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RESEARCH ARTICLE

Social regulation of maternal traits in nest-founding bumble bee (*Bombus terrestris*) queens

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SUMMARY

During the nest-founding phase of the bumble bee colony cycle, queens undergo striking changes in maternal care behavior. Early in the founding phase, prior to the emergence of workers in the nest, queens are reproductive and also provision and feed their offspring. However, later in the founding phase, queens reduce their feeding of larvae and become specialized on reproduction. This transition is synchronized with the emergence of workers in the colony, who assume the task of feeding their siblings. Using a social manipulation experiment with the bumble bee *Bombus terrestris*, we tested the hypothesis that workers regulate the transition from feeding brood to specialization on reproduction in nest-founding bumble bee queens. Consistent with this hypothesis, we found that early-stage nest-founding queens with workers prematurely added to their nests reduce their brood-feeding behavior and increase egg laying, and likewise, late-stage nest-founding queens increase their brood-feeding behavior and decrease egg-laying when workers are removed from their nests. Further, brood-feeding and egg-laying behaviors were negatively correlated. We used Agilent microarrays designed from *B. terrestris* brain expressed sequenced tags (ESTs) to explore a second hypothesis, that workers alter brain gene expression in nest-founding queens. We found evidence that brain gene expression in nest-founding queens is altered by the presence of workers, with the effect being much stronger in late-stage founding queens. This study provides new insights into how the transition from feeding brood to specialization on reproduction in queen bumble bees is regulated during the nest initiation phase of the colony cycle.

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INTRODUCTION

A common feature of animal societies is social regulation, where individuals regulate the behavior and physiology of other group members (Wilson, 2000). Social regulation serves to promote cohesion between society members, for example it organizes reproductive efforts in cichlid fish (White et al., 2002) and meerkats (O'Riain et al., 2000), and reduces conflict (Ratnieks et al., 2006) and coordinates the labor force (Robinson, 1992) in insect societies.

The eusocial bumble bees (genus *Bombus*, family Apidae) possess an annual colony cycle wherein each spring, queens emerge from overwintering and initiate new nests (Free and Butler, 1959). As the season progresses, workers emerge in the nest and perform work-related tasks, with the queen performing most reproduction. Various forms of social regulation have been demonstrated in bumble bees at different stages in the colony cycle, including the regulation of circadian rhythmicity in queens by the brood early in colony development (Eban-Rothschild et al., 2011) and after worker emergence, the pheromonal regulation of worker foraging activity (Dornhaus et al., 2003) and the inhibition of worker reproduction by both queens (Alaux et al., 2004) and workers (Bloch, 1999).

For bumble bee queens, one of the most dramatic behavioral and physiological changes occurs during the nest founding-phase of the colony cycle. Early in the nest-founding phase, prior to the emergence of workers in the nest, queens are reproductive but also do all work-related tasks in the nest, such as foraging and progressively feeding their larval offspring. Later in the founding phase, following the emergence of workers, queens significantly decrease their brood-feeding activity and become largely specialized on reproduction (Free and Butler, 1959), as workers take over most brood-feeding activity in the colony. Exploring the changes in maternal care that occur over the bumble bee queen life cycle may provide insights into how maternal traits have changed in the social insects over an evolutionary time scale. These extreme changes in bumble bee queens also make them an excellent system for studying the social regulation and plasticity of behavior and physiology in social insects.

We performed a social manipulation experiment to test the hypothesis that there is social (worker) regulation of the transition from mainly brood feeding to specialization on reproduction in nest-founding bumble bee queens. This hypothesis was based on two previous findings in bumble bees: (1) the transition coincides with the emergence of workers in the colony (Free and Butler, 1959); and (2) workers stimulate egg-laying behavior in nest-founding queens (Gretenkord and Drescher, 1997; Gurel and Gosterit, 2008;

Kwon et al., 2006). Based on this hypothesis, we predicted that artificial addition and removal of workers from recently founded nests (Fig. 1) would alter both maternal care and reproductive behaviors in nest-founding queens. More specifically, we predicted that (1) addition of workers to young nests in which workers had not yet emerged ('early-stage' nests) would cause a decrease in brood-feeding behavior and an increase in egg-laying behavior in queens; and (2) removal of all workers from nests in which workers were beginning to emerge ('late-stage' nests) would cause an increase in brood-feeding behavior and a decrease in egg-laying behavior in queens.

We also explored the hypothesis that changes in brain gene expression are associated with the transition away from brood feeding in nest-founding queens. In many cases of social regulation, social signals, which are initially received and processed by sensory and neural systems, ultimately lead to behavioral changes *via* changes in brain gene expression (Robinson et al., 2008). To test this hypothesis, we studied patterns of brain gene expression in early- and late-stage founding queens with and without workers (Fig. 1) using an Agilent microarray made specifically for the bumble bee used in this study, *Bombus terrestris*, which was based on a large-scale brain transcriptomic project (Woodard et al., 2011). Based on previous studies on social regulation in bees (Alaux et al., 2009a; Alaux et al., 2009b; Grozinger et al., 2003), which have shown major effects of the social environment on brain gene expression, we predicted that a large number of genes would be differentially expressed in the brains of founding queens with *versus* without workers. Such genes may represent components of the transcriptional architecture underlying social regulation of maternal care and reproduction in bumble bee queens. Understanding the ways in which social signals are transduced *via* transcriptional mechanisms is important because these mechanisms can provide important insights into how sociality evolved at the molecular level (Robinson et al., 2005).

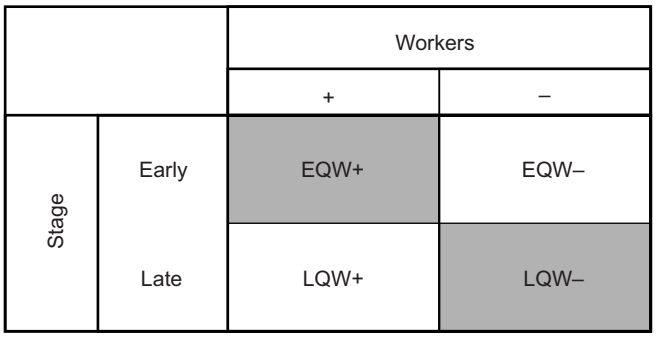

Fig. 1. Factorial design of the experiment. For the 'Workers' axis, each group of queens either did (+) or did not (–) have workers present in the nest. For the 'Stage' axis, each group of queens was either in the earliest stages of the nest-founding period, in which workers had not yet begun to emerge in the nest ('Early') or in the later stages of the founding phase, when workers had begun to emerge ('Late'). EQW+, early-stage queens with workers; EQW–, early-stage queens without workers; LQW–, late-stage queens without workers; LQW+, late-stage queens with workers.

MATERIALS AND METHODS

Bees

Bombus terrestris (Linnaeus 1758) is a well-studied, temperate bumble bee that is commercially managed for pollination services (Velthuis and Van Doorn, 2006). Bees were purchased from Polyam Industries (Kibbutz Yad-Mordechai, Israel) and all experiments were performed in the Bee Research Facility in Givat Ram, the Hebrew University of Jerusalem, Jerusalem, Israel. Forty-seven recently initiated (i.e. eggs present but no larvae) nests were brought into the laboratory and kept under conditions described elsewhere (Geva et al., 2005; Yerushalmi et al., 2006). To synchronize the treatment groups, 22 of the 47 nests were brought to the laboratory and allowed to develop until 2 days after the first workers emerged (~20 days prior to day 1 of the experiment); queens in these nests were 'late-stage' founding queens (LQ). Fourteen days later, 25 additional nests were brought into the laboratory and allowed to develop until larvae were present (~5 days prior to day 1 of the experiment) but no workers had yet emerged; queens in these nests were 'early-stage' founding queens (EQ).

Social manipulation

For 12 of the 25 EQ nests, callow workers (<24 h old, identified by the lack of yellow pigmentation) were added to simulate the premature emergence of workers ('EQW+' group). For the remaining 13 EQ nests, workers were not added and thus these nests were without workers for the duration of the experiment ('EQW–' group). For 11 of the 22 LQ nests, any workers that emerged were removed <24 h after their emergence ('LQW–' group). For the other 11 LQ nests, any workers that emerged were removed <24 h post-emergence and replaced with callow workers as a sham control ('LQW+' group). To maintain the same number of workers in all '+W' nests, the following numbers of workers were added to EQW+ and LQW+ nests on days 1–7: 1, 2, 1, 1, 0, 0, 0; these numbers were based on the average number of workers that emerged in a subset of colonies (data not shown). All callow workers added to nests originated from the same source colonies to control for potential effects of worker genotype.

Observations of maternal care behavior

Forty-two of the 47 nest-founding queens were observed for a single 10 min period on days 3, 5 and 7, for a total of 30 min of observation per nest across the 3 days. The five queens not observed were from the EQW+ (N=2) and EQW– (N=3) groups; these queens were included in the other analyses in this study (colony development and brain gene expression analyses, described below). During each observation period the queens were continuously observed and the occurrence of any brood-feeding event was recorded. Brood feeding is an easily observable, discrete behavioral sequence that lasts ~5–15 s. Larvae are clumped together spatially, and when bees feed the brood, they open and regurgitate food into the larval cells, which can be observed when a bee places her mouthparts into an open cell and her abdomen contracts (Free and Butler, 1959).

Reproductive behavior and colony development

At the end of day 7, all nests were collected and stored at −20°C until nest dissections occurred. The number of eggs in the nest at the end of the experiment was recorded as a proxy for queen reproductive behavior; it is unlikely that any eggs were laid by workers, as the nests were very young and queens are highly effective at policing worker reproduction, even when nests are much older and larger (Free et al., 1969; Duchateau, 1989; Bloch, 1999). The number of larvae and pupae present in the nests at the end of the experiment was also recorded as a measure of colony development.

Statistical analyses of behavior and colony development data

Data on feeding behavior and the number of eggs, larvae and pupae in the nest were analyzed using ANOVA performed in R (R

Development Core Team, 2011). Because ANOVA revealed a significant effect of both stage of queen and presence/absence of workers on the number of eggs in the nest, pairwise *t*-tests (two-tailed) were used to compare the mean number of eggs in nests of the four groups. Additional *t*-tests (two-tailed) were used to compare the mean number of larvae and pupae in early *versus* late queen nests, as ANOVA also revealed a significant effect of stage of queen on these stages of brood. To test for an association between maternal care and reproductive behaviors in the queens, pairwise Pearson correlation coefficients were calculated between the total number of feeding events performed across the 3 days of observation and the number of eggs and larvae in the nest, using data from the 42 observed queens.

RNA preparation

At the end of day 7, all queens were collected within 1 h to control for circadian effects on gene expression. The queens were collected directly onto dry ice and their heads were immediately removed, placed in liquid nitrogen, and stored at −80°C to ensure RNA preservation. For brain dissections, whole queen heads were partially lyophilized and dissections were performed over dry ice (Schulz and Robinson, 1999). RNA was isolated from dissected brains using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the kit protocol except that the initial homogenization was performed in a 500 µl microfuge tube using 100 µl extraction buffer.

Microarrays

An Agilent 4×44K *B. terrestris* brain expressed sequence tag (EST)-based microarray was designed using sequence data obtained from 454/Roche pyrosequencing of *B. terrestris* mRNA derived primarily from brain tissue (90% and 10% abdomen) (Woodard et al., 2011). Total RNA, 250–1000 ng per sample, was reverse transcribed and linear amplified according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). Samples (one per array) were hybridized on the microarray slide and washed according to the Agilent protocol. Slides were scanned using an Axon 4000B scanner and images were analyzed with GENEPIX software (Agilent Technologies). Methods are described in greater detail elsewhere (Alaux et al., 2009b; Sen Sarma et al., 2009). The microarray results from this study can be accessed at the ArrayExpress website (http://www.ebi.ac.uk/arrayexpress/).

Microarray data pre-processing and statistical analyses were carried out in R (R Development Core Team, 2011) using the limma package (Smyth, 2005). Median foreground and median background values from the 47 .gpr files were read into R and any spots manually flagged (−100 values) were given a weight of zero. Background values were ignored because the use of these values to adjust for background fluorescence added more noise to the data, and the background was low and even for all arrays. Expression values were normalized with the quantile method (Smyth and Speed, 2003) then $\log_2$ transformed. Coefficient of variation (CV) values were calculated across all 47 samples; the distribution of CV values was strongly bimodal, separating at a value of 0.015 (data not shown), suggesting a subset of oligos with little variation overall and hence no useful information; nearly all of the positive and negative control spots had low CV values as well. All control spots and oligos with CV values <0.015 were removed from subsequent analyses, leaving 36,869 spots out of 45,220.

Because the microarray was designed from EST sequence rather than genomic data, stringent annotation criteria based on orthology to the honey bee (*Apis mellifera*) genome (Honeybee Genome Sequencing Consortium, 2006) were used to minimize the chances of including data from oligos that did not represent true *B. terrestris* genes in the subsequent statistical analyses. *Apis mellifera* orthologs were identified using the basic local alignment search tool (BLAST) to the non-redundant nucleotide database in GenBank; annotated oligos had strongly supported (E-value <0.0001) hits to genes in the Official Honey Bee Gene Set Version 2 (Honeybee Genome Sequencing Consortium, 2006). A total of 9924 oligos (putatively representing 4468 unique *A. mellifera* genes) met the annotation criteria and were included in subsequent analyses. Results are reported at the level of genes.

Statistical analyses of microarray data

ANOVA

To identify genes associated with the presence of workers and with stage of the queen (early *versus* late), a 2×2 factorial model (R Development Core Team, 2011) fitted in limma (Smyth, 2005) was used to compare expression values across the four groups, taking into account which slide the array was on. Using ANOVA, the main effects of presence of workers and stage of the queen and the interaction term were estimated, as well as mean expression levels for the four groups. Multiple-test adjustment was performed using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995) and probes with FDR $P<0.05$ were considered significant.

Pairwise tests

Four pairwise tests were performed to identify genes differentially expressed in the following contrasts: (i) EQW+ *versus* EQW–, (ii) LQW+ *versus* LQW–, (iii) LQW+ *versus* EQW+ and (iv) LQW– *versus* EQW–. Here also, multiple test adjustment was done separately for each contrast using the FDR method (Benjamini and Hochberg, 1995) and probes with FDR $P<0.05$ were considered significant.

Rank product tests

An additional, non-parametric analysis based on the rank product test was performed using the RankProd package (Breitling et al., 2004; Hong et al., 2006). The rank product test ranks oligos in each replicate according to expression value, with the underlying assumption that oligos that change expression level will change in rank. This additional analysis was used because of the small number of significant oligos in the ANOVA and pairwise tests, and also because within-group variation in expression values was high for many oligos. A rank sum analysis, which compares the sums of the ranks between two groups, was used because it requires greater consistency between replicates; the large numbers of replicates within the four groups (N=11–13) led to spurious results using the more traditional rank product analysis. To correct for the effect of which slide the array was on, all normalized expression values from all treatment groups were batch-corrected using the removeBatchEffect function.

RankProd tests upregulation and downregulation separately and corrects for multiple hypothesis testing using a permutation-based approach (N=100) to calculate the percentage of false-positives (PFP), which is an estimate of the false discovery rate (Hong et al., 2006). Therefore, the four pairwise comparisons between treatment groups yielded eight significant (PFP<0.05) rank product test gene lists: (i) upregulated in EQW+ *versus* EQW–; (ii) downregulated in EQW+ *versus* EQW–; (iii) upregulated in LQW+ *versus* LQW–; (iv) downregulated in LQW+ *versus* LQW–; (*v*) upregulated in LQW+ *versus* EQW+; (vi) downregulated in LQW+ *versus* EQW+;

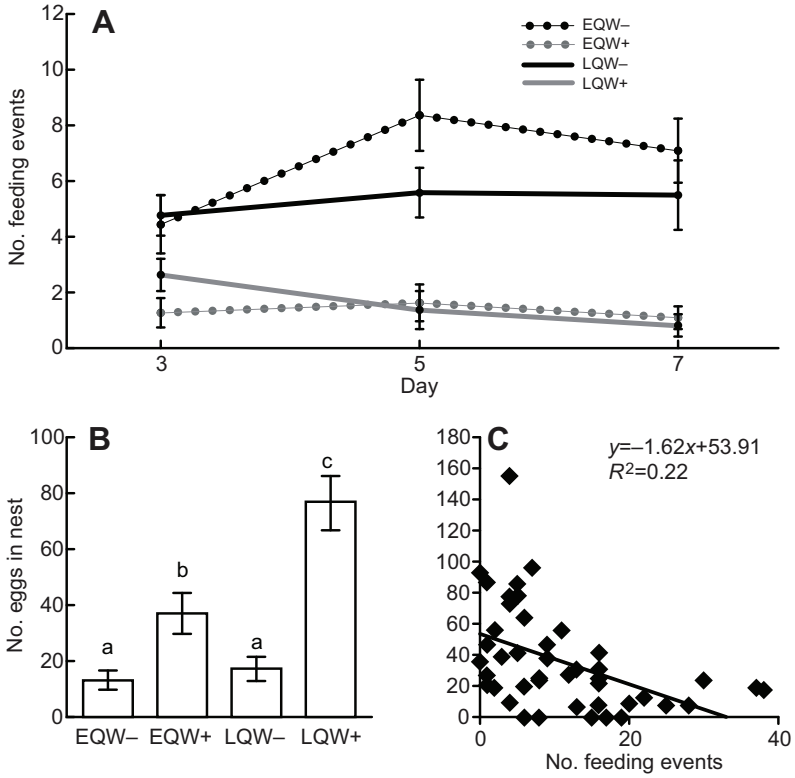

Fig. 2. Effect of worker number on queen behavior. (A) Effect of manipulating worker number on brood-feeding behavior; *x*-axis, day of the experiment; *y*-axis, total number of brood-feeding events observed per day. Values are group means ± s.e.m.; 3-way ANOVA with stage of queen (early *versus* late), workers (+/−) and day (3,5,7) as factors: $P_{workers(d.f.=1)}<0.0001$, $P_{workers\times day(d.f.=1)}<0.05$, other comparisons N.S. (B) Effect of manipulating worker number on queen egg-laying behavior; *x*-axis, group; *y*-axis, number of eggs in nests on day 7. Values shown are group means ± s.e.m.; letters represent results of *post hoc* pairwise *t*-tests; 2-way ANOVA with stage of queen and workers as factors: $P_{stage(d.f.=1)}<0.01$, $P_{workers(d.f.=1)}<0.001$, $P_{stage\times workers(d.f.=1)}<0.01$. (C) Association between brood-feeding and reproductive behavior; *x*-axis, total number of brood-feeding events observed (summed across all days); *y*-axis, number of eggs in nests on day 7.

(vii) upregulated in LQW– *versus* EQW–; and (viii) downregulated in LQW– *versus* EQW–.

Technical validation of microarray results

Real-time, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on all samples using an ABI Prism 7900 sequence detector and the SYBR green detection method (Applied Biosystems, Foster City, CA, USA). To validate microarray results, qRT-PCR was performed using primers designed for a subset (N=6) of genes showing differential expression between groups using microarrays (supplementary material Figs S1–S6) and a subset (N=2) of genes that did not show differential expression between groups using microarrays (supplementary material Figs S7, S8). All samples used for microrrays were used for qRT-PCR (N=45). As a loading control, qRT-PCR was also performed using primers for exogenous *root cap protein 1* (*rcp1*), which was added to RNA prior to cDNA synthesis (supplementary material Fig. S9). qRT-PCR was also performed using primers designed for the endogenous housekeeping gene *S8*, whose expression values did not vary in the microarray experiment; these data were used to normalize qRT-PCR expression data for experimental genes (supplementary material Fig. S10). Overall, the majority of individual gene qRT-PCR results were consistent with microarray results; however, others were not consistent. Additional details are provided in supplementary material Figs S1–S10 and Tables S1 and S2.

Associations between brain gene expression and behavior

Using data from the 42 observed queens, Pearson correlation coefficients were calculated between the normalized expression values for all 9924 annotated oligos on the microarray and the total number of brood-feeding events observed on day 7, as well as between expression values and the number of eggs in the nest. Because no genes were significantly correlated with behavioral data at FDR P<0.05, results reported for this test are significant at an uncorrected P<0.001.

Functional analysis of gene lists

Gene ontology (GO) enrichment analysis was performed on all gene lists of sufficient size (more than five genes) using the GO functional annotation tool (GOFat level) on the database for annotation, visualization and integrated discovery (DAVID) website (Huang et al., 2009). For a more direct comparison to the rank product test gene lists, each of the four pairwise test gene lists was separated into upregulated and downregulated lists for GO enrichment analysis. All gene lists were transformed into lists of *Drosophila melanogaster* orthologs using a previously published *A. mellifera–D. melanogaster* ortholog list (Honeybee Genome Sequencing Consortium, 2006). Results reported are 'biological process' and 'molecular function' terms enriched within the lists of *D. melanogaster* orthologs at an uncorrected P<0.05, with terms with fewer than five genes excluded. For the background list used for all tests of enrichment, *D. melanogaster* orthologs (N=3369) were identified for all 9924 annotated probes on the *B. terrestris* microarray.

RESULTS

Effect of treatment on brood-feeding behavior

Queens with workers in their nests (EQW+, LQW+) fed their brood significantly fewer times than queens without workers (EQW–, LQW–) (Fig. 2A). Differences in brood-feeding behavior between queens of the same stage with *versus* without workers could not be attributed to differences in the number of larvae in the nests (Fig. 3A). The stage of the queen had no effect on the amount of brood-feeding performed, despite the fact that LQ nests had nearly four times the number of larvae as EQ nests (P<0.001) (Fig. 3A). The number of pupae did not differ between EQW+ and EQW– nests (P=0.7);

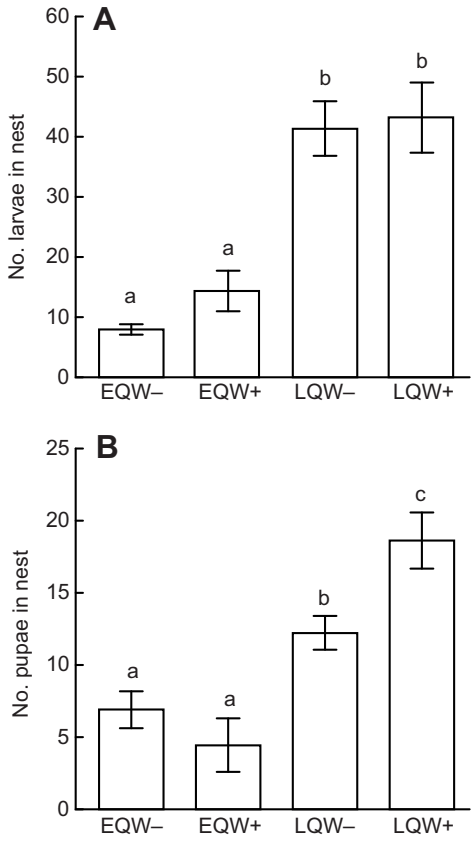

Fig. 3. Effect of social manipulation on colony development. (A) Effect of treatment on the number of larvae in nests; *x*-axis, group; *y*-axis, number of larvae in nests on day 7; 2-way ANOVA with stage of queen and workers as factors: $P_{stage(d.f.=1)}<0.0001$, other comparisons N.S. (B) Effect of treatment on number of pupae in nests; *x*-axis, group; *y*-axis, number of pupae in nests on day 7; 2-way ANOVA with stage of queen and workers as factors: $P_{stage(d.f.=1)}<0.0001$, $P_{stage\times workers(d.f.=1)}<0.01$. Values shown in A and B are group means ± s.e.m.; letters represent results of pairwise *t*-tests.

however, there were more pupae in LQW+ nests relative to LQW– nests ($P<0.05$) (Fig. 3B), which may reflect an influence of workers on developmental rates of the brood, specifically in the later stages of development.

Effect of workers on queen egg laying

Artificial manipulation of the number of workers also had a strong effect on egg laying in nest-founding queens (Fig. 2B). EQW+ nests had more than three times the number of eggs as EQW– nests ($P<0.01$). Likewise, LQW+ nests had more than four times the number of eggs as LQW– nests ($P<0.001$). LQW– and EQW– nests had a similar number of eggs ($P=0.3$), whereas LQW+ nests had more eggs than EQW+ nests ($P<0.05$).

Association between feeding and egg laying

Across the 42 observed queens, there was a significant negative correlation ($P=0.002$; $r=-0.47$) between the total number of brood-feeding events performed across all 3 days of observations and the number of eggs in the nest (Fig. 2C). The number of eggs in the nest and total amount of brood feeding were not correlated within any of the four groups. There was no significant correlation between the total number of brood-feeding events performed and the number of larvae in the nest ($P=0.5$), or between the number of larvae and the number of eggs in the nest ($P=0.3$).

Gene expression: ANOVA, pairwise tests and rank product tests

Table 1 shows the numbers of oligos and putative orthologous genes in *A. mellifera* and *D. melanogaster* that were significantly differentially expressed between groups using ANOVA, pairwise tests, and rank product tests. A subset of genes differentially expressed between groups that have previously been identified as important in insect behavior are highlighted in Table 2. Across all tests, not all gene list sizes were large enough for GO analysis; for the lists of adequate size, the enrichment results are listed in supplementary material Table S1. A direct comparison of the genes significant for the pairwise and rank product tests, which performed the same group comparisons, is shown in supplementary material Fig. S11.

Associations between brain gene expression and behavior

Across the 42 observed queens, 17 genes had expression levels that were significantly correlated with the total number of brood-feeding events performed on day 7 and eight genes had levels of expression significantly correlated with the number of eggs in the nest (Table 3).

DISCUSSION

We used a social manipulation experiment to provide evidence that workers socially regulate queen maternal traits during the nest initiation phase of the bumble bee *B. terrestris*. The transition from feeding their brood to specialization on reproduction with little brood feeding in nest-founding queens appears to be highly plastic in both directions. Depending on social conditions, queens can increase brood feeding and decrease egg laying beyond the point when workers emerge in the nest and, likewise, queens can decrease brood feeding and increase egg laying prior to the natural emergence of workers in the nest. Two other major transitions in the bumble bee queen life cycle also appear to be socially regulated by workers: the 'switch point', where queens switch from laying diploid (i.e. female-destined) to haploid (i.e. male-destined) eggs, and later on the 'competition point', which is typically followed by a dramatic increase in queen–worker conflict in the nest (Alaux et al., 2005; Bloch, 1999).

Despite exhibiting considerable behavioral plasticity, there appear to be limits to the amount of brood feeding and reproduction that queens can perform. Although the nests of late-stage founding queens without workers had far more larvae than nests of early-stage queens without workers, queens in these nests performed similar amounts of brood feeding, suggesting that a maximum rate of brood feeding may have been reached. Additionally, the negative association between brood feeding and reproduction across queens in all four groups suggests that a trade-off may exist, perhaps due to constraints stemming from physiological, pleiotropic or other connections between the two behaviors.

Surprisingly, despite the strong effect of treatment on behavior, the number of genes associated with the presence of workers was relatively small compared with the findings of other studies on the social regulation of gene expression, many of which used similar methods to measure expression (Alaux et al., 2009a; Alaux et al., 2009b; Grozinger et al., 2003; Cummings et al., 2008; Replogle et al., 2008). In addition, the effect of workers on queen brain gene expression was highly dependent on the stage of the queen. Far more genes were differentially expressed between late-stage queens that varied in worker number relative to the early-stage queens.

Table 1. Comparison of gene list sizes from ANOVA, pairwise tests, and rank product tests of differential brain gene expression

	List	Oligos	Unique *A. mellifera* orthologs	Unique *D. melanogaster* orthologs
ANOVA	Main effect of workers (+/–)	4	4	4
	Main effect of stage of queen	394	328	253
Pairwise tests	LQW+ *vs* EQW+	0	0	0
	LQW– *vs* EQW–	1101	884	824
	EQW+ *vs* EQW–	0	0	0
	LQW+ *vs* LQW–	37	37	34
Rank product tests	Upregulated in LQW+ *vs* EQW+	39	28	26
	Downregulated in LQW+ *vs* EQW+	21	18	18
	Upregulated in LQW– *vs* EQW–	575	453	430
	Downregulated in LQW– *vs* EQW–	685	566	528
	Upregulated in EQW+ *vs* EQW–	9	8	8
	Downregulated in EQW+ *vs* EQW–	0	0	0
	Upregulated in LQW+ *vs* LQW–	338	304	286
	Downregulated in LQW+ *vs* LQW–	149	132	123

'List', gene list name; 'Oligos', number of oligos significant for test; 'Unique *A. mellifera* orthologs', number of non-redundant *A. mellifera* orthologs, derived from oligo lists; 'Unique *D. melanogaster* orthologs', number of non-redundant *D. melanogaster* orthologs, derived from *A. mellifera* ortholog lists. All oligos are significant at false discovery rate (FDR)-corrected $P<0.05$.

Given that worker loss occurs in the wild and is likely detrimental for nests, perhaps bumble bee queens have evolved the ability to respond to worker loss in a way that involves changes in gene expression. Another possibility is that quantitative changes in behavior involve changes in gene expression to a lesser extent than do changes in behavioral state (e.g. the nurse to forager transition in honey bees), which involves a more complete change in behavioral repertoire. Alternatively, the unnatural, premature addition of workers to early-stage nests may have precipitated a more 'opportunistic' response by queens in these nests. An opportunistic response may not have required changes in brain gene expression, or may have involved expression changes that were not detectable at the time point that we sampled (Robinson et al., 2008). In cichlid fish, dramatic behavioral changes that occur in response to changes in social environment precede detectable changes in gene expression (Burmeister et al., 2005), suggesting that gene expression changes are not necessary to initiate behavioral responses in this system.

The gene *cycle* is a strong candidate for future studies on the molecular basis of social regulation of queen maternal traits. *cycle*, which is a molecular component of the circadian clock, was differentially expressed between late-stage queens with *versus* without workers. This finding suggests that workers may alter queen behavior *via* changes in circadian gene expression. A previous study on *B. terrestris* (Eban-Rothschild et al., 2011) found that rhythmicity in nest-founding queens is influenced by social environment, although this study manipulated developing brood in the colony rather than worker number. The influences on circadian rhythm in locomotor activity previously found (Eban Rothschild et al., 2011) and on *cycle* expression in the current study may be mediated by changes in maternal or reproductive physiology that are associated with oogenesis or brood care.

Table 2. Differentially expressed genes with known functions in insect behavior

Gene	Functional information	Result	Test
arrestin 2	Phototransduction in bees (Sasagawa et al., 2003)	Upregulated in queens with workers (*vs* without)	ANOVA
calcium/calmodulin-dependent protein kinase	Learning and memory in flies (Waddell and Quinn, 2001)	Upregulated in late-stage queens (*vs* early)	ANOVA
inositol 1,4,5,-trisphosphate receptor	Feeding- and flight-related behaviors in flies (Agrawal et al., 2010; Banerjee et al., 2004); behavioral maturation in honey bees (Kucharski and Maleszka, 2002; Whitfield et al., 2003); nest-provisioning behavior in *Polistes* wasps (Toth et al., 2010)	Downregulated in late-stage queens (*vs* early)	ANOVA
nejire	Circadian rhythmicity in flies (Hung et al., 2007)	Upregulated in late-stage queens (*vs* early)	ANOVA
cycle	Circadian activity, socially regulated expression in honey bees (Shemesh et al., 2007)	Upregulated in late-stage queens with workers (*vs* LQW–)	Pairwise tests
NMDA receptor 1	Learning and memory in flies (Qin and Dubnau, 2010)	Upregulated in late-stage queens (*vs* early)	ANOVA
		Upregulated in late-stage queens with workers (*vs* LQW–)	Pairwise tests; rank product tests
target of rapamycin (*tor*)	Regulation of behavioral maturation and aging in honey bees (*via* the insulin signaling pathway) (Ament et al., 2008; Page and Amdam, 2007; Corona et al., 2007)	Upregulated in late-stage queens without workers (*vs* EQW–)	Pairwise tests; rank product tests
foraging (*for*)	Regulation of feeding- and foraging-related behaviors in honey bees and flies (Ben-Shahar et al., 2002)	Downregulated in late-stage queens without workers (*vs* EQW–)	Rank product tests

Bombus terrestris gene identities are based on orthology to *A. mellifera* genes.

Table 3. List of genes with expression values correlated with maternal traits

Apis mellifera gene ID	Gene name	*P*-value	*r*
A. Expression correlated with brood-feeding behavior			
GB19602	*sodium-coupled neutral amino acid transporter 7*	0.00020	0.53
GB13198	Hypothetical protein LOC411299	0.00037	–0.51
GB12627	Hypothetical LOC551126	0.00039	0.51
GB14934	*tryptophanyl-tRNA synthetase*	0.00042	–0.50
GB16129	*succinate dehydrogenase cytochrome b small subunit, mitochondrial*	0.00044	0.50
GB16868	*feminizer*	0.00047	0.50
GB17688	*inosine triphosphate pyrophosphatase*	0.00050	0.50
GB17957	*ras-like protein 2*	0.00058	0.49
GB18234	*high affinity copper uptake protein 1*	0.00058	0.49
GB13850	*cleavage and polyadenylation specificity factor 100*	0.00061	0.49
GB16412	*translationally controlled tumor protein*	0.00063	0.49
GB17536	Unknown	0.00066	–0.49
GB19751	*α-mannosidase II*	0.00068	–0.49
GB18025	*β-catenin-like protein 1*	0.00085	–0.48
GB15724	*zinc finger C4H2 domain-containing protein*	0.00087	0.48
GB18632	*uncharacterized MFS-type transporter C09D4.1*	0.00088	–0.48
GB12825	*cleavage and polyadenylation factor subunit*	0.00092	0.48
B. Expression correlated with egg-laying behavior			
GB10161	*venom acid phosphatase Acph*	0.00018	0.53
GB18510	*threonine aspartase 1*	0.00024	–0.52
GB13341	*THO complex subunit 6*	0.00033	–0.51
GB16201	Hypothetical LOC408320	0.00065	0.49
GB13722	*glucocerebrosidase*	0.00080	0.48
GB12816	*teneurin-3*	0.00084	0.48
GB13833	*thrombospondin*	0.00088	–0.48
GB10483	*venom acid phosphatase Acph-1*	0.00090	0.48

Bombus terrestris gene identities are based on orthology to *A. mellifera* genes. '*Apis mellifera* gene ID', *A. mellifera* orthologs derived from oligos. *P*-values reported are for Pearson correlation coefficients.

In contrast to the small number of genes associated with the presence of workers, many genes were associated with stage of the queen. In terms of absolute time, the difference between early- and late-stage founding queens in the experiment was small (on the order of weeks) relative to their lifespan (Free and Butler, 1959). Regardless, queens may undergo important internal developmental changes during this critical period in the life cycle, which occur irrespective of the social environment or their brood-feeding and egg-laying behavior. The finding that genes involved in circadian rhythm, learning and memory, and aging were differentially expressed between early- and late-stage queens is consistent with the idea that intrinsic developmental changes may be occurring in association with gene transcriptional changes during this transitional period in the queen life cycle.

Although we have attributed the differences in queen behavior and gene expression in this experiment to the social effect of workers, social signals originating from the brood may have also influenced these traits, as the number of eggs and pupae differed between nests with and without workers. Previous studies in bumble bees suggest that pupae do influence egg-laying behavior in queens (Gretenkord and Drescher, 1997; Gurel and Gosterit, 2008; Kwon et al., 2006). However, the number of larvae in the nests of queens of the same stage did not differ in our study, suggesting that this factor did not influence differences in behavior or gene expression between queens of the same stage.

A small number of genes had levels of expression that were correlated with brood-feeding or egg-laying behavior across all queens in the experiment. Previous studies in fish (Trainor and Hofmann, 2007; Sanogo et al., 2012) and monkeys (Sabatini et al., 2007) have demonstrated correlations between social behaviors and gene expression in brain tissue. The set of genes with levels of expression correlated with behavior in this experiment includes the gene *feminizer*, which is involved in sex determination, as well as genes involved in glycolipid metabolism and putative zinc finger proteins. These genes are an excellent set of candidates for future studies on the molecular regulation of maternal and reproductive behaviors in bumble bees.

Understanding the behavioral and molecular bases of the transition from the solitary to eusocial phase has important implications in both evolutionary and conservation contexts. With wild bumble bee populations in decline (Cameron et al., 2011), understanding the transition from brood feeding to specialization on reproduction in nest-founding queens may be important for predicting how bumble bees can respond to environmental changes. This includes understanding how the transition is regulated and the limits of behavioral plasticity in queens of different ages. Although queens in this study responded to changes in social environment, the absence of workers was associated with a significant decline in queen reproductive output, suggesting that maintaining a substantial worker population is important for the viability of young colonies. Additionally, the results of this study suggest that artificially adding workers to young nests may be a valuable strategy for minimizing the loss of colonies reared for pollination, a finding that is consistent with previous studies (Gretenkord and Drescher, 1997; Gurel and Gosterit, 2008; Kwon et al., 2006).

The results of this study may also provide insights into how complex sociality evolved in insects. In bee lineages with a more highly organized division of labor (e.g. stingless bees and honey bees), queens do not undergo a life stage in which they found nests as solitary individuals. However, it is possible, and perhaps likely (Cardinal and Danforth, 2011), that such a life history stage was present at some point in the evolutionary history of these 'highly'

eusocial lineages. As such, extant primitively eusocial species can serve as models for exploring the evolutionary transition from solitary to eusocial in bees. For example, molecular mechanisms involved in transitioning from the 'solitary-like' nest-founding stage to specialization on reproduction in bumble bee queens may also have played a role in the evolutionary transition from a solitary to a eusocial lifestyle in this lineage. The genes identified in this study may be used for direct comparisons between eusocial and non-eusocial insects to explore whether, at the molecular level (Robinson et al., 2005), there were shared routes to eusociality across disparate lineages.

LIST OF SYMBOLS AND ABBREVIATIONS

EQW–	early-stage founding queens without workers
EQW+	early-stage founding queens with workers
EST	expressed sequence tag
FDR	false discovery rate
GO	gene ontology
LQW–	late-stage founding queens without workers
LQW+	late-stage founding queens with workers

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AUTHOR CONTRIBUTIONS

S.H.W. designed and performed the research, analyzed the data and wrote the paper; G.B. designed the research and wrote the paper; M.R.B. designed the microarrays, performed the research and analyzed the data; and G.E.R. designed the research and wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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