Genes, Brain and Behavior
Official publication of the International Behavioural and Neural Genetics Society

Genes, Brain and Behavior (2009) **8:** 309–319

Regulation of brain gene expression in honey bees by brood pheromone

C. Alaux*[†,‡], Y. Le Conte[§], H. A. Adams[¶], S. Rodriguez-Zas[¶], C. M. Grozinger[**], S. Sinha[††] and G. E. Robinson[†,‡,‡‡]

[†]Department of Entomology and [‡]Institute for Genomic Biology, University of Illinois at Urbana–Champaign, Urbana, IL, USA, [§]INRA, UMR 406 Abeilles et Environnement, Laboratoire Biologie et Protection de l'abeille, Institut National de la Recherche Agronomique, Domaine Saint-Paul, Avignon, France, [¶]Department of Animal Sciences, University of Illinois at Urbana–Champaign, Urbana, IL, USA, [**]Departments of Entomology and Genetics, W.M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC, USA, and [††]Department of Computer Science and [‡‡]Neuroscience Program, University of Illinois at Urbana–Champaign, Urbana, IL 61801, USA

**Corresponding author: C. Alaux, INRA, UMR 406 Abeilles et Environnement, Laboratoire Biologie et Protection de l'abeille, Domaine Saint-Paul, 84914 Avignon, France. E-mail: cedric.alaux@avignon.inra.fr*

Pheromones are very important in animal communication. To learn more about the molecular basis of pheromone action, we studied the effects of a potent honey bee pheromone on brain gene expression. Brood pheromone (BP) caused changes in the expression of hundreds of genes in the bee brain in a manner consistent with its known effects on behavioral maturation. Brood pheromone exposure in young bees causes a delay in the transition from working in the hive to foraging, and we found that BP treatment tended to upregulate genes in the brain that are upregulated in bees specialized on brood care but downregulate genes that are upregulated in foragers. However, the effects of BP were age dependent; this pattern was reversed when older bees were tested, consistent with the stimulation of foraging by BP in older bees already competent to forage. These results support the idea that one way that pheromones influence behavior is by orchestrating large-scale changes in brain gene expression. We also found evidence for a relationship between *cis* and BP regulation of brain gene expression, with several *cis*-regulatory motifs statistically overrepresented in the promoter regions of genes regulated by BP. Transcription factors that target a few of these motifs have already been implicated in the regulation of bee behavior. Together these results demonstrate strong connections between pheromone effects, behavior, and regulation of brain gene expression.

Keywords: Behavioral maturation, *cis*-regulatory element, gene expression, honey bee, pheromone

Received 8 August 2008, revised 16 October 2008, accepted for publication 9 December 2008

Data deposition: Gene expression data meet Minimum Information About a Microarray Experiment (MIAME) standards and have been deposited at ArrayExpress (http://www.ebi.ac.uk/arrayexpress): E-TABM-512, E-TABM-658

Pheromones are important for understanding the mechanisms by which behavior is shaped by the environment because they induce behavioral plasticity through shifts in neural response thresholds to environmental conditions (Wyatt 2003). Discovery of pheromone receptors (Dulac & Axel 1995; Kurtovic *et al.* 2007; Wanner *et al.* 2007) and neural pathways and olfactory sensory maps in the brain has given new insights about pheromone processing (Jefferis *et al.* 2007; Kimchi *et al.* 2007, Sandoz 2006). However, understanding how pheromones affect neural and behavioral plasticity also requires tracing the molecular changes that occur throughout the brain in response to pheromone perception.

The honey bee is an important model for such studies because its behavior and pheromones are unusually well characterized (Le Conte & Hefetz 2008, Slessor *et al.* 2005) and its genome has been sequenced (Honey Bee Genome Sequencing Consortium 2006). Because of known similarities in pheromone transduction across organisms, it is reasonable to assume that studies of models such as the honey bee will have general significance.

Grozinger *et al.* (2003) used microarray analysis to show that honey bee queen mandibular pheromone (QMP) affects the expression of hundreds of genes in the brain of worker bees. Pheromone regulation of brain gene expression has also been reported in other organisms (Brennan *et al.* 1999; Halem *et al.* 2001). Consistent with its effect on behavior (delays transition from brood care –'nursing'– to foraging for food), QMP tends to upregulate genes in the brain that are upregulated in nurses but downregulate genes that are upregulated in foragers. These results suggest that one way that a pheromone can influence behavior is by orchestrating large-scale changes in brain gene expression.

We tested this idea by studying another pheromone, brood pheromone (BP). Brood pheromone affects a variety of physiological and behavioral processes, including age-related division of labor (Le Conte *et al.* 2001). It also can delay the onset of foraging, as does QMP, but it is composed of entirely different chemicals than QMP. We determined whether BP

doi: 10.1111/j.1601-183X.2009.00480.x

differentially regulates genes in the brain that also are associated with honey bee behavioral maturation.

Another reason we studied BP was because it has age-dependent effects on bee behavior. In addition to primer effects on behavioral maturation, BP quickly stimulates the foraging activity of older bees that are competent to forage (Pankiw 2004; Pankiw & Page 2001). As it is not known whether pheromone processing is hard-wired independent of age or is modulated in some way by maturational processes in any animal, we used microarray analysis to determine whether there is a robust brain 'molecular signature' of age-dependent effects of BP.

We also determined whether pheromone-regulated genes are associated with transcription factor-binding *cis*-regulatory motifs in their promoter regions, and if so, whether these patterns of association are also age-dependent. Some transcription factors have been identified in the context of behavior in mammals, songbirds and fish (Robinson *et al.* 2008), but connections with pheromone effects are not well established. Identification and analysis of regulatory motifs associated with pheromone-induced genes will provide the framework to better understand transcriptional regulation of brain and behavior.

Materials and methods

Pheromone treatment

Experiments were performed in the field at the Laboratoire Biologie et Protection de l'Abeille, Avignon, France, with colonies of *Apis mellifera ligustica* bees. Large typical colonies (source colonies) were maintained according to standard commercial procedures. These studies were conducted with triple-cohort colonies made with 1-day-old adult marked bees (focal cohort), nurses and foragers ($n = 500$ per cohort) as in Le Conte *et al.* (2001). Triple-cohort colonies allow for standardization of some factors such as adult population and demography that might affect brain gene expression (Whitfield *et al.* 2003, 2006) and are often used in studies of pheromones and behavior (Leoncini *et al.* 2004). To provide bees of known age for triple-cohort colonies, honeycombs containing late-stage pupae were removed from source colonies and placed in an incubator to emerge (33°C, 95% relative humidity). The source colonies were headed by queens that were naturally mated. Each triple-cohort colony was placed in a small beehive that contained two honeycomb frames (one full with honey and one empty). The colonies were transferred to a different apiary >3 km away from the source colony so the bees would not return to the site of their natal hive.

Treatments were as follows: One triple-cohort colony had a caged queen, no brood and a dose (see below) of brood pheromone (BP+), and the other one had a caged queen, no brood and no brood pheromone (BP−). Each colony was made from the same source colony to control for genotypic variation and in addition was made as similar as possible to each other in every respect (size, demography, honeycomb number and contents, and location in the field). Early in the morning, before foraging activity, marked bees from the focal cohort were collected 5 and 15 days later ('BP5' and 'BP15' for treated bees, respectively) for gene expression analysis. This approach allowed us to randomly collect marked bees that were walking on the combs to avoid any bias toward a particular behavior; behaviorally related effects on brain gene expression are extensive in honey bees (Cash *et al.* 2005; Whitfield *et al.* 2003, 2006). Bees were collected into liquid nitrogen, and heads were stored at −80°C before brain dissection. Two replicates were conducted, each with a different pair of triple-cohort colonies.

BP was made by mixing the 10 identified components in the proportions found on 4- to 5-day-old larvae (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% and ethyl linolenate 10% (Sigma Chemical Co., Saint Quentin Fallavier, France). A 4.1 larval equivalents per bee of BP was administered daily in fresh sugar candy as in Le Conte *et al.* (2001). This dose has a strong and consistent effect on behavioral maturation, causing a significant delay in the onset age of foraging (Le Conte *et al.* 2001). It also represents a natural exposure as it has been shown that bees consume about 4.1 larval equivalents per bee/day in field experiments (Le Conte *et al.* 2001), and bees repeatedly visit cells containing larvae (Robinson 1987a), feeding them approximately five times per hour (Huang & Otis 1991).

The effect of BP exposure on bees in this study was verified by determining the age at onset of foraging for the first 50 foragers from each of the experimental colonies (Le Conte *et al.*, 2001). Foragers were identified as bees returning to the entrance of their colony with either pollen loads in their corbiculae or distended abdomens. They were then removed from the experiment so that each bee was counted only once.

Sample preparation, microarray and data analysis

Bee heads were partially lyophilized to facilitate brain dissection (Schulz & Robinson 1999) and dissections were performed as in Grozinger *et al.* (2003). We analyzed four bees per treatment (BP−, BP+) per trial, each trial involving a different, unrelated, source colony, using a total of 48 arrays. Messenger RNA (mRNA) extraction, mRNA amplification and microarray hybridization are described in the supporting information. We used a new honey bee oligonucleotide microarray based on the recently sequenced honey bee genome (Honey Bee Genome Sequencing Consortium 2006) that contains roughly twice as many genes as the first generation (Whitfield *et al.* 2003) array. The microarray contained oligonucleotides representing about 13 440 different genes (with duplicate spots), based on gene predictions and annotation from the honey bee genome sequencing project (Honey Bee Genome Sequencing Consortium 2006). This new honey bee microarray has been validated (Kocher *et al.* 2008).

The following filtering protocols were used (Whitfield *et al.* 2003, 2006). Spots were removed from analysis if flagged by the GENEPIX Pro 6.1.0.27 software or if the fluorescence intensity was less than the median intensity of the negative control spots. Fluorescence intensities were normalized using a LOWESS transformation, duplicate spots for each gene were averaged, and adjusted for microarray and dye effects. A total of 8160 and 7810 genes passed these filters for the BP5 and BP15 data sets, respectively, approximately 60% of the genes so far identified from the honey bee genome.

Differences in brain gene expression between nurses and foragers

To determine whether BP differentially regulates genes in the brain that are associated with behavioral maturation, we performed a study of brain gene expression in nurses and foragers, also with the new microarray, to provide the basis for comparative analysis with the pheromone results. This experiment was modeled after the original nurse/forager microarray study, conducted with the first generation [complementary DNA (cDNA)] array, which was based on a brain EST project (Whitfield *et al.* 2003). The study was conducted at the University of Illinois Bee Research Facility, Urbana, IL. Bees were a typical mixture of European races, predominantly *Apis mellifera ligustica*. As in Whitfield *et al.* (2003), we collected 7- to 10-day-old nurses and 21- to 24-day-old foragers from both typical colonies (mixed age structure, population approximately 25 000 bees) and single-cohort colonies (1500 bees); this allowed us to dissociate effects of age and behavior. We measured brain gene expression for young nurses (YN) and old foragers (OF) from typical colonies ($n = 6$ bees/group, three unrelated colonies, 36 bees total) and YN, OF, old nurses (ON) and young foragers (YF) from single-cohort colonies ($n = 3$ bees/group, three single-cohort colonies made from the above three typical colonies, 36 bees total). A total of 90 microarrays were used in direct comparisons (Figure S1). We used the results of the nurse/forager comparison to create a 'top 100' list of

genes showing the most consistent differences in brain expression between nurses and foragers (Whitfield *et al.* 2003). This top 100 list was determined by a 'leave-one-out' cross-validated class prediction using zero-centered, $\log_2$-transformed normalized values for individual brains as in Whitfield *et al.* (2003) with GeneSpring v.5.0 (Agilent, Foster City, CA, USA). We compared forager/nurse brain gene expression ratios from this set with ratios for the same genes in the BP experiment to determine whether pheromone treatment causes patterns of expression that are more forager-like, more nurse-like or dissimilar to either (Whitfield *et al.* 2006).

Statistical analysis

A linear mixed effects model implemented using restricted maximum likelihood was used to analyze the normalized $\log_2$-transformed fluorescence intensities for each gene, accounting for the effects of dye, treatment, bee and microarray. Treatment effects were evaluated with *F*-test statistics.

We calculated the number of overlapping genes between BP5 and BP15 divided by the expected number of genes (number of genes in BP5 $\times$ number of genes in BP15)/total number of genes expressed) (Kim *et al.* 2001) and used an exact hypergeometric probability test to determine whether the overlap between the gene sets regulated by BP in young and old bees was significant.

Verification by real-time quantitative reverse transcriptase polymerase chain reaction

Confirmation of some of the results obtained from microarray analysis was performed with real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in individual brains (independent samples, not used for arrays). Expression levels were measured for two genes (*ox:* downregulated by BP5; *CG6178:* upregulated by BP5 and in nurses) with an ABI Prism 7900 sequence detector and the SYBR green detection method (Applied Biosystems, Foster City, CA, USA). *eIF3-S8*, a housekeeping gene that did not vary in expression levels on these microarrays and in the qRT-PCR (permutation test: $P = 0.426$), was used as loading control (Grozinger *et al.* 2003). Quantification was as described (Grozinger *et al.* 2007). The sequences for the primers used are given in Table S1. Results are consistent with the microarray results (Figure S2). We did not have enough samples to also validate genes associated with BP15 effects.

Functional analysis

After generating lists of differentially expressed genes, we explored whether any particular molecular functions or biological processes were represented by larger numbers of genes than expected on the basis of chance. *Drosophila melanogaster* orthologs were identified by reciprocal best BLASTX match to bee genes, and Gene Ontology (GO) terms were assigned based on annotation of *Drosophila* genes. GOToolBox (Martin *et al.* 2004) was used to perform these analyses; hypergeometric tests followed by the Benjamini Hochberg correction for multiple testing were used to identify overrepresented terms (GO categories at $P < 0.05$ are shown).

Analysis of cis-regulatory DNA motifs associated with BP regulation

We used the honey bee genome (Honey Bee Genome Sequencing Consortium 2006) to scan for *cis*-regulatory motifs in the promoter regions of genes identified here as BP regulated. We searched for 134 motifs that were previously well characterized in *D. melanogaster*, representing 71 transcription factors [10% of 753 known transcription factors (Adryan & Teichmann 2006)]. These motifs include a partially redundant list of 52 known motifs from the FlyREG database (Bergman *et al.* 2005; Matys *et al.* 2006; Noyes *et al.* 2008), as well as 82 computationally predicted motifs (Stark *et al.* 2007), that were highly similar to at least one of the known motifs. To determine if a given motif M is highly similar to a known motif K, we calculated the per-column relative entropy between M and K, for every window of width 7, allowing for relative shifts between the position-specific weight matrix. We then used a threshold of 0.1 or below on this similarity measure to decide if M and K are highly similar. We scanned a 5-kb upstream region of each gene to score the gene for each motif, using methods from Sinha *et al.* (2006) with modification. The score was used as a basis for deciding if a gene's promoter contains the motif or not ('target' or 'non-target', respectively). For each gene set G and its 'opposite' H (e.g. up- and downregulated, respectively, by a pheromone), a 2×2 contingency table was constructed with columns representing G & H, and rows representing the number of targets and non-targets, respectively, in each gene set (see supporting information). A one-sided Fisher exact test (test1) was performed with this table, thereby giving a *P* value for the association between motif and gene set G. A *Q* value was calculated for each significant motif ($P < 0.01$) to adjust for multiple testing.

As our promoter scans have used motifs characterized in *Drosophila*, we determined next if these motifs are likely to be conserved in *Apis*, that is whether the DNA-binding domains of the respective proteins are conserved at the DNA-binding residues, between *Drosophila* and *Apis*. As shown in Table S2, most transcription factors are completely unchanged.

The honey bee genome is extremely A/T rich compared with *Drosophila* (Honey Bee Genome Sequencing Consortium 2006). But some of our motif-gene set associations were confounded by a significant association of motifs with the high G/C content of the promoter regions, as was previously observed (Sinha *et al.* 2006). Interestingly, brain-specific genes tend to have high G/C content promoters in humans (Vinogradov 2003). Therefore, to eliminate motif-gene set associations that occurred solely because of the high promoter G/C content and identify motif–gene set associations on the basis of the specific identity of the motifs, we controlled for the effect of G/C content. We mimicked gene set G with a set G_r of random non-coding sequences from the genome, keeping the number, lengths and G/C content of the sequences the same as that of the sequences in G. We then constructed (for each motif) another 2×2 contingency table with the counts of targets and non-targets in G and G_r, and performed a Fisher exact test (test2). Rejection of the null hypothesis in this test indicates that the ratio of targets to non-targets in G is significantly greater than that in G_r, thereby controlling for the effect of gene set-specific G/C content. A motif–gene set association is reported here only if the *P* value of 'test1' is <0.01 and the *P* value of 'test2' is <0.05. Further details are presented in supporting information and Tables S3 and S4.

Results

Inhibition of behavioral maturation by BP

Brood pheromone exposure caused a delay in the age at onset of foraging in both trials (Fig. 1) (mean $\pm$ SD given, trial 1: BP$-$:17.7 $\pm$ 4.61, BP+: 27.02 $\pm$ 9.38, $P < 0.001$; trial 2: BP$-$:21.48 $\pm$ 3.65, BP+: 25.98 $\pm$ 7.81, $P = 0.018$, $n = 50$ bees/treatment for each trial, Mann-Whitney rank sum test). In trial 2, the pheromone effect on behavior was only apparent at older ages. It is not clear how this difference might affect the results because physiological changes that trigger the onset of foraging occur early in adult life (Whitfield et al. 2006). Significant effects of BP on age at onset of foraging in both trials validate the BP treatment for microarray analysis.

Age-dependent differences in effects of BP on brain gene expression

A total of 227 (96 upregulated + 131 downregulated) genes were differentially expressed between BP+ and BP$-$ bees

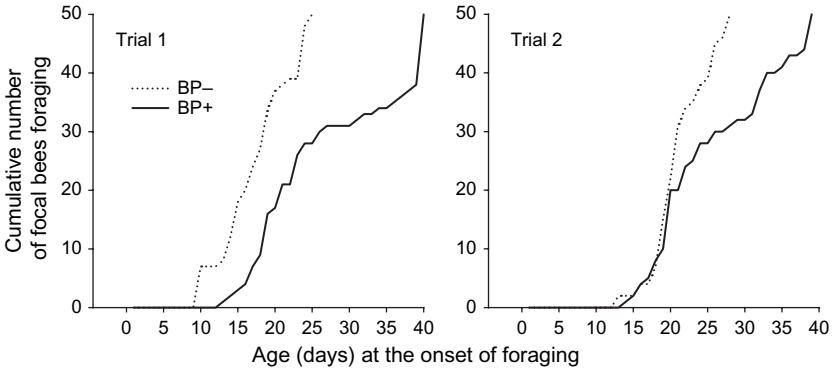
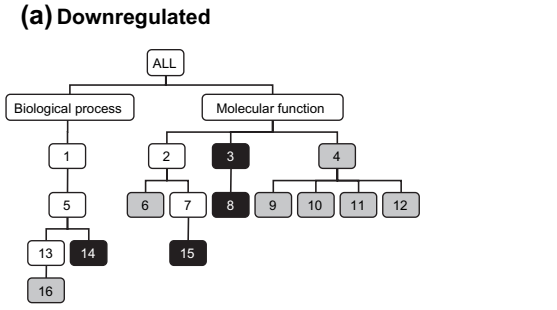


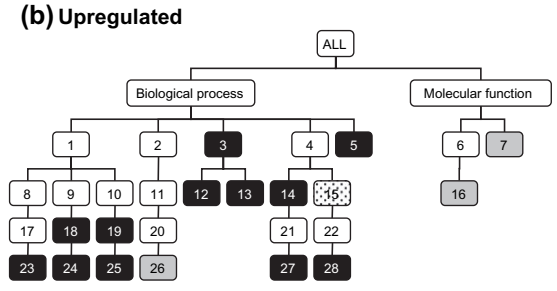
Figure 1: Effect of BP on honey bee behavioral maturation, measured as age at onset of foraging. BP−, no brood pheromone; BP+, with brood pheromone. Results of statistical analyses are given in text.

after 5 days of exposure ('BP5 genes'; $P < 0.01$, False Discovery Rate (FDR) < 0.3) (Table S5). A total of 228 (122 upregulated + 106 downregulated) genes were differentially expressed between BP+ and BP− bees after 15 days of exposure ('BP15 genes'; $P < 0.01$, FDR < 0.3). Nineteen genes were chronically regulated, that is, showed significant differences after both 5 and 15 days of exposure. This represents a significant overlap between the gene sets regulated by BP in young and old bees ($P < 0.0001$, exact hypergeometric probability test). However, the expression levels of these 19 genes are not significantly correlated between young and old bees ($r = 0.097$, $P = 0.693$) showing that many are not overall regulated in the same direction.

Age-dependent differences in the effects of BP on brain gene expression were further revealed by GO-based functional analysis. The BP5 and BP15 gene lists mostly reflect different GO categories for molecular function and biological process in the brain (Fig. 2). The 'multicellular organismal process' category was the only one in which genes were overrepresented in both young and older bees (upregulated in both). Among the enriched GO categories for genes downregulated by BP5 were 'oxidoreductase activity' and



1 Cellular process
2 Binding
3 Structural molecule activity
4 Catalytic activity
5 Metabolic process
6 Nucleotide binding
7 Protein binding
8 Structural constituent of ribosome
9 Helicase activity
10 Oxidoreductase activity
11 Hydrolase activity
12 Pyrophosphatase activity
13 Cellular metabolic process
14 Catabolic process
15 Cell surface receptor–linked signal transduction
16 Generation of precursor metabolites and energy



1 Cellular process
2 Metabolic process
3 Response to stimulus
4 Developmental process
5 Immune system process
6 Transcription regulator activity
7 Enzyme regulator activity
8 Cell communication
9 Cellular component organization and biogenesis
10 Cellular development process
11 Macromolecule metabolic process
12 Response to external stimulus
13 Behavior
14 Anatomical structure development
15 Multicellular organismal development
16 RNA polymerase II transcription factor activity
17 Cell–cell signaling
18 Cellular structure morphogenesis
19 Cell differentiation
20 Macromolecular complex assembly
21 System development
22 System process
23 Synaptic transmission
24 Cell morphogenesis
25 Cell development
26 Protein–DNA complex assembly
27 Nervous system development
28 Neurological system process

Figure 2: Functional (Gene Ontology) analysis of genes regulated by BP. The lists of genes regulated by BP at 5 and 15 days of age ($P < 0.05$) were analyzed for statistical enrichment of associated GO terms, relative to the representation of these terms for all genes on the array. The grey and black colored boxes indicate significant enrichment in the BP5 and BP15 gene lists, respectively. The spotted boxes indicate significant enrichment in both BP5 and BP15 gene lists. Categories at the highest level of the Gene Ontology hierarchy are located on the top of the graph and those at the lowest level of the hierarchy at the bottom of the graph (panel a, downregulated genes, panel b, upregulated genes).

'generation of precursor metabolites and energy' and for BP15: 'structural constituent of ribosome' and 'catabolic process'. Among the enriched GO categories for genes upregulated by BP5 were 'RNA polymerase II transcription factor activity' and for BP15: 'synaptic transmission', 'nervous system development' and 'response to stimulus'.

Differences between nurses and foragers in brain gene expression

A total of 1692 genes were differentially expressed between nurses and foragers ($P < 0.01$, FDR < 0.07); 1055 were upregulated in nurses and 637 upregulated in foragers (Table S6). A total of 370 genes were found to be differentially expressed between nurses and foragers in both the present (oligo) study and an earlier cDNA array study (Whitfield *et al.* 2003). The expression level of these genes is highly significantly correlated between the two array platforms ($r = 0.79$, $P < 1e{-}06$).

The top 100 most predictive genes of behavioral state are listed in Table S7. Leave-one-out cross-validation using these genes correctly classified 67 bees of 72 to behavioral phenotype (nurse or forager) on the basis of individual brain gene expression profiles. This high percentage (93%) is similar to what was seen in Whitfield *et al.* (2003). The expression levels of these genes is strongly correlated with the corresponding values from the cDNA array ($r = 0.88$, $P < 1e{-}06$).

Six genes found in our top 100 behavior predictive genes were also found in the top 100 of Whitfield *et al.* (2003); we did not necessarily expect high congruence between these two lists because the present list is derived from a different microarray and the 'leave-one-out' algorithm that generates theses lists works with whatever starting set is available. Five of the six have fly orthologs: *Inos*, *PebIII*, *BM-40-SPARC*, *CG11334*, *CG11791* and one does not: *GB15474*. These six overlapping genes were regulated in the same directions in the present experiment and in Whitfield *et al.* (2003). The new list of top 100 behavior predictive genes was used in analyses described in the following section.

Behaviorally relevant BP-mediated brain gene expression

Exposure to BP for 5 days caused a brain gene expression profile that was similar to the profile of nurse bees. Bees exposed to BP for 5 days tended to upregulate 'nurse genes' (those upregulated in nurses) and downregulate 'forager genes'. This pattern was significant for 5-day-old bees (Table 1a). Moreover, a correlation analysis performed on forager/nurse brain expression ratios for the top 100 behavior predictive genes and the expression ratios for the same genes in the BP experiment showed a significant negative correlation for BP5 genes ($r = -0.4485$, $P = 3e{-}06$).

There was no such similarity to nurse bees for bees exposed to BP for 15 days. A correlation analysis performed on forager/nurse brain expression ratios for the top 100 behavior predictive genes and the expression ratios for the same genes in the BP experiment showed a significant positive correlation ($r = 0.346$, $P < 0.001$). Genes downregulated in nurses and upregulated in foragers are down- and upregulated, respectively, by BP after 15 days of exposure. *malvolio* (*mvl*) was upregulated by BP in 15-day-old bees, but not in 5-day-old bees. *mvl* is upregulated in foragers relative to nurses, and treatment that activates the *mvl* pathway causes precocious foraging (Ben-Shahar *et al.* 2004).

Table 1: Overlap of genes regulated in the brain by behavioral state and pheromone. (a) Number of genes regulated in the brain by behavioral state and pheromone. (b) Number of genes regulated by BP and QMP.

(a)	Nurse ↑ (1055)	Forager ↑ (637)	Significance
BP5↑ (96)	25	8	$\chi^2 = 4.01$
BP5↓ (131)	10	12	$P = 0.0452$
BP15↑ (122)	25	15	$\chi^2 = 0.1$
BP15↓ (106)	20	14	$P = 0.9331$
QMP↑ (374)	42	17	$\chi^2 = 11.16$
QMP↓ (323)	15	27	$P = 0.0008$

(b)	QMP↑ (374)	QMP↓ (323)
BP5↑ (96)	3	3
BP5↓ (131)	0	1
BP15↑ (122)	2	4
BP15↓ (106)	2	0

Table 1a gives the number of genes that show significant differences in brain gene expression between nurses and foragers and are regulated by BP after either 5 or 15 days of exposure or QMP in colonies (Grozinger et al. 2003). In Table 1b, numbers in parentheses are the total number of genes on each list.

Chi-square tests with Yates correction were performed to determine if BP- and QMP-regulated genes in a manner consistent with the nurse/forager differences, based on pheromones causing a delay in onset of foraging. No statistical analysis was performed on Table 1b because the number of genes is too low.

We also compared our results with those from a previous study performed with a brain cDNA microarray that identified a total of 697 genes in the worker honey bee brain that were significantly regulated by QMP in experiments performed on colonies in the field (Grozinger et al. 2003). QMP is composed of different chemicals than BP but also delays the onset of foraging. Very few genes were regulated by both BP and QMP, showing no obvious patterns of common regulation (Table 1b). However, as for BP5, QMP tended to upregulate nurse genes and downregulate foragers genes identified with the genome oligonucleotide microarray. As for BP, the pattern was significant for QMP (Table 1a). Few of the behavioral genes were regulated by both pheromones (Table S5).

cis-*regulatory DNA motifs associated with BP regulation*

Table 2 lists the statistically significant motif–gene set associations. The *Dorsal* motif was chronically overrepresented in

Table 2: *cis*-regulatory DNA motifs associated with BP regulation. Lists of the statistically significant motif–gene set associations

Positive gene set	Negative gene set	Motif	Similar known motif	Transcription factor or group	*P* value of test1	*Q* value of test1	*P* value of test2
BP up vs. down							
b5u	b5d	HSRGAAAAHYV	dl(0.12)	Dorsal	0.0007355	0.0074	0.0003945
b5u	b5d	GGGWWWWCCA	Dl_10(0.10)	Dorsal	0.006475	0.0340	0.003149
b5u	b5d	MGAADHTTCKMGAAD	Hsf(0.09)	Heat shock factor	7.84E−05	0.0018	0.005107
b5u	b5d	TAAT	Homeodomain	Homeodomain group	1.03E−06	0.0001	0.04908
b5u	b5d	CCTTTGATCTT	I_dTCF_1(0.01)	T-cell factor	0.004636	0.0301	0.03317
b5u	b5d	br-Z4		Broad complex Z4	0.0001543	0.0028	0.005139
b5u	b5d	GGGWWWWCCM	Dl_10(0.10)	Dorsal	0.0001767	0.0028	0.009343
b5d	b5u	VHRRCAGGTGYM	I_SN_01(0.04)	Snail	0.008133	0.2883	0.007625
b5d	b5u	WCATTWMM	I_ZEN_Q6(0.08)	Zernknullt	0.004907	0.2883	3.89E−08
b5d	b5u	Tin		Tinman	0.008542	0.2883	0.01714
b5d	b5u	CAGSTG	I_SN_01(0.12)	Snail	0.005124	0.2883	0.006346
b15u	b15d	KGGGWWWHCYV	Dl_10(0.14)	Dorsal	0.0009504	0.1283	0.0002203
b15d	b15u	AANTNTAATGACA	Antp Homeodomain group	Antp Homeodomain	0.0002827	0.0378	2.22E−05
b15d	b15u	Abd-B		Abdominal-B	0.007143	0.4769	0.02498
BP 5 vs. 15							
b5u	b15u	AANTNTAATGACA	Antp Homeodomain	Antp Homeodomain	0.00611	0.3675	0.0002535
b5u	b15u	Adf1		Adf1	0.005294	0.3675	0.01578
b5d	b15d	Ovo		Ovo	0.007453	0.2563	0.00709
b5d	b15d	VHRRCAGGTGYM	I_SN_01(0.04)	Snail	0.007593	0.2563	0.003718
b5d	b15d	CAGSTG	I_SN_01(0.12)	Snail	0.001994	0.2563	0.00297
b5d	b15d	Pho		Pleiohomeotic	0.004608	0.2563	3.00E−06

Nomenclature for gene sets: 'bX[u/d]': genes [u]p/[d]own regulated by BP at day X. Each test measures the overrepresentation of a motif in the 'positive' gene set compared with the 'negative' gene set. For a computationally predicted motif (those with consensus strings in place of names in column 'Motif'), the experimentally verified motif that is most similar to it is indicated in column 'Similar known motif'. The last three columns report the *P* value and *Q* value of the motif-gene set association (test1, $P < 0.01$), and the *P* value of the test to control for G/C association (test2, $P < 0.05$), respectively. See Methods for additional details.

gene sets upregulated by BP, at both ages (5 at 15) compared with the corresponding downregulated gene sets. Motifs highly resembling the *Snail* motif 'CAGSTG' were enriched in genes downregulated by BP at day 5, compared with upregulated genes at day 5 or downregulated genes at day 15. But this motif was weakly enriched in genes upregulated by BP at day 15 ($P = 0.06$), marking an age-dependent change in *cis*-element association. Another case of age-specific regulatory association is the motif *Adf1*, which is enriched in BP-upregulated genes at day 5 (Table 2), but weakly enriched ($P = 0.03$) in downregulated genes at day 15 (data not shown). The *Adf1* motif was previously found to have an association with genes regulated by juvenile hormone (Sinha *et al.* 2006). Similarly, the *Abd-B* motif is enriched in the downregulated genes at day 15 ($P = 0.007$, Table 2), and in the upregulated genes at day 5 ($P = 0.01$, data not shown). Another notable finding in Table 2 is the strong association of the pattern 'TAAT', the canonical *homeodomain*-binding motif, with BP-induced genes at day 5. Interestingly, a subgroup of the Homeodomain family formed by *Antennapedia* (*Antp*) and related proteins has its motif (AANTNTAATGACA) underrepresented in the BP-induced genes at day 15, suggesting another instance of age-dependent differences in the effects of BP on the regulation of brain gene expression.

We also attempted to find *cis*-elements that are associated with genes regulated as a function of both BP and behavioral state (nurse or forager). This was performed by analyzing combined gene lists, that is genes over- or underrepresented by BP on day 5 (or day 15) and genes differentially expressed between nurses and foragers. We additionally required that these associations were *not present* in the original BP-regulated gene sets alone to implicate the *cis*-elements as being specific to the intersected gene lists. We found seven motifs that fit one of these two patterns (Table 3). The *P* value threshold used here is weaker than in Table 2 because these tests were performed with smaller gene sets, resulting in lower statistical power.

Discussion

Results of this study support the idea that one way that pheromones influence behavior is by orchestrating changes in brain gene expression. We found that BP, administered in a way that leads to a delay in the onset of honey bee foraging, tended to upregulate in the brain nurse genes but downregulate forager genes. These results do not indicate that these genes are only regulated by BP; they may also be sensitive to other extrinsic factors. Our findings are consistent with results for QMP, another pheromone that also delays onset of foraging, also apparently by effects on brain gene expression (Grozinger *et al.* 2003).

We also demonstrate for the first time age-dependent effects of a pheromone on brain gene expression, with the following four results. First, although there was a significant overlap between the gene sets regulated by BP in young and old bees, many were regulated in opposite directions. Second, the GO categories with genes overrepresented in the BP5 and BP15 gene lists were mostly different, with only one category showing overrepresentation at both ages. Third, the pattern noted above – negative correlation with the 100 behavior predictive genes – was only seen in 5-day-old bees. 15-day-old bees showed a positive correlation. Fourth, most of the *cis*-regulatory DNA motif–gene set associations detected here were age specific. Understanding how the same pheromone provokes such different molecular responses in the brain is an important topic for future analysis.

Results from both BP and a previous QMP study (Grozinger *et al.* 2003), when compared with our nurse/forager gene list, support the idea that pheromones regulate behavior by modifying expression of behaviorally relevant genes in the brain. However, our results show that the overlap between BP and QMP on brain gene expression is very weak and that both pheromones affect very few behaviorally relevant genes in common. One explanation is that the two pheromones were analyzed with different microarray platforms and in different types of colonies (triple-cohort for BP and typical field colonies for QMP). Another explanation is that the distinct chemical compositions of BP and QMP elicit effects

Table 3: *cis*-regulatory DNA motifs associated with genes that are regulated by both BP and behavioral state (nurse or forager)

Positive gene set	Negative gene set	Motif	Similar known motif	Transcription factor or group	*P*-val1	*P*-val2	*P*-val3
b5d.f	b5u.n	bin		Biniou	0.0393	0.5813	0.00586
b15d.n	b15u.f	TAATTAA	Lim1.new.7(0.01)	En Homeodomain	0.0159	0.6623	6.30E−06
b15u.f	b15d.n	BHTAAKCYSBV	bcd(0.05)	Bcd Homeodomain	0.01881	0.478	0.03125
b15u.f	b15d.n	RAAMGRGTT	kruppel(0.09)	Kruppel	0.01881	0.1265	0.002059
b15u.f	b15d.n	SGGATTAW	Ptx1.new.7(0.00)	Bcd Homeodomain	0.009369	0.3782	0.01111
b15u.f	b15d.n	HWAAKCYB	bcd(0.07)	Bcd Homeodomain	0.001396	0.4106	0.000717
b15u.f	b15d.n	CAYRTGTG	twi(0.19)	Twist	0.01387	0.4072	0.001274

'bX[u/d].[n/f]': genes [u]p/[d]own-regulated at day X and induced in [n]urses/[f]oragers. Shown are the motif associations that were significant (*P*-val1 = 'test1 *P*-value' < 0.05; *P*-val3 = 'test2 *P*-value' < 0.05) when BP-regulated gene sets were intersected with nurse/forager gene sets, but not significant (*P*-val2 > 0.1) when considering the BP-regulated sets alone. These motif associations are thus specific to genes regulated by both BP and behavioral state.

on different sets of genes in the brain. The two pheromones likely use different peripheral receptors (Robertson & Wanner 2006; Wanner *et al.* 2007), which apparently trigger different neural and molecular pathways. However, firm conclusions cannot be drawn until both pheromones are studied under identical conditions.

The effects of BP on foraging behavior are complex, with both dose-dependent and age-dependent effects. Our gene expression findings mirror this complexity. If BP causes a delay in onset of foraging, with many of the bees in our study showing a BP-caused behavioral effect that resulted in foraging at ages older than 15 days, then why do we see foraging-like profile already at 15 days of age? One possible explanation is that the effects of BP on rate of behavioral maturation only occur early in life. Whitfield *et al.* (2006) showed that most age-related changes in brain gene expression are essentially completed by 8 days of age. It is known that 15-day-old bees are generally competent to forage (Robinson 1992), but the precise age at onset of foraging depends on a variety of environmental factors. Brood pheromone might affect brain gene expression in 15-day-old bees in a manner consistent with its role as a releaser pheromone, triggering foraging behavior in bees that are competent to forage. Although releaser pheromones cause relatively quick behavioral responses, they can also affect brain gene expression (Alaux & Robinson 2007). This speculation is consistent with the effects of BP on foraging. Brood pheromone acts as a primer pheromone and delays the age at onset of foraging in young bees (Le Conte *et al.* 2001) but also acts as a releaser pheromone, increasing foraging activity in older bees that are already competent to forage (Pankiw & Page 2001; Pankiw 2004).

A possible explanation for the finding of a more foraging-like profile of brain gene expression in 15-day-old bees relates to the dose-dependent effects of BP. Relatively 'high doses' of BP delay the onset of foraging, but 'low doses' accelerate it (Le Conte *et al.* 2001). The mechanisms underlying this difference are unknown, but it is possible that they are related to age-related changes in BP perception or responsiveness. If so, then a given exposure affects a young bee like a 'high dose' (delaying age at onset of foraging) and an older bee as a 'low dose' (triggering foraging). Age-related changes in responsiveness to pheromones are known in honey bees (Grozinger & Robinson 2007, Robinson 1987b). Consistent with this speculation, among the upregulated GO categories that showed overrepresentation of genes regulated by BP in older workers in our study were 'nervous system development' and 'response to stimulus'. This is interesting because structural changes in neurons (Farris *et al.* 2001) but not neurogenesis (Fahrbach *et al.* 1995) have been observed during behavioral maturation in honey bees. Perhaps changes in these biological processes are related to age-related changes in sensitivity to BP. It has been shown that the adult insect brain is plastic and that pheromone processing can be hormonally modulated. For example, juvenile hormone modulates age-related changes in sensitivity of antennal lobes and behavioral responsiveness in moths and locusts (Anton & Gadenne 1999; Anton *et al.* 2007). Many studies in vertebrates also showed that responses to pheromone are influenced endocrine factors (Wyatt 2003). Perhaps the age-related decrease in sensitivity to BP in honey bees is endocrine mediated (Robinson 1992).

One of the genes that contributes strongly to the conclusion that BP causes a foraging-like profile of brain gene expression in 15-day-old bees is *malvolio.* Brood pheromone caused an upregulation of *malvolio* in 15-day-old bees. *malvolio*, which encodes a manganese transmembrane transporter, is upregulated in forager brain compared with nurse and treatment with manganese causes precocious foraging (Ben-Shahar *et al.* 2004). This treatment also causes an increase in sucrose responsiveness (Ben-Shahar *et al.* 2004), an endophenotype associated both with an early age at onset of foraging and the tendency to specialize on pollen rather than nectar while foraging (Page *et al.* 1998). These dual effects of *malvolio* are intriguing when considered in light of BP's dual effects on age at onset of foraging and the tendency to specialize on pollen foraging. *malvolio* might represent a key component of the regulation of foraging behavior by BP. This gene is implicated in the feeding behavior of *Drosophila* (Rodrigues *et al.* 1995; Orgad *et al.* 1998). This is more evidence that genes regulating behavior of solitary species have been used for the evolution of social behavior, something that has been discovered for both invertebrates and vertebrates (Robinson & Ben-Shahar 2002).

Plasticity of pheromone-guided behavior is widespread in vertebrates (Meredith 1986; Moncho-Bogani *et al.* 2002) and invertebrates (Anderson *et al.* 2007; Anton *et al.* 2007; Daly & Figueredo 2000; Judd *et al.* 2005) and usually involves an increase or a decrease in the behavioral response to the same pheromone. But opposite effects of the same pheromone have not been reported. By contrast, we found that the same pheromone can cause opposite effects in gene expression and behavior at different ages. One explanation might relate to our finding that transcription factors were overrepresented among genes regulated by BP, and similar results for QMP (Grozinger *et al.* 2003). In addition, our analysis of *cis*-regulatory elements showed that most of the *cis*-regulatory DNA motif-gene set associations were age specific. The opposite pattern of motif usage could explain the opposite patterns of gene expression reported here. One motif showing such an association that could explain in part the opposite pheromone responsiveness is *Adf1*, which has been shown to have an association with genes regulated by juvenile hormone (Sinha *et al.* 2006), and juvenile hormone has been implicated in pheromone sensitivity in insects, as discussed above, in addition to involvement in olfactory learning (Roman & Davis, 2001) and synapse formation in adults (DeZazzo *et al.* 2000). The identification and analysis of the promoters and transcription factors that regulate pheromone-induced genes suggests that the relationship between *cis* and BP regulation of brain gene expression will be very important to elucidate further. An important first step will be biological evidence for the functional significance of these findings, to confirm the *in silico* results we report.

Cis-regulation of single genes has been shown to be important in the regulation of social behavior in rodents (Insel & Young 2001; Weaver *et al.* 2002) and *D. melanogaster* (Drapeau *et al.* 2006). Our analysis found several *cis*-elements that are associated with genes that are regulated as a function of both pheromone and behavioral state. Among these, the *snail* motif was enriched in genes downregulated by BP in young workers, and in a previous study this motif

was enriched in genes upregulated by cyclic guanosine-3′,5′-monophosphate (cGMP) (Sinha *et al.* 2006). The onset of foraging is associated with an increase in brain expression of the *foraging* gene, which encodes cGMP-dependent protein kinase (PKG), and treatment with cGMP, which activates PKG, leads to precocious foraging (Ben-Shahar *et al.* 2002). In *Drosophila*, the transcription factor encoded by *snail* plays a role in nervous system regulation (Ashraf *et al.* 1999). These connections are provocative and suggest functional linkages between pheromone regulation, *cis* regulation and the action of genes known to be involved in the regulation of bee behavior.

The findings presented here indicate that exploiting the wealth of information available on pheromone regulation of behavior (Wyatt 2003) can provide powerful experimental systems for elucidating the molecular basis of behavioral plasticity.

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Acknowledgments

We thank M. Chabaut, A. Maisonnasse and D. Crauser for field assistance with the BP experiment, K. Pruiett and S.A. Ament for collecting the samples for the nurse/forager experiment; S. Zhang and T. Newman for assistance with microarray analysis; members of the Robinson laboratory and two anonymous reviewers for helpful comments on the manuscript. This is part of a series of papers arising from an NSF Frontiers in Biological Research grant (B.R. Schatz, PI) that uses large-scale analysis to explore the influences of heredity and the environment on brain gene expression and behavior. Additional funding from the Fyssen Foundation (C.A.), the Illinois Sociogenomics Initiative (G.E.R.) and USDA-NRI 2004-35604-14277 (G.E.R.).

Supporting Information

The following supplementary information is available for this article:

Figure S1: Nurse/Forager microarray experimental design. First number indicates host colony, and second number indicates bees. Each arrow represents a single microarray. Arrow tail indicates Cy3-labeled sample and arrowhead indicates Cy5-labeled sample. Each brain was analyzed on two or four microarrays, balanced for dye labelling. After Whitfield *et al.* (2003).

Figure S2: Validation of microarray results with real-time quantitative (q) RT-PCR. (A)Brain expression levels of 2 genes identified from the microarray study as regulated by BP. Gene expression differences between bees exposed to BP ($n = 8$) and control bees ($n = 7$) were analyzed using qRT-PCR. Data are normalized to expression levels of *eIF3-S8*. (B) Ratio of mean expression levels (BP/control) from array and qRT-PCR analyses are shown. Functions based on Gene Ontology information for *Drosophila* orthologues. Samples were biological replicates not used for arrays. Means $\pm$ SE are shown. Significant differences were determined using a permutation test ($^{*}P < 0.05$, $^{**}P < 0.01$).

Table S1: Primer sequences

Table S2: Changes in DNA-binding domain of transcription factors. 'Some change' implies two or fewer changes at DNA-contacting residues. 'Large change' implies more than two changes. Among DNA-binding domains, 'H' refers to Homeobox and 'Z' refers to Zf-C2H2

Table S3: Contingency table for testing motif association (A) and controlling for effect of G/C content (B)

Table S4: Motifs found to be significantly discriminative for pairs of gene sets. (Additional information beyond that provided in Table 2). Nomenclature for gene sets: 'bX[u/d]': genes [u]p/[d]own regulated by BP at day X. Each test measures the overrepresentation of a motif in the 'positive' gene set compared with the 'negative' gene set. For

a computationally predicted motif (those with consensus strings in place of names in column 'Motif'), the experimentally verified motif that is most similar to it is indicated in column 'Similar known motif'. The columns report the *P* value and *Q* value of a motif-gene set association (test1, $P < 0.01$), and the *P* value of the test to control for G/C association (test2, $P < 0.05$), respectively. Columns *n1* through *n4* and *n1r* through *n4r* are the sizes of various sets that were used in performing the Fisher test, and are explained in the text (*Materials and Methods*)

Table S5: List of genes regulated by the brood pheromone. Overlapping QMP-regulated genes (Grozinger *et al.* 2003) are shown

Table S6: List of genes differentially expressed between young nurse and old forager. Overlapping QMP (Grozinger *et al.* 2003) and BP-regulated genes are shown

Table S7: Top 100 most predictive genes for nurse/forager behavior. N, gene upregulated in nurse; F, gene upregulated in forager

Additional Supporting Information may be found in the online version of this article.

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