pheromone.

Keywords: *Apis mellifera*; brood pheromone; division of labour; juvenile hormone; social regulation

1. INTRODUCTION

Primer pheromones are thought to play central regulatory roles in many animal societies, exerting relatively slow effects on endocrine and neural systems to coordinate physiological and behavioural development in response to prevailing social conditions (Wilson 1971). The actions of many primer pheromones have been shown in a variety of vertebrates and invertebrates but, to date, only a few have been chemically identified (Robinson 1996). In contrast, hundreds of faster-acting releaser pheromones have been identified since the discovery of the first sex pheromone in the silk moth (Fabre 1879).

Brood pheromone is a blend of ten fatty-acid esters found on the cuticles of honeybee larvae. It was first identified as a kairomone that attracts the parasitic mite, *Varroa jacobsoni* (Le Conte *et al.* 1989). Later, it was found that some components of this blend have releaser-like effects on various aspects of brood care (Le Conte *et al.* 1990, 1994, 1995). Some components are more active than others, but all ten individual compounds show some releaser activity, leading to their being called, collectively, brood pheromone. Brood pheromone also inhibits ovary development in worker honeybees, indicating a primer effect, which may be involved in the regulation of reproductive division of labour (Mohammedi *et al.* 1998).

Two components of brood pheromone, methyl palmitate and ethyl oleate, were shown in laboratory studies (Mohammedi *et al.* 1996) to increase the activity of the hypopharyngeal glands, which produce proteinaceous material that is fed by nurse bees to larvae. Differences in the activity of the hypopharyngeal glands are associated with honeybee division of labour (Robinson 1992) suggesting that brood pheromone might also act as a primer pheromone in division of labour among worker bees.

Division of labour in honeybee colonies is based on a pattern of worker-bee behavioural development (Robinson 1992). Young bees work in the hive performing brood care and other tasks for the first two to three weeks of adult life and then begin to forage outside the hive for the remaining one to three weeks of their life. One proximate factor that influences the division of labour in honeybee colonies is juvenile hormone (JH). JH blood titres and rates of biosynthesis are low in nurse bees and high in foragers, and JH treatments cause precocious foraging (Robinson 1992; Robinson & Vargo 1997). Removal of the corpora allata, the only known source of JH in the honeybee, results in a delay in foraging that is eliminated with hormone replacement (Sullivan *et al.* 2000).

Behavioural development in honeybees is also influenced by the needs of the colony. A shortage of foragers leads to accelerated behavioural development and precocious foraging, while a shortage of nurse bees leads to delayed behavioural development and overage nursing (Robinson 1992). The presence of old bees delays the onset of foraging and depresses the JH titres of younger bees (Robinson *et al.* 1989; Huang & Robinson 1992, 1996); this social inhibition requires physical contact, suggesting the possibility of a worker inhibitory pheromone (Huang *et al.* 1998). Honeybee queen mandibular pheromone also exerts similar effects; colonies given supplemental doses of synthetic pheromone show a delayed onset of foraging (Pankiw *et al.* 1998) and lower JH titres (Pankiw *et al.* 1998; Kaatz *et al.* 1992) relative to control colonies. This is in addition to the other effects of queen mandibular pheromone as a sex attractant, a releaser of retinue behaviour and an inhibitor of queen-rearing behaviour (Winston & Slessor 1992).

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Nurse bees come into contact with the queen most frequently, leading Pankiw *et al.* (1998) to propose that exposure to queen mandibular pheromone can extend the duration of the nursing phase to ensure more efficient brood rearing.

The amount of brood reared by a colony of honeybees depends on variations in worker genotype (Ruttner 1986), adult population size, weather, availability of floral resources and the season (Lavie 1968). It is not known how colonies adjust the division of labour in response to an increased need for brood rearing. Possibilities include delayed behavioural development and a longer period of time devoted to brood care, as suggested by Pankiw *et al.* (1998), or increased brood-rearing activity by nurse bees without extending their brood-care phase. If one component of the colonial response to an increased need for brood rearing involves delayed worker behavioural development, this may be mediated by signals from the brood. We studied whether brood pheromone delays the rate of worker behavioural development and, if so, whether it also depresses JH titres.

2. MATERIAL AND METHODS

(a) *Bees*

Bees were from colonies maintained according to standard commercial bee-keeping techniques. Experiments 1 and 3 were performed in France with *Apis mellifera mellifera* bees and experiment 2 was performed in Illinois with bees that are considered to be a mixture of European races, predominantly *Apis mellifera ligustica* (Pellett 1938).

(b) *Experimental colonies*

Field experiments were performed with 'triple-cohort' colonies (Giray & Robinson 1994). Each triple-cohort colony was initially made up of three cohorts of bees ($n = 500$ per cohort): one-day-old adults, nurse bees and foragers. Nurses and foragers were behaviourally identified but their ages were not known. They were collected with a modified portable vacuum cleaner from a typical field ('source') colony headed by a naturally mated queen. One-day-old (0–18 h old) bees were obtained by removing honeycomb frames with maturing pupae from the same source colony and placing them in an incubator (34 °C). They were each marked with a spot of paint on the thorax and were the focal animals for our experiments.

Triple-cohort colonies each contained two honeycomb frames with honey, pollen and empty cells. Each colony was moved 10 km away from the source colony to prevent nurse bees and foragers from 'drifting' back into the source colony. A fourth cohort of one-day-old bees ($n = 500$) was added seven days after colony establishment to roughly approximate the condition in typical colonies, in which new bees continually emerge.

The population of a triple-cohort colony is smaller and better characterized than in typical colonies (*ca.* > 20 000 bees). We used triple-cohort colonies so as to be able to perform each replicate of an experiment with a set of colonies in which there was precise control of important variables that can affect behavioural development such as colony age demography, genotypic structure and food reserves (Giray & Robinson 1994; Huang & Robinson 1996; Schulz *et al.* 1998). Bees in triple-cohort colonies show normal rates of behavioural development (Giray & Robinson 1994), which was the behavioural variable we measured in this study.

(c) *Pheromone treatment*

Brood pheromone was made by mixing the ten previously identified components (Sigma Chemical Co., Saint Quentin Fallavier, France) in the proportions found on bees during the fourth and fifth days of larval development (Trouiller *et al.* 1992): methyl palmitate 5%, methyl oleate 18%, methyl stearate 8.5%, methyl linoleate 6%, methyl linolenate 10.5%, ethyl palmitate 7.5%, ethyl oleate 21%, ethyl stearate 11%, ethyl linoleate 2%, ethyl linolenate 10%.

Brood pheromone was administered daily in fresh sugar-candy. We thought that candy was necessary because it is not known whether brood pheromone alone is attractive to bees. This form of treatment allowed for chronic treatment with minimal disturbance but does raise the question of whether brood pheromone is transmitted naturally more by antennal contact than by oral ingestion. Bees heavily antennate their food when ingesting it, so it is not clear by which route activity might be achieved using this method. Fortunately, a previous study (Arnold *et al.* 1994) has shown that brood pheromone administered in this way is active for honeybees in a different behavioural and physiological context (Arnold *et al.* 1994). In addition, another honeybee primer pheromone, queen mandibular pheromone, is also active when used in this way.

In experiment 1, a field experiment, the low dose was 1 mg brood pheromone per gram sugar-candy, which corresponds to 620 larval equivalents (LEqu) per day. Assuming equal consumption by all bees in the colony, this resulted in an estimated dose of 0.41 LEqu per bee per day. The high dose (10 mg brood pheromone per gram candy) was 6200 LEqu, or 4.1 LEqu per bee per day. It is unlikely that this dose represents an unnaturally high exposure to brood pheromone; nurse bees repeatedly visit cells containing larvae (Robinson 1987) and each larva is fed about five times per hour (Huang & Otis 1991). Equal consumption was assumed because extensive food exchange takes place in a bee colony (Winston 1987).

In experiments 2 and 3 only the high dose was used. In experiment 2, which involved caged bees in the laboratory, bees were estimated to consume 5.2 LEqu per bee per day. In experiment 3, a field experiment, bees were estimated to consume 4.1 LEqu per bee per day. The 1 g of pheromone-containing sugar-candy was always absent on the following day, suggesting that it was completely consumed.

In experiments 1 and 3, colonies contained no brood and the queen was caged to prevent the confounding effects of natural brood. The queen cage was made of 'queen-excluder' material, which allowed workers to freely contact the queen.

(d) *Measurement of behavioural development*

Rates of behavioural development were measured by determining the age at onset of foraging for bees from the focal cohort (one day old at the time the experiment began). This was done by recording the age at first foraging for the first 50 bees from the focal cohort that initiated foraging. Daily observations of foraging behaviour started when the focal bees were seven days old, at least several days before any bees were expected to be seen as foragers (Giray & Robinson 1994). Each colony was observed for two 1 h periods per day, once in the morning (between 10.00 and 12.00) and once in the afternoon (between 15.00 and 17.00). Bees returning to the hive with a pollen load or distended abdomen were identified as foragers (Huang &

Robinson 1992). They were then collected and removed from the experiment, so that each bee was counted only once. Observations were performed blind with respect to treatment. Comparison of results from other studies (Robinson 1987; Robinson *et al.* 1989; Giray *et al.* 2000) indicates that sampling the first 50 foragers from a larger cohort provides an accurate reflection of the rate of behavioural development of the entire cohort.

(e) *Measurement of juvenile hormone titres*

JH titres were determined for individual bees. Blood (1.0–8.0 μg) was collected in a calibrated capillary tube from an incision in the penultimate abdominal intersegmental membrane, mixed with acetonitrile and stored at −20 °C. Samples were extracted with hexane. The radioimmunoassay uses a chiral-specific antibody to JH III, specifically validated for adult honeybees (Huang *et al.* 1994). All solvents were high-performance liquid chromatography grade, obtained from either EM Science (Haawthorne, NY, USA) or Fisher Scientific (Pittsburg, PA, USA). The sensitivity of the radioimmunoassay is about 5 pg R(−) JH III per sample and typical inter- and intra-assay variation is about 10% (Huang & Robinson 1996; Sullivan *et al.* 2000). For additional details see Huang *et al.* (1994).

(f) *Experiment 1: effect of brood pheromone on behavioural development*

Four triple-cohort colonies were made from a single source colony and treated as follows: one had a caged queen and a low dose of brood pheromone (BPL); one had a caged queen and a high dose of brood pheromone (BPH); one had a caged queen and no brood pheromone (BP−); and one had a freely laying queen (B+), which resulted in about 2000 larvae of different ages, or about 1.33 larvae per bee during the course of the experiment. Five trials were performed, each time with bees from a different, unrelated, source colony. In each trial, the four queens used (one per colony) were half-sisters. Data were analysed for all trials together by ANOVA (SAS (SAS Institute, Inc 1985), PROC GLM and planned contrasts based on the hypothesis that brood pheromone delays the rate of behavioural development).

(g) *Experiment 2: effect of brood pheromone on juvenile hormone titres in the laboratory*

Three groups of 100 one-day-old bees were established from the same source colony. The groups were maintained in Plexiglas cages (12 cm × 10 cm × 12 cm) (Pain 1966) in a darkened incubator at 34 °C with water, candy and pollen *ad libitum*. Two queenright (Q+) groups were given a high dose of brood pheromone (BPH) and no brood pheromone (BP−), respectively, and one queenless group (Q−) was not given brood pheromone (BP−). Queens again were half-sisters but they were not caged since there was no honeycomb in which to lay eggs. Bees ($n=10$) were taken at 1, 2, 3, 5, 7 and 21 days of age for hormone analyses. Four trials were performed, each with a different unrelated source colony. Statistical analyses were performed with Kruskal–Wallis and Mann–Whitney *U*-tests. Data were pooled for all trials because no differences were found between them.

(h) *Experiment 3: effect of brood pheromone on behavioural development and juvenile hormone titres in the field*

Experiment 1 was repeated with the following changes. We used just two treatments (BPH and BP−) and focal bees ($n=20$) were collected at 2, 5 and 15 days of age for JH analysis.

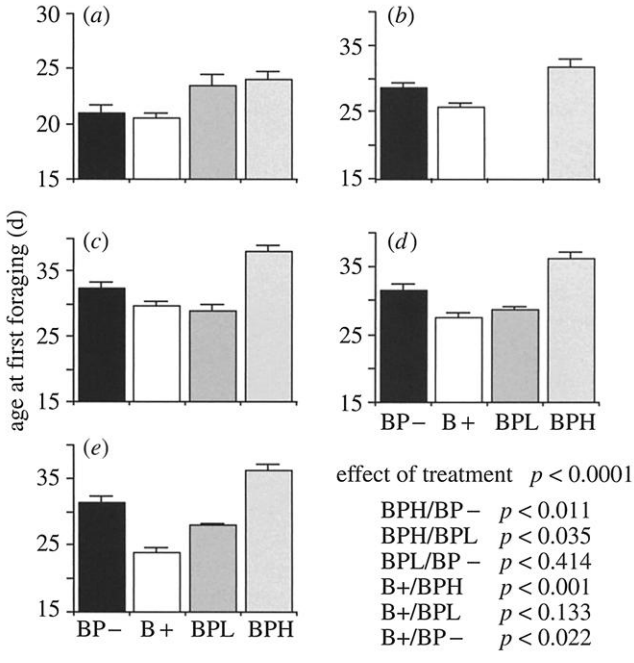

Figure 1. Effects of low (BPL) and high (BPH) doses of brood pheromone on honeybee behavioural development, measured as the mean (±s.e.m.) age at onset of foraging. (*a*) Trial 1, (*b*) trial 3, (*c*) trial 2, (*d*) trial 4 and (*e*) trial 5. Other abbreviations: BP−, control without pheromone; B+, control with real brood. Bars with different letters are significantly different ($p < 0.05$, planned contrasts, preceded by two-way ANOVA, $p < 0.001$; $n=50$ for each treatment group). Missing bar in (*b*) trial 3: this treatment group was not formed due to a shortage of bees at the time colonies were established.

Collections were made blind with respect to behaviour by opening hives early in the morning, when all bees were still in the hive. Three trials were performed, each with a different unrelated source colony. Data were analysed for each trial separately in order to better relate the behavioural and hormonal analyses to each other. We acknowledge that this is a less conservative way to analyse the data than in experiment 1, even though colonies within each trial are as similar as possible to each other.

3. RESULTS

(a) *Experiment 1: effect of brood pheromone on behavioural development*

ANOVA revealed significant overall treatment effects (figure 1). Comparing BP− and B+ colonies, the presence of brood resulted in a significantly earlier onset of foraging (figure 1). The low dose of brood pheromone (BPL) caused weak and inconsistent effects with no significant difference in age at first foraging relative to BP− colonies. The high dose of brood pheromone (BPH) exerted a stronger and more consistent effect, causing a significant age at onset of foraging relative to both BP− and B+ colonies.

(b) *Experiment 2: effect of brood pheromone on juvenile hormone titres in the laboratory*

JH titres varied significantly with worker age ($p < 0.0001$) and pheromone treatment ($p < 0.0001$)

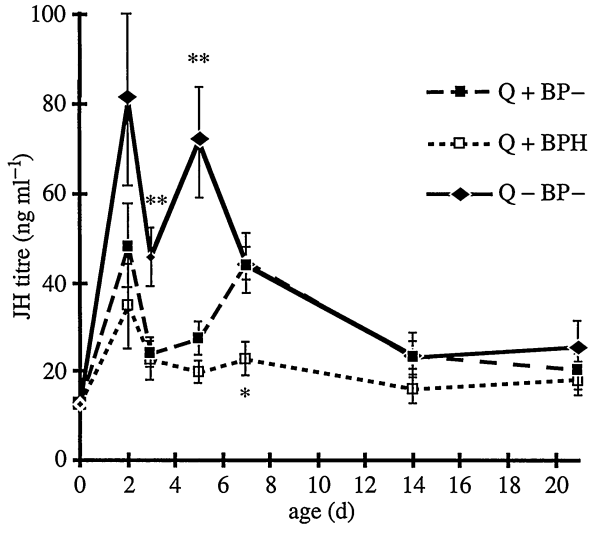

Figure 2. Effects of brood pheromone on mean (±s.e.m.) juvenile-hormone blood titres in adult worker honeybees in laboratory cages. Abbreviations: Q+, queenright bees; Q−, queenless bees. Results shown are grand means ($n = 10$ bees per data point per trial, four trials). $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the Q+ BP− group, Mann–Whitney *U*-tests (preceded by Kruskal–Wallis analysis as reported in §2(g)).

(figure 2). Bees in BPH groups tended to have the lowest JH titres; this effect was significant for seven-day-old bees (BPH versus BP−, $p < 0.02$). Bees in BPH groups had significantly lower JH titres than did bees in BP− groups on the third and fifth days ($p < 0.003$). The presence of a queen also had an inhibitory effect on JH (as in Kaatz *et al.* 1992; Pankiw *et al.* 1998).

(c) *Experiment 3: effect of brood pheromone on behavioural development and juvenile hormone titres in the field*

The inhibitory effects of a high dose of brood pheromone on the rate of behavioural development were confirmed (figure 3). Bees in BPH colonies had a significantly later age at onset of foraging than bees in BP− colonies in three out of three trials. Bees from the same BPH colonies also had significantly lower JH titres than their BP− counterparts on two out of three sampling dates in two out of three trials. The lack of an age-related increase in JH is not consistent with most previous studies (reviewed in Robinson & Vargo 1997) but has been observed before and is probably due to seasonal effects (Huang & Robinson 1996; Pankiw *et al.* 1998).

4. DISCUSSION

Our results demonstrate that honeybee brood pheromone acts as a primer pheromone in the regulation of honeybee behavioural development. To our knowledge, honeybee brood pheromone and honeybee queen mandibular pheromone are the only two chemically identified pheromones shown to act as both releasers and primers in any animal species. Because primer pheromones are notoriously difficult to identify (Slessor *et al.* 1998), we suggest that it may be fruitful to explore the possibility that other releaser pheromones, particularly those that are not highly volatile, also act as primer pheromones.

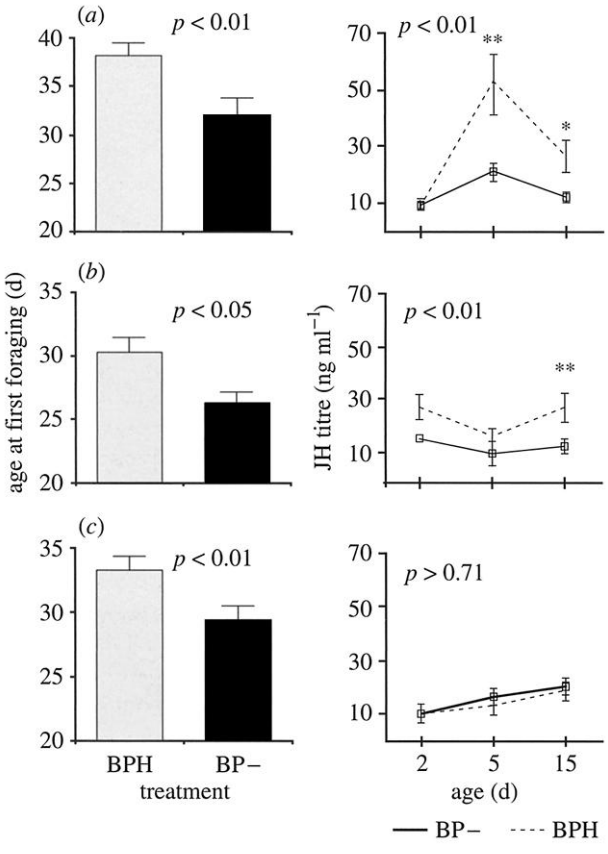

Figure 3. Effects of brood pheromone on (mean ± s.e.m.) behavioural development and juvenile hormone blood titres in adult worker honeybees in the field. (*a*) Trial 1, (*b*) trial 2 and (*c*) trial 3. Statistical analyses performed with Kruskal–Wallis and Mann–Whitney *U*-tests.

Alternatively, the varied releaser and primer functions of 'brood pheromone' may actually reflect distinct pheromones, composed of different combinations of the ten component esters. In the absence of this information, we chose to study the full ten-component blend, because previous studies have shown all ten components to have pheromonal activity (Le Conte *et al.* 1990, 1994, 1994/95, 1995). Detailed analyses are beginning to reveal that different components of brood pheromone may be more active in one social context than another (Mohammedi *et al.* 1996) but the other components are also active in these contexts (Le Conte *et al.* 1990, 1994, 1994/1995, 1995; Mohammedi *et al.* 1996, 1998).

The effects of brood pheromone varied with dose. The low dose caused weaker and less consistent effects that sometimes appeared to involve a slightly earlier age at onset of foraging. The effects of a high dose of brood pheromone were stronger and more consistent. The high dose was 4.1 LEqu per bee per day, which is unlikely to represent an unnaturally high exposure to brood pheromone because nurse bees repeatedly visit cells containing larvae (Robinson 1987; Huang & Otis 1991). We speculate that the delay in age at onset of foraging caused by exposure to the high dose reflects the fact that exposure to more larvae in a natural context would cause a pheromone-mediated delay in behavioural development, thus leading to a lengthening of the nursing phase. This speculation is based on the assumption that one component of

the colony's response to an increased need for brood rearing involves a lengthening of the nursing phase; this has not been determined.

It is clear that brood pheromone caused primer-type effects on behavioural development. However, the effects of brood pheromone on JH titres were not as strong. A high dose of brood pheromone depressed JH titres in both laboratory and field experiments, consistent with its inhibitory effect on behavioural development. These results agree with previous findings (Robinson 1992; Robinson & Vargo 1997; Sullivan *et al.* 2000) showing that JH acts to help pace behavioural development in honeybees. However, pheromone-mediated differences in JH were detected on only a few of the sampling days and overall effects on behaviour were stronger than those on JH. The weaker effects on JH titres do not undermine the claim of brood pheromone being a primer pheromone. The behavioural results alone demonstrate it to be a primer pheromone because treatment is followed by a slow change in behavioural probabilities rather than a rapid change in behaviour. What is uncertain is whether the primer effects of brood pheromone on honeybee behavioural development act via the JH system or via some other unidentified proximate system. Alternatively, perhaps transient effects of brood pheromone on the JH system are sufficient to influence behavioural development.

In this study, bees were exposed to brood pheromone on sugar-candy, for reasons outlined in § 2(c). This means that bees may have been exposed to brood pheromone either via antennal contact (performed extensively during feeding) or via ingestion, or both. Queen mandibular pheromone, even when applied without sugar-candy on glass slides, is both antennated and ingested (Naumann *et al.* 1991) suggesting that pheromone transfer occurs via both routes. In view of the fact that brood pheromone can modulate feeding behaviour (Le Conte *et al.* 1995), it would be interesting to determine whether similar results are obtained in both behavioural and endocrine analyses when bees are exposed to brood pheromone by antennal contact alone, if such a method could be devised. In addition, even though bees in triple-cohort colonies show normal rates of behavioural development (Giray & Robinson 1994), which was the behavioural variable we measured in this study, it would also be useful to determine the effects of brood pheromone on larger, more typical colonies of honeybees.

The number of social factors known to influence the rate of worker behavioural development in honeybee colonies is now three: worker–worker interactions, queen mandibular pheromone and brood pheromone. We believe that worker–worker inhibition plays a primary role in enabling colonies to reallocate labour in response to changing colony conditions because division of labour is extremely sensitive to changes in the age demography of the adult colony population (Robinson 1992; Huang & Robinson 1992, 1996). Changes in demography are more likely to affect a worker's pattern of interactions with other workers than a worker's exposure to queen mandibular pheromone or brood pheromone. Worker–worker interactions that influence the rate of behavioural development may also involve chemical communication (Huang *et al.* 1998). An important topic for future study is how worker–worker inhibition, queen mandibular pheromone and brood pheromone interact to regulate division of labour.

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