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Brood pheromone suppresses physiology of extreme longevity in honeybees (*Apis mellifera*)

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SUMMARY

Honeybee (*Apis mellifera*) society is characterized by a helper caste of essentially sterile female bees called workers. Workers show striking changes in lifespan that correlate with changes in colony demography. When rearing sibling sisters (brood), workers survive for 3–6 weeks. When brood rearing declines, worker lifespan is 20 weeks or longer. Insects can survive unfavorable periods on endogenous stores of protein and lipid. The glyco-lipoprotein vitellogenin extends worker bee lifespan by functioning in free radical defense, immunity and behavioral control. Workers use vitellogenin in brood food synthesis, and the metabolic cost of brood rearing (nurse load) may consume vitellogenin stores and reduce worker longevity. Yet, in addition to consuming resources, brood secretes a primer pheromone that affects worker physiology and behavior. Odors and odor perception can influence invertebrate longevity but it is unknown whether brood pheromone modulates vitellogenin stores and survival. We address this question with a 2-factorial experiment where 12 colonies are exposed to combinations of absence *vs* presence of brood and brood pheromone. Over an age-course of 24 days, we monitor the amount of vitellogenin stored in workers' fat body (adipose tissue). Thereafter, we track colony survival for 200 days. We demonstrate that brood rearing reduces worker vitellogenin stores and colony long-term survival. Yet also, we establish that the effects can result solely from exposure to brood pheromone. These findings indicate that molecular systems of extreme lifespan regulation are integrated with the sensory system of honeybees to respond to variation in a primer pheromone secreted from larvae.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/23/3795/DC1

Key words: vitellogenin, fat body, nurse load, lifespan regulation, colony survival.

INTRODUCTION

Feral honeybees live in a wide range of climates, from tropical Africa to the temperate zone of Northern Europe (Ruttner, 1988). A colony usually consists of one reproductive queen, some hundred drones (males) and many thousand sister workers that perform a variety of tasks, including cleaning, building comb, nursing brood and foraging for nectar and pollen (Winston, 1987). In temperate climates, the lifespan distribution of worker bees is strongly bimodal (Fukuda and Sekiguchi, 1966; Fluri and Imdorf, 1989). During favorable conditions in summer, young workers conduct tasks inside the nest such as nursing, and 2–3 weeks later they initiate foraging (Seeley, 1982). The majority of bees die within 1–2 weeks of their first foraging flight (Neukirch, 1982; Visscher and Dukas, 1997) with a resulting adult lifespan of 3–6 weeks for summer workers. However, when the favorable season ends, brood rearing and foraging ceases. Instead of dividing labor between nest tasks and foraging activities, the workers enter the *diutinus* 'winter bee' stage (Amdam and Omholt, 2002), and can survive for 20 weeks or longer (Maurizio, 1950).

The bimodal distribution of worker bee lifespan (with peaks around 3–6 weeks *vs* 20 weeks or longer) can be an adaptation to a strongly seasonal environment; during favorable conditions, summer bees sustain colony growth and reproduction by raising drones, sister workers and virgin queens and by taking part in swarming whereas *diutinus* workers allow the society to endure cold winter months when resources are limited (Omholt, 1988). Determining how adult bees can enter the *diutinus* stage is important, not only for understanding the bee's biology of survival but for improving our general understanding of aging processes (Muench et al., 2008). Honeybees have received increasing attention in aging research (Brandt et al., 2005; Remolina and Hughes, 2008) because some aspects of their lifespan regulation (Amdam and Omholt, 2002; Guidugli et al., 2005; Seehuus et al., 2006; Nelson et al., 2007) can differ from the molecular signaling systems of senescence studied, e.g. in *Caenorhabditis elegans* (reviewed by Kenyon, 2005) and *Drosophila melanogaster* (reviewed by Toivonen and Partridge, 2009). Many studies have addressed how *diutinus* bees develop. Cessation of flight activity, decreasing daylength and falling temperatures may play a role (Kefuss and Nye, 1970; Huang and Robinson, 1995) but the vast majority of research has focused on how reduced brood rearing influences worker physiology (Maurizio, 1950; Haydak, 1963; Free and Racey, 1968; Avitabile, 1978; Winston, 1980; Omholt, 1988).

The extended lifespan of *diutinus* bees correlates with an increased amount of stored lipids and proteins in their hemolymph (blood) and fat body [analogous to liver and adipose tissue (Koehler, 1921; Maurizio, 1954; Fluri et al., 1977; Shehata et al., 1981; Fluri et al., 1982)]. Fat body nutrient content correlates with survival capability in insects (Haunerland and Shirk, 1995; Hahn and Denlinger, 2007). Honeybee fat body also influences worker lifespan by acting as the synthesis organ of vitellogenin, a glyco-lipoprotein (Seehuus et al., 2006). Vitellogenin is a female-specific yolk

precursor in many oviparous species that has taken on new functions in honeybees. The protein is utilized in brood food production (Amdam et al., 2003) and in the regulation of foraging behavior. It can also extend worker lifespan independent of behavior (Nelson et al., 2007), possibly by scavenging free radicals (Seehuus et al., 2006) and enhancing innate immunity (Amdam et al., 2004b; Amdam et al., 2005a).

In the absence of brood, vitellogenin accumulates in the hemolymph of workers, and this accumulation characterizes *diutinus* bees (Amdam et al., 2004a; Amdam et al., 2005b). We hypothesized that the *diutinus* bees' physiology of extreme longevity could develop because less vitellogenin was lost to the production of brood food (Amdam and Omholt, 2002; Amdam et al., 2009). This proposition builds on previous work by Omholt (Omholt, 1988), who suggested that the nurse load of young honeybee workers affects longevity: a low nurse load can increase lifespan whereas a high nurse load has the opposite effect. In addition to consuming colony resources, however, larval brood secrete a primer brood pheromone, a blend of 10 fatty acid methyl and ethyl esters (Le Conte et al., 1994) produced by the salivary glands (Le Conte et al., 2006). This pheromone affects worker brain gene expression (Alaux et al., 2009), gland physiology (Pankiw et al., 2008) and behavior (Pankiw, 2004). Odors and odor perception influence longevity in *C. elegans* (Alcedo and Kenyon, 2004) and *D. melanogaster* (Libert et al., 2007) but the effect of brood pheromone on worker bee survival is unknown.

As brood rearing and nurse load decline in colonies toward the end of summer, so does the amount of brood pheromone. Previous work that explains *diutinus* bee development as a function of brood rearing does not fully account for this fact. To resolve how the amount of brood in honeybee colonies can affect worker lifespan, it is necessary to decouple the effects of nurse load and brood pheromone. To achieve this, we used a 2-factorial design that took into account that workers are exposed to nurse load and pheromone from larvae when brood is present, while they encounter neither when brood is absent. Workers were exposed to brood pheromone in the absence of brood by using an established synthetic pheromone blend that consists of the same mixture of 10 fatty acid methyl and ethyl esters as is secreted by fourth–fifth instar larvae (Le Conte et al., 2001). We measured stored vitellogenin in biopsies from fat body of 3–4-, 7–8- and 23–24-day-old workers, and we monitored the long-term survival of colonies. Our data confirm that brood rearing reduces the amount of stored vitellogenin in fat body and the long-term survival of colonies. Yet, we also demonstrate that brood pheromone interacts with brood rearing and worker age to influence vitellogenin, and that the decline in vitellogenin content and colony survivorship is achieved by exposure to brood pheromone alone. These results establish a new understanding of how brood rearing regulates honeybee lifespan.

MATERIALS AND METHODS

Our factorial experiment was composed of four treatments that corresponded to all possible combinations of presence *vs* absence of larval brood (factor='brood') and synthetic brood pheromone (factor='BP'). We replicated the design three times at colony level, using 12 honeybee colonies in total (Fig. 1).

Honeybees

In August 2007, 12 colonies with about 3000 worker bees each (*Apis mellifera carnica* L.) were prepared in six 2-compartment hive bodies at the apiary of the University of Life Sciences, Aas, Norway. Every hive box was divided in the middle by an excluder wall (wood) to form the two equally sized compartments that each accommodated one colony (Fig. 1). The colonies were prepared as described before (Amdam et al., 2004a). Briefly, to ensure that the distribution of queen pheromone (central to colony integrity) (Winston, 1987) and brood could be carefully controlled during the experiment, each colony was given one queen that was caged on a wax comb in the center of the nest. The 12 queens were sisters, freely mated at the same certified *A. m. carnica* mating station. By caging, the queens' motility and egg-laying behavior were constrained so that colonies would not produce their own brood. In parallel, all colonies received synthetic queen pheromone for the duration of the experiment (following the manufacturer's instructions, BeeBoost, Pherotech International, Delta, BC, Canada) (Amdam et al., 2004a).

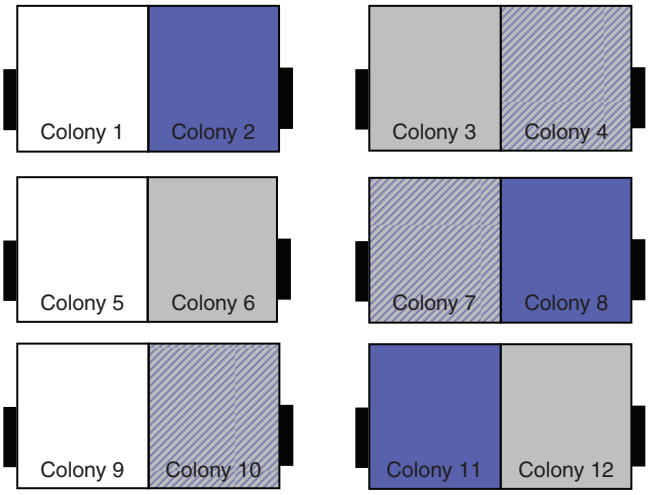

Fig. 1. The 2-factorial design that represented all possible combinations of the presence *vs* absence of larval brood ('brood') and synthetic brood pheromone blend ('BP'), resulting in four experimental treatments. Each treatment combination was applied to an entire honeybee colony, and the design was replicated three times. The resulting 12 colonies were maintained in 2-compartment hive boxes. Treatments were quasi-randomized between boxes so the same set of treatments never co-occurred more than once between colonies in a single hive box, as indicated by color codes: white='brood' and 'BP' absent; blue='brood' present, 'BP' absent; gray='brood' absent, 'BP' present; blue/gray upward diagonal='brood' and 'BP' present. Black bars on either side of each hive unit indicate the entrances of the colonies.

Three colonies were assigned to each of the four treatments from the 2-factorial experimental design (Fig. 1). Overall, the four factorial treatments were quasi-randomized between hive boxes, so that the same set of treatments never co-occurred more than once between two colonies in a single hive body (Fig. 1). Presence of brood was achieved by adding two combs with eggs and young larvae to colonies every fifth day of the experiment (egg-to-larval development is three days, while larval-to-pupal development is six days). Each set of brood comb replaced the previous set, which ensured the continuous presence of open brood and prevented brood from emerging as adults in the colonies. Through the duration of the experiment, we made an effort to match the relative treatment amount of larval brood to the relative treatment amount of synthetic brood pheromone (below) by using a similar number of larval equivalents (LEqu) per colony (exposure to about 1000 larvae daily). Colonies assigned absence of brood treatment received sham handling. The presence of synthetic pheromone blend was achieved by treating colonies with an established mix of 10 fatty acid methyl

and ethyl esters (Le Conte et al., 1994). For application, small glass dispensers were filled with 250 ml sugar syrup (Bifor®, Danisco, Denmark) and the brood pheromone was mixed in by careful stirring. For honeybee colonies, experimental delivery of pheromones is routinely done by feeding (e.g. Le Conte et al., 2001; Leoncini et al., 2004). All colonies received one dispenser of syrup freshly mixed each day at 10:00 h. Colonies assigned absence of brood pheromone treatment received dispensers with sugar syrup only.

As soon as this experimental setup was established, each colony received 700 newly emerged (<24 h old) adult worker bees (*A. m. carnica*) that were marked with paint on the thorax to convey host colony identity. Each group of 700 bees consisted of an equal mix of workers from five different colony sources. This mixture ensured an equal and broad genotypic distribution of marked bees among all the colonies of the experiment. The hives were monitored daily to ensure that the caged queens were alive, that the treatment scheme was applied consistently and that colonies were similar in strength (number of workers) and remained healthy (without symptoms of known honeybee pathogens or disease).

Bees were sampled until day 24 of the experiment and the four factorial treatments were continued for two more weeks. Thereafter, treatments were discontinued by releasing the queens from the cages, and by removing brood and brood pheromone blend. The 12 colonies were wintered in a controlled environment room at 0°C and 50–60% RH (relative humidity). Over the next three weeks, colonies were observed every fifth day to verify that worker mortality declined, a pattern consistent with successful overwintering (Fluri and Imdorf, 1989; Mattila et al., 2001). This basic validation was facilitated by replacing the standard bottom-boards of the hives with a custom-made set that had built-in drawers. As workers died, they fell from the colony cluster into the drawers, and could be counted. After the three weeks, we inspected the colonies one final time to confirm that the queens were alive and that all hive units were healthy (see above). The colonies were scored as alive or dead after 200 days.

Synthetic brood pheromone blend

This blend was as described before (Le Conte et al., 1994), i.e. 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% and ethyl linolenate 10% (Fluka, Buchs, Switzerland). Exposure to brood pheromone was estimated in LEqu according to Le Conte et al. (Le Conte et al., 1994). Each treated colony of about 3700 bees received 1000 LEqu daily, corresponding to about 1/4–1/3 LEqu bee^{-1} day^{-1}. After the blend was prepared, it was aliquoted into microcentrifuge tubes and stored at –20°C until applied (described above).

Sampling of worker bees and tissue fixation

Starting on day 3 and ending on day 24 of the experiment, marked worker bees were sampled from all colonies. Bees were taken quickly from the apiary to the laboratory and dissected on ice. The sting apparatus and gut were removed before the abdomen was stored in PP-test tubes (Greiner Bio-One, Monroe, NC, USA), fixed in formaldehyde and embedded in London Resin White (Electron Microscopy Science), as described before (Seehuus et al., 2007), to provide resin blocks with embedded abdominal tissue ready for sectioning.

Immunofluorescence

Semi-thin sections (1–2 μm) of resin-embedded material were cut with a diamond knife using a Reichert Jung ultra-microtome (Ontario, Canada). For each of the four treatment groups of the factorial design, five independent sections (biopsies) were collected from the fat bodies of each of 15 individuals, which were selected randomly from the three replicate colonies that received each treatment (summing to a total of 60 bees). Biopsies were from ages 3–4, 7–8 and 23–24 days. The five separate biopsies from each of the 60 workers were processed independently during five separate and unique replicate rounds of immunostaining to ensure that inference from the material overall was not confounded by the potentially great intra-individual heterogeneity of insect fat body tissue (Jensen and Borgesen, 2000) and by potential errors linked to technical replication in immunofluorescence analysis.

For each staining round, tissue sections were dried onto SuperFrost®Plus slides, rinsed with PBS-T (phosphate buffer salt-solution pH=7.2, with 0.02% Triton X100) 3×5 min, washed 3×5 min with PBS-T and blocked with 2% BSA (Sigma-Aldrich, Steinheim, Germany) in PBS-T for 60 min at room temperature. After 3×15 min washes with PBS-T, sections were incubated overnight at 4°C with a polyclonal (rabbit) anti-vitellogenin antibody at 1:500 (raised against 180 kDa honeybee vitellogenin, Pacific Immunology, Ramona, CA, USA); specificity was tested and confirmed previously (Seehuus et al., 2007). The negative control was incubated with PBS-T, as verified before (Seehuus et al., 2007). After 3×5 min washes in PBS-T, the samples were incubated with a polyclonal anti-rabbit antibody coupled to the fluorochrome Cy3 (AffiniPure Goat Anti-Rabbit IgG, Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK), dilution 1:200, overnight at 4°C. For negative control staining, sections were incubated with the secondary antibody only (Seehuus et al., 2007). Finally, samples were washed 3×5 min in PBS-T and subsequently mounted in 50% glycerol/PBS. Images were acquired with a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany). In total, this design of sample processing resulted in 300 images.

Semi-quantitative scoring of fat body vitellogenin storage and statistic analysis

A scoring key was developed by choosing images that represented incremental differences in signal intensity. Increments were discerned as ascending from the least to the most intense positive staining among the 300 images. This approach identified seven images that were assigned a relative gray-scale intensity from 1 (least intense image) to 7 (most intense image). The resulting scoring key (supplementary material Fig. S1) was evaluated and approved by two additional subjects that did not take part in the subsequent scoring of the sample material. Four independent observers without prior expectations of image intensities were asked to score the intensity of the remaining 293 images on a scale from 1 to 7 based on the scoring key. Observers were blind to the treatment identity of the images. After scoring, one consensus value was calculated for each image as the mean of the four independent scores that it had received.

A factorial analysis of variance (ANOVA) model was used to analyze the resulting dataset of 300 image intensities. ‘brood’, ‘BP’ (each with two levels) and ‘age’ (three levels) were coded as fixed main effects. ‘Staining round’ (five replicate staining series on biopsies from each of 60 workers) was coded as a random factor. The dataset adhered to the assumptions of ANOVA, after the criteria of normality (estimated by a normal probability plot of residuals from the analysis) and homogeneity of variances (determined by Levene’s test). *Post-hoc* comparisons were made with Fisher’s LSD test.

The data on colony long-term survival were analyzed with factorial ANOVA using a one-sided test criterion (see Results).

'Brood' and 'BP' were fixed main effects and the dependent variable, survivorship, was coded as 0 (dead) or 1 (alive). ANOVA is not an optimal model for such categorical outcome variables but the approach can accommodate factorial designs and is widely in use (Jaeger, 2008). One colony was excluded because it did not winter (see above) (Fluri and Imdorf, 1989; Mattila et al., 2001). All statistics used Statistica 6.0 (StatSoft, Tulsa, OK, USA).

RESULTS

Vitellogenin stored in individual fat bodies

Stored vitellogenin protein was detected as immunostained granules (Seehuus et al., 2007) in anterior tissue-sections (biopsies) of workers' abdominal fat body (Fig. 2A–C). We found that the factors 'brood' and 'age' significantly influenced the mean amount of vitellogenin stored in the fat body (F=19.75, P<0.0001 and F=77.33, P<0.0001, respectively; d.f.=238). The presence of brood in colonies reduced the amount of vitellogenin detected in biopsies from 7–8- and 23–24-day-old bees (Fisher's LSD test, P<0.001), while increasing worker age led to a greater amount of stored protein: fat body tissue from 23–24-day-old workers contained more vitellogenin on average than fat body tissue collected from younger bees (Fisher's LSD test, P<0.0005) (Fig. 2D–F). This time-course of vitellogenin increase is in line with previous data on how the hemolymph vitellogenin titer can develop with worker age (Amdam et al., 2004a; Amdam et al., 2005b), particularly during autumn (Fluri et al., 1982).

As an independent factor in ANOVA, 'BP' did not explain variation in vitellogenin storage (F=1.42, P=0.23). Vitellogenin accumulation, however, was significantly affected by 'BP' in interaction with 'brood' (F=13.73, P<0.0005) and with 'age' (F=7.52, P<0.001), implying that 'BP's influence on physiology was conditional on the social environment (i.e. if brood was present or not) and worker ontogeny. Explicitly, in fat body from 3–4-day-old workers, exposure to brood pheromone tended to increase the amount of vitellogenin (Fisher's LSD test, P<0.05). By contrast, the presence of brood pheromone strongly reduced stored vitellogenin in biopsies taken from 23–24-day-old bees (Fisher's LSD test, P<0.0001). This level of suppression was indistinguishable from the negative influence brood rearing had on the amount of granules with positive immunostaining in biopsies from the same age group (Fisher's LSD test, P=0.11). In fact, as long as 23–24-day-old workers had been exposed to brood pheromone, the mean amount of vitellogenin stored in their fat bodies was the same regardless of whether larval brood was present or absent in colonies (Fisher's LSD test, P=0.88) (Fig. 2D–F). In line with this many-sided interplay between treatment factors and worker age, the dataset confirmed a significant three-factor interaction between 'brood', 'BP' and 'age' (F=7.55, P<0.001).

Summing the data, our experimental design successfully decoupled effects of nurse load and brood pheromone as the fat body's physiology developed with worker age. The experiment showed that a repression of stored vitellogenin occurs when brood

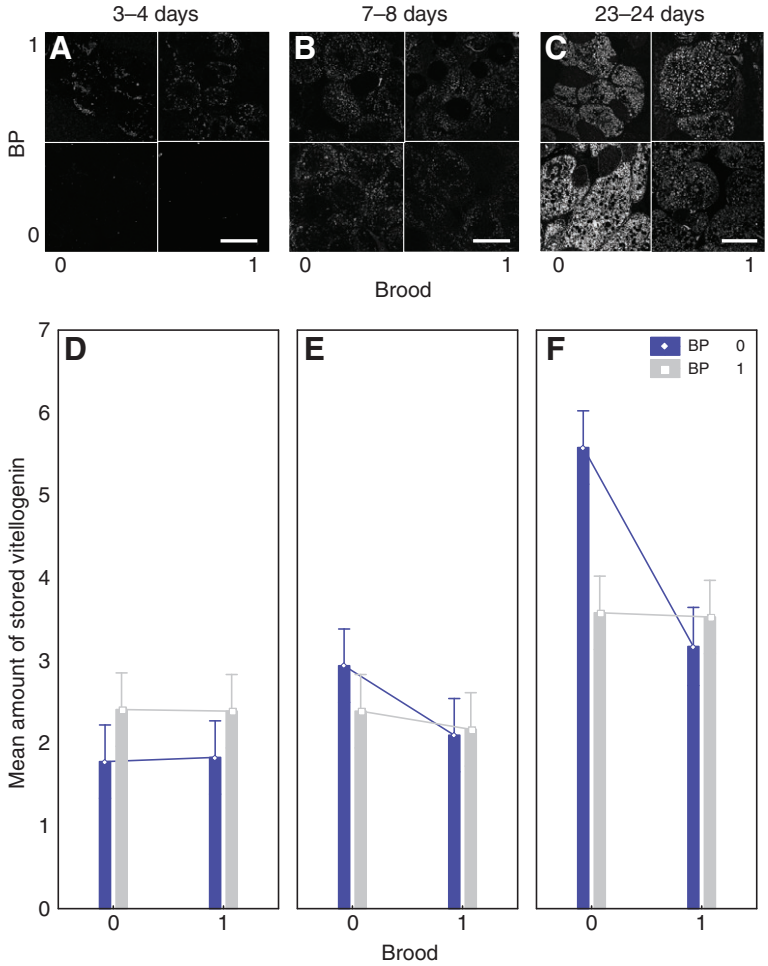

Fig. 2. Semi-quantitative levels of vitellogenin stored in fat body (adipose tissue). The amount of vitellogenin was scored in 300 sections (biopsies) taken from the anterior abdominal fat bodies of 60 worker honeybees. (A–C) Image micrographs of fat body cells immunostained for vitellogenin (white), as obtained from 3–4-, 7–8- and 23–24-day-old bees. Each image represents the mean score given to its assigned treatment group. The four treatment groups of the experiment are identified by the factorial combinations of '0' and '1' indicators below and to the left of each panel. Zero represents the absence of brood or synthetic brood pheromone blend, and 1 indicates presence. Scale bar=50 μm. (D–F). Factorial bar graphs (d.f.=238) of corresponding mean + 95% confidence interval, showing the semi-quantitative amount of vitellogenin stored in the sample-groups exemplified by panels A–C. For each time-point (3–4, 7–8 and 23–24 days), panels D–F show the relative quantities of stored vitellogenin achieved in the absence *vs* presence of brood (assigned as 0 and 1 on the *x*-axes), and the absence and presence of synthetic pheromone blend (assigned as blue *vs* gray bars, respectively). Trend lines connect corresponding means.

is present, which represents a combined influence of nurse load and brood pheromone. Yet, the experiment also revealed that a similar repression is achieved with brood pheromone alone. The greatest mean amount of stored vitellogenin, accordingly, characterized the fat bodies of 23–24-day-old workers from colonies treated with neither brood nor brood pheromone (Fig. 2F), conditions that replicate the colony setting where *diutinus* bees develop (Maurizio, 1950; Fluri and Imdorf, 1989).

Long-term survival of colonies

The 12 colonies were wintered in a controlled environment room and scored as alive or dead after 200 days. Two hundred days is an extended but not uncommon duration of *diutinus* bee lifespan (reviewed by Amdam and Omholt, 2002). From our knowledge on vitellogenin stored in fat bodies (see above), we expected *a priori* that pre-wintering exposure to brood or brood pheromone could only reduce the survival probability of colonies. Therefore, the dataset was analyzed with factorial ANOVA using a one-sided test criterion, which assumes that the null hypothesis is rejected only by outcomes located entirely at one end of the probability distribution (Montgomery, 1997). We found that 'brood' (F=3.81, P<0.05) and 'BP' (F=3.81, P<0.05) influenced colony survival (d.f.=8). As predicted, the ability to survive 200 days was reduced after pre-wintering exposure to brood or brood pheromone (Fig. 3). Colonies treated with both factors, furthermore, were significantly less likely to survive compared with colonies exposed to neither brood nor synthetic brood pheromone blend (Fisher's LSD test, P<0.03). This latter group also tended to outlive colonies that were exposed to either brood or synthetic pheromone before overwintering (Fisher's LSD test, P=0.05). The survival probability of the latter two treatment groups (either brood or synthetic blend) was identical (Fisher's LSD test, P=0.22).

DISCUSSION

Our data provide new insights into mechanisms that allow worker bees to develop extreme longevity potential. We show that changes in storage protein physiology, which can affect worker survival directly (Koehler, 1921; Maurizio, 1950; Maurizio, 1954; Haunerland, 1996; Seehuus et al., 2006), are influenced by a primer pheromone secreted by larval brood. This finding calls for a revised understanding of how *diutinus* bees develop and of how successful overwintering is achieved by honeybee colonies.

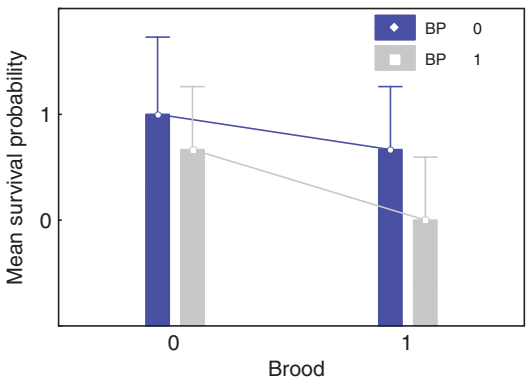

Fig. 3. Factorial bar graphs (d.f.=8) for colony long-term survival. After 200 days, colony survivorship was scored as 0 (dead) or 1 (alive). The resulting mean + 95% confidence interval for colony survival probability is graphed for each treatment group. The absence *vs* presence of brood is assigned on the *x*-axis as 0 and 1, respectively. The absence and presence of synthetic pheromone blend is each indicated by blue *vs* gray bars, respectively.

Exposure to synthetic brood pheromone blend tended to increase the amount of vitellogenin in the fat bodies from young 3–4-day-old bees. This result may be understood in light of a recent study by Pankiw and coworkers (Pankiw et al., 2008), who showed that brood-rearing colonies treated with synthetic brood pheromone blend consumed more pollen substitute. Pollen is the major protein source of honeybees (Winston, 1987). In parallel with the increased consumption of pollen substitute by colonies, Pankiw and coworkers found that the hypopharyngeal head glands of nest workers had a higher protein content. Hypopharyngeal glands are active in brood food synthesis (reviewed by Crailsheim, 1990). Pankiw et al. (Pankiw et al., 2008) hypothesized that brood pheromone stimulates young workers to consume more pollen to ensure a brood-food-production-capacity that is ramped up to balance the perceived demand. Increased consumption of pollen by young bees also correlates with increased hemolymph levels of vitellogenin (Bitondi and Simões, 1996). Thus, we propose that the greater amount of vitellogenin granules seen after 3–4 days of synthetic brood pheromone treatment (Fig. 2A,D) results from increased production of vitellogenin enabled by a higher consumption of pollen.

In 23–24-day-old workers, our treatment scheme revealed a significant interaction between factors 'brood' and 'BP' on the amount of vitellogenin stored in fat body. Brood suppressed vitellogenin stores in the absence of synthetic pheromone blend, and synthetic pheromone blend suppressed vitellogenin stores in the absence of brood; yet, there was no further reduction (no additive effect) seen in fat bodies from workers that were exposed to both factors (Fig. 2C,F). The lack of an additive effect of brood and synthetic pheromone blend was apparent also in biopsies from 3–4-day-olds (no further increase in storage in the presence of both factors) (Fig. 2D) and 7–8-day day-olds (no further decrease in the presence of both factors) (Fig. 2E). After the 23–24 days, thus, the same mean vitellogenin stores were achieved regardless of whether worker bees were exposed to brood treatment, brood pheromone or both. How is this pattern explained? The brood treatment of our experiment consisted of larvae that secreted brood pheromone. A primer pheromone treatment constituent, therefore, was shared between 'brood' and 'BP'. Because exposure to either factor had the same physiological outcome after 23–24 days, we hypothesize that brood pheromone is the causal element that suppresses further vitellogenin storage as workers grow older. The lack of an additive effect in the presence of both factors, regardless of worker age, could be due to sensory adaptation (Wark et al., 2007) in the insects' dose-response to pheromone (Marion-Poll and Tobin, 1992).

The positive effect of synthetic brood pheromone on vitellogenin stores in 3–4-day-old bees and the negative effect in later life (as suggested in 7–8-day-olds and significant after 23–24 days) (Fig. 2E,F) indicate that brood pheromone has a dual function. Brood pheromone may increase pollen consumption in workers (Pankiw et al., 2008), enhancing their capacity to produce brood food and to store a surplus from vitellogenin synthesis. But, the pheromone may also inhibit workers from developing a physiology of extensive vitellogenin storage, ensuring that more vitellogenin remains free in hemolymph (Amdam and Omholt, 2002) and ready to be converted into brood food (Amdam et al., 2003). This effect on the stored amount of protein would impact the young workers (our 3–4-day-olds) less than the more mature worker bees (our 7–8- and 23–24-day-olds), because the cumulative amount of vitellogenin that potentially can be stored

increases with time (Amdam and Omholt, 2002). These putative functions of the brood pheromone are in line with recent data from Fischer and Grozinger, who proposed that honeybee queen mandibular primer pheromone can modify nutrient storage pathways in worker fat body (Fischer and Grozinger, 2008).

We found that pre-wintering exposure to brood and brood pheromone decreased the long-term survival probability of the experimental colonies (Fig. 3). In contrast to the data from tissues, the colony-level results point to an additive effect of the treatment scheme, in that colonies kept in the presence of both brood and synthetic pheromone blend did less well in our survival test (all died before the completion of the experiment). The negative influence of brood rearing was indistinguishable from the negative effect of synthetic brood pheromone blend. This outcome supports our hypothesis that worker longevity potential, which translates into colony-level survival capability (Amdam and Omholt, 2002), is influenced by primer brood pheromone rather than the nurse load placed on workers. It has already been proposed that brood rearing shortens worker life and can lead to colony deaths in winter (Eischen et al., 1984; Omholt, 1988; Fluri and Imdorf, 1989; Amdam and Omholt, 2002) but the cause–effect relationship was previously explained by the metabolic costs of caregiving (Amdam et al., 2009).

Our colony-level experiment revealed an additive effect of the treatment combinations on survival but a similar summation was absent for the vitellogenin storage physiology of the fat body. How does this pattern emerge? As noted already, the treatment scheme was extended for two weeks after we collected the final set of histological samples (see Materials and methods). Furthermore, our study focused on a single predictor of survival, fat body vitellogenin stores, while other physiological variables that influence worker survival might also have been affected by the treatments. These factors include the amount of stored lipid and carbohydrate (Koehler, 1921; Shehata et al., 1981; Toth and Robinson, 2005). Thus, the work presented here does not exclude that our factorial treatment scheme had additive effects on worker physiology. We speculate that we observed the colony-level summation of these latent and many-sided traits of workers indirectly – through the outcome of our survival test.

Taken together, our data show for the first time that exposure to brood pheromone is sufficient to explain variation in fat body vitellogenin stores that may predict age at death in honeybee workers. This effect of pheromone alone is supported by data on the long-term survival of colonies. Our results revise the current knowledge base on how *diutinus* workers develop in temperate climates, and therefore, through our work, survival patterns of honeybees can be better understood. Adding to this, our findings provide evidence for how a primer pheromone from young siblings can act on older sisters to shorten lifespan. This relationship should be of interest to research centered on the interface between sensory systems and aging regulation (Alcedo and Kenyon, 2004; Libert et al., 2007), which currently is poorly understood.

ABBREVIATIONS

BP	brood pheromone (factorial treatment factor)
LEqu	larval equivalents
LSD	least significant difference

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