Published in final edited form as:

J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 2011 September ; 197(9): 939–948. doi: 10.1007/s00359-011-0654-5.

cGMP modulates responses to queen mandibular pheromone in worker honey bees

Brendon L. Fussnecker^{1,4,6}, Alexander M. McKenzie², and Christina M. Grozinger^{1,3,4,5}

¹Department of Genetics, Box 7614, 1566A Gardner Hall, MC 7613, North Carolina State University, Raleigh NC 27695

²Department of Biology, Box 7617, 1566A Gardner Hall, MC 7613, North Carolina State University, Raleigh NC 27695

³Department of Entomology, Box 7613, 1566A Gardner Hall, MC 7613, North Carolina State University, Raleigh NC 27695

⁴W.M. Keck Center for Behavioral Biology, 1566A Gardner Hall, MC 7613, North Carolina State University, Raleigh NC 27695

Abstract

Responses to social cues, such as pheromones, can be modified by genotype, physiology, or environmental context. Honey bee queens produce a pheromone (queen mandibular pheromone; QMP) which regulates aspects of worker bee behavior and physiology. Forager bees are less responsive to QMP than young bees engaged in brood care, suggesting that physiological changes associated with behavioral maturation modulate response to this pheromone. Since 3', 5'-cyclic guanosine monophosphate (cGMP) is a major regulator of behavioral maturation in workers, we examined its role in modulating worker responses to QMP. Treatment with a cGMP analog resulted in significant reductions in both behavioral and physiological responses to QMP in young caged workers. Treatment significantly reduced attraction to QMP and inhibited the QMP-mediated increase in *vitellogenin* RNA levels in the fat bodies of worker bees. Genome-wide analysis of brain gene expression patterns demonstrated that cGMP has a larger effect on expression levels than QMP, and that QMP has specific effects in the presence of cGMP, suggesting that some responses to QMP may be dependent on an individual bees' physiological state. Our data suggest that cGMP-mediated processes play a role in modulating responses to QMP in honey bees at the behavioral, physiological, and molecular levels.

Keywords

behavioral genetics; neuroethology; honey bees; pheromone; communication

Corresponding Author: Brendon Fussnecker. 536 Broadway, 4th Floor, New York, NY 10012. brendon.fussnecker@flywheelpartners.com, 201-937-6383.

⁵Current address: Department of Entomology, Center for Pollinator Research, Center for Chemical Ecology, Huck Institutes of the Life Sciences, Pennsylvania State University, University Park PA 16802

⁶Current address: Flywheel Partners, 536 Broadway 4th Floor, New York, NY 10012.

The authors declare no competing interests.

Introduction

Pheromones mediate a myriad of intraspecific social interactions, including mating behaviors, social hierarchies, and coordinating defense of nests or colonies (reviewed in Wyatt 2003). Releaser pheromones result in an immediate behavioral response in the receiver, while primer pheromones cause long-term physiological and behavioral changes in the receiver, which may be mediated by changes in gene expression. While pheromones are classically considered to be fixed chemical blends that cause stereotyped behavioral and physiological responses, it is clear that genotype, physiological state, and environmental context can modulate responses to pheromones (e.g., see Linn 1984; Linn et al. 1986; Linn et al. 1992; Gadenne et al. 1993; Linn et al. 1996; Gadenne and Anton 2000; Pophof 2000; Zhukovskaya and Kapitsky 2006). Furthermore, the primer effects of a pheromone can persist even in the absence of any releaser effect and *vice versa* (Grozing et al 2007), demonstrating that the two major divisions of pheromone action can be uncoupled. Though the neural networks that process pheromone cues are well-characterized (reviewed in Touhara and Vosshall 2009), little is known about the underlying molecular pathways that translate the detection of these cues to behavioral responses or how responses to pheromones are modulated at a molecular level.

Honey bee (*Apis mellifera*) queen mandibular pheromone (QMP) is arguably the best characterized pheromone in honey bees, and serves as an important means of communication between the honey bee queen and her workers (reviewed in Slessor et al. 2005). QMP is a five-component blend, the major compound of which is (E)-9-oxodec-2-enoic acid (9-ODA) (Slessor et al. 1988). Though QMP does not represent the honey bee queen's full pheromone blend, these five components can stimulate many of the responses produced by a live queen. QMP elicits both primer and releaser effects from worker bees. As a releaser pheromone, it attracts young workers to the queen, a behavior known as the *retinue response* (Slessor et al. 1988). As a primer pheromone, QMP delays worker behavioral maturation from brood care (nursing) to foraging behavior (Pankiw et al. 1998), increases brain expression of nurse-related genes (Grozing et al. 2003), and causes young caged bees to develop nurse-like phenotypes, including higher lipid levels and higher *vitellogenin* (*Vg*) RNA expression levels in the fat bodies (Fluri et al. 1982; Toth et al. 2005; Fischer and Grozing et al 2008).

Interestingly, unlike nurse bees, forager bees are not responsive to QMP in terms of the retinue response and brain gene expression (Pham-Delegue et al. 1991; Grozing et al 2007). Physiological differences between nurse and forager bees may be responsible for these differences in responsiveness. Foragers have higher juvenile hormone titers, and treatment with juvenile hormone analogs accelerates the transition from nursing to foraging (Robinson 1987). Treatment with these analogs modulates primer, but not releaser, responses to QMP in young bees (Grozing et al 2007; Grozing et al 2007). Foragers also have higher levels of the biogenic amine octopamine, and treatment with octopamine also accelerates behavioral maturation (Schulz and Robinson 2001). However, octopamine does not alter retinue responses to queens (Barron et al. 2002).

Another key factor in the regulation of the behavioral maturation process is the *foraging* gene, *Amfor*. *Amfor* codes for a 3', 5'-cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) and its orthologs have been associated with food-related locomotion in many organisms including fruit flies (Pereira and Sokolowski 1993), *C. elegans* (Fujiwara et al. 2002), mouse (Morley et al. 1996; Morley et al. 1999), bumble bees (G. Bloch, H. Patch and C.M. Grozing et al, unpublished data), harvester ants (Ingram et al. 2005), and honey bees (Ben-Shahar et al. 2002). In addition, PKG has also been associated with learning and

memory (Kaun et al. 2007a; Kaun et al. 2007b; Mery et al. 2007; Wang et al. 2008) and sucrose responsiveness (Scheiner et al. 2004; Belay et al. 2007) in *Drosophila melanogaster*. This gene is more highly expressed in the brains of honey bee foragers than nurses, and treatment of young workers with 8-Bromo-cGMP (8-Br-cGMP, an analog of cGMP) leads to increased PKG activity, increased phototactic behavior, and an accelerated transition from nursing to foraging behavior (Ben-Shahar et al. 2002; Ben-Shahar et al. 2003). 8-Br-cGMP treatment has also been shown to affect brain gene expression, by up-regulating expression of genes associated with the foraging behavioral state (Whitfield et al. 2006; Fussnecker and Grozinger 2008). Thus, PKG-regulated pathways may be key regulators of foraging behavior and behavioral maturation in honey bees.

As a major regulator of the transition from nursing to foraging, we hypothesized that cGMP pathways play a role in modulating behavioral, physiological and brain gene expression responses to QMP. To test this prediction, we examined whether 8-Br-cGMP treatment reduces the retinue response to QMP, inhibits QMP-induced changes in *Vg* levels and alters global brain gene expression responses to QMP.

Materials and methods

General bee rearing

Honey bee colonies were maintained at the North Carolina State University Lake Wheeler Honey Bee Research Facility according to standard commercial procedures. Worker bees were derived either from queens that were instrumentally inseminated with semen from a single, different drone (single drone inseminated, or SDI, queens, *Apis mellifera carnica*, Glenn Apiaries, CA), according to established procedures (Laidlaw 1987), or with naturally mated queens, which are typically inseminated by multiple drones (MDI queens; Buckfast-SMR bees, Weaver Apiaries, TX). Sources of bees will be noted in their respective sections below. To provide bees of known age, honeycombs containing late-stage pupae were removed from a source colony and placed in an incubator overnight to emerge (33°C, 30% relative humidity). Bees less than 24 hours old were collected off the frames and placed into Plexiglas cages (10×10×7cm). Bees were provided with 50% sucrose and ground pollen; sucrose was changed daily, while pollen was changed every 3 days. Cages were maintained in an incubator under red light at 33°C, 30% relative humidity for the duration of the experiments. The initial retinue experiment was performed in the summer of 2007, while the microarray experiment and its associated analyses were performed during the summer and early fall of 2008. Details about specific experiments are below.

Pheromone treatment

Experimental cages were treated with either synthetic QMP (0.1 queen equivalents in 10 µL of 1% water/isopropanol; Pherotech, Canada) or control slide containing solvent only (1% water/isopropanol) as in Grozinger et al. (2003), Grozinger and Robinson (2007), and Fischer and Grozinger (2008). Each treatment was administered by placing 10 µL of either QMP or solvent onto a microscope coverslip, which was allowed to evaporate before being placed in its respective cage. Fresh treatment was applied daily, at the same time each day. Number of days of treatment for each experiment will be described in their respective sections below.

8-Br-cGMP treatment

Experimental cages were treated with 2 mL total volume of 8-Br-cGMP (Sigma Aldrich, St. Louis, MO) or a sucrose control, as in Ben-Shahar et al. (2002) and Fussnecker and Grozinger (2008). 8-Br-cGMP was fed to bees as a 500µM solution in 50% sucrose.

Treatments were replaced daily and mortality of cGMP-treated bees was less than 5%. Number of days of treatment for each experiment will be described in their respective sections below.

Retinue response

The effect of 8-Br-cGMP treatment on retinue responses to QMP was assessed using bees from one SDI and two naturally mated source colonies in three different trials (SDI6, MDI20, and MDI21). Bees were reared as described above, with 20 bees/cage, 3 cages/treatment group in each trial. There were four experimental treatments: sucrose only with solvent (NN), sucrose only with QMP (NQ), 8-Br-cGMP with solvent (GN), and 8-Br-cGMP with QMP (GQ). Retinue counts were performed at the same time each day (1 pm) by counting the number of bees contacting (antennating or licking) the QMP or solvent slide every 5 minutes for 15 minutes each day. This was repeated from 3-8 days post-emergence. Retinue response was monitored for days 1-2, but no retinue responses were observed. This is typical of newly-emerged bees. Data was analyzed in SAS 8.0 (SAS, Cary, NC) using a repeated measures ANOVA that fit QMP and cGMP treatments separately. The data was analyzed as the arcsine-transformed mean frequency of responses per day for each cage. Day was a repeated measure, QMP, cGMP and their interactions were set as fixed factors, and colony was set as a random effect. The model was as follows:

$$Y_{ghik} = \mu + cGMP_g + QMP_h + DAY_j + \sim COLONY_k + (cGMP * QMP)_{gh} + (cGMP * QMP * DAY)_{ghj} + \epsilon_{ghjk}$$

where g indexes the cGMP treatment, h indexes the QMP treatment, j indexes the day, and k indexes the colony; $(QMP * cGMP)_{gh}$ is the fixed QMP by cGMP interaction, $(cGMP * QMP * day)_{ghj}$ is the fixed cGMP by QMP by day interaction, colony indexes that colony where $\sim$ denotes a random effect, and ϵ_{ghjk} is the normally distributed error. Because assays were measured repeatedly in time, a first-order autoregressive model was used to accommodate correlations among measurements from the same cage.

Quantification of Vg RNA levels in fat bodies

In order to determine if 8-Br-cGMP affected *Vg* expression levels in abdominal fat bodies, bees were collected from the microarray experiment described below (MDI5). Total RNA was extracted from eviscerated carcasses (cuticle with attached fat bodies) using the RNeasy kit (Qiagen, Valencia, CA). 150 ng of total RNA from each sample were reverse-transcribed using random primers to produce cDNA. Reaction mix included 10× ArrayScript buffer (Ambion, Austin, TX), RNaseOUT (Invitrogen, Carlsbad, CA), and ArrayScript RT enzyme (Ambion, Austin, TX). Quantitative real time PCR (qRT-PCR) was performed with an ABI Prism 7900 sequence detector and the SYBR green detection method (Applied Biosystems, Foster City, CA). *eIF-58*, a housekeeping gene, was used as a loading control as in Fischer and Grozinger (2008) and Grozinger et al. (2003). The sequences for the primers are found in Fischer and Grozinger (2008). Individual sample/primer sets were used in triplicate reactions. Quantification was performed using a standard curve with genomic DNA. A negative control containing no cDNA was used to check for genomic contamination of the RNA samples. A dissociation step was added to the qRT-PCR reactions to confirm primer fidelity. Quantification was based on the number of PCR cycles (C_T) required to cross a threshold of fluorescence intensity (ABI User Bulletin 2) described in Bloch et al (2001). Data was analyzed in JMP 7 (SAS, Cary, NC) using a standard least squares ANOVA with treatment as a fixed factor and colony and treatment by colony set as random factors. The number of bees in each sample group is indicated in Fig. 2.

Collections for microarray analysis

Bees from a single source colony, headed by a naturally mated queen (MDI5), were used for the microarray collections. As before, there were four experimental treatments: sucrose only with solvent (NN), sucrose only with QMP (NQ), 8-Br-cGMP with solvent (GN), and 8-Br-cGMP with QMP (GQ). Bees were reared as described above, with 20 bees/cage and 4 cages/treatment for the NQ and GQ groups and two cages/treatment for the NN and GN. Bees were treated as above, but were treated over 9 days and collected on day 10 into liquid nitrogen. Retinue response, *vitellogenin* levels, and *AmOr11* levels were measured as described in their respective sections. For analysis of the brain expression levels, heads of bees were freeze-dried to facilitate dissection. Brains were dissected on dry ice. Brains were pooled in groups of 4, with 6 pools per treatment. These pools were made from the two cages of each treatment with the highest retinue response.

AmOr11 quantification by qRT-PCR

Antennae were collected on dry ice from bees treated in microarray experiment. Eight antennae were pooled to form one sample (corresponding to four bees); pooled antennae were from bees used in microarray experiment. Six pools of antennae were used for both the NQ and GQ groups. Samples were extracted and analyzed as described for *vitellogenin*. *AmOr2* was used as a loading control because it is a known ortholog to *Or83b* (Robertson and Wanner 2006), an odorant receptor that is expressed in all olfactory neurons (Touhara and Vosshall 2009). The sequences for the primers (5' to 3') are as follows:

AmOr11 Fwd: TCCTTTTACCGAACAACATGACA

AmOr11 Rvr: GGTGTGGCTTGCTCGTTCA

AmOr2 Fwd: GGACATGGATCTTCGAGGGATA

AmOr2 Rvr: TTGAACGTCATTCCAGCAGTTG

Data was analyzed in JMP 7 (SAS, Cary, NC) using a Student's t-test.

Microarray extraction and hybridization

RNA extractions from brains were performed using the Qiagen RNeasy Isolation kit (Qiagen, Valencia, CA). 500 ng RNA was amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX). 5 µg of amplified RNA was labeled with Cy3 or Cy5 dye (Kreatech, Amsterdam, Netherlands). Two sets of labeled probe were then hybridized to the whole-genome oligonucleotide arrays supplied from the W.M. Keck Center for Functional Genomics (University of Illinois, Urbana-Champaign). Brains were hybridized using a loop design with dye-swaps incorporated (n = 6 for each group, 24 arrays total). Arrays were scanned using the Axon Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA) using GENEPIX software (Agilent Technologies, Santa Clara, CA).

Microarray data analysis

Any spots with an intensity of less than 300 (the background level on the arrays) were removed from the analysis. Also, spots present on less than 10 out of 24 arrays were excluded from the data set as well. Expression data was log-transformed and normalized using a mixed-model ANOVA (proc MIXED, SAS, Cary, NC) with the following model:

$$Y = \mu + \text{dye} + \text{array} + \text{block} + \text{dye} * \text{array} + \text{array} * \text{block} + \varepsilon$$

where Y is expression, dye is a fixed effect, and array, block, and their interactions are random effects. Genes with significant expression differences between groups were detected by using a mixed-model ANOVA with the model:

$$Y = \mu + \text{treatment} + \text{dye} + \text{array} + \text{dye} * \text{array} + \varepsilon$$

where Y represents the residual from the previous model. The fixed effects are behavior and dye and the random effects are array and dye*array. p-values were corrected for multiple testing using a false discovery rate < 0.01 (proc MULTTEST, SAS). Hierarchical clustering and principal components analysis were performed in JMP 7 (SAS, Cary, NC). Gene ontology analysis was performed using DAVID (Dennis et al. 2003; Huang et al. 2009) with a cutoff of p < 0.05. Analysis of overlap between the significantly regulated genes in our study and previous array studies was performed using statistical significance of the overlap between two groups of genes (Dr. Jim Lund, University of Kentucky, http://elegans.uky.edu/MA/progs/overlap_stats.html). The array data has been deposited on the ArrayExpress website according to MIAME standards (E-MEXP-3135).

Results

8-Br-cGMP decreases retinue responses to QMP

Honey bees from three different source colonies (SDI6, MDI20, and MDI21) were reared with and without QMP, and with and without 8-Br-cGMP. Responses to the QMP or solvent control were recorded daily, starting at day 3 of the experiment. There was an overall significant effect of both QMP and cGMP treatment ($p < 0.0001$, Fig. 1a). Untreated (NN) and 8-Br-cGMP treated (GN) bees were not attracted to the solvent lure. QMP treated bees (NQ) were more attracted to a QMP lure than 8-Br-cGMP treated bees (GQ) by day 4 of the experiment, and this difference continued throughout the rest of the experiment (Fig. 1a). We repeated this experiment for the bees used in the microarray analysis (MDI5) and found that there was a significant effect of QMP treatment ($p = 0.0012$, Fig. 1b) on the retinue response, but not of cGMP treatment ($p = 0.2629$, Fig. 1b). Note that the number of bees attracted to the QMP lure was substantially lower in this trial overall. These bees were from a different colony than those used in Fig. 1a, but did demonstrate responsiveness to QMP for *vitellogenin* levels (see results below). Previous studies have found that there can be substantial effects of both genotype and season on retinue response (Pankiw et al. 1994).

8-Br-cGMP reduces physiological responses to QMP

To determine if 8-Br-cGMP treatment affects *Vg* expression, we performed qRT-PCR on RNA extracted from the fat bodies of young workers with the four treatment groups used in the microarray experiment (MDI5) and found that *Vg* expression is significantly affected by treatment (ANOVA, $p < 0.0001$, Fig. 2). Expression is significantly higher in bees exposed to QMP but not treated with 8-Br-cGMP (NQ) than any other group (Tukey HSD $p < 0.05$). Bees treated with 8-Br-cGMP (GN, GQ) have slightly lower levels than untreated controls (NN), but this difference is not significant.

8-Br-cGMP treatment does not affect *AmOR11* expression levels

The AmOR11 odorant receptor is responsive to 9-ODA (Wanner et al. 2007), a major component of QMP. To determine if 8-Br-cGMP affects the expression of *AmOr11*, we performed qRT-PCR on RNA extracted from pools of antennae from workers after

treatment (MDI5). 8-Br-cGMP treatment had no significant effect on *AmOr11* expression (NQ mean $\pm$ SE: 0.83 $\pm$ 0.01; GQ mean $\pm$ SE: 0.09 $\pm$ 0.01; t-test, $p=0.32$).

Brain gene expression profiles results

Both QMP exposure and 8-Br-cGMP treatment significantly affect brain expression patterns. QMP exposure causes workers to display nurse-like brain expression patterns (Grozinger et al. 2003), while 8-Br-cGMP treatment causes workers to develop foragerlike brain expression patterns (Whitfield et al. 2006). Statistical analysis showed that 300 transcripts out of the 9,218 transcripts included in the analysis were differentially expressed in our treatment groups ($n=6$, all groups, $FDR < 0.01$; see Supplementary Table 1 for a listing of all the genes, including genes that differed significantly in pairwise comparisons). Hierarchical clustering analysis grouped NN and NQ together and GN and GQ together, suggesting that 8-Br-cGMP treatment is the major driver of brain gene expression in this study (Fig. 3). Principal component analysis was used to detect any underlying expression patterns across all four groups (Fig. 4). The first principal component accounts for 43% of the variation and again shows a substantial effect of 8-Br-cGMP treatment. The second principal component explains 30% of the variation and demonstrates that the NQ group is markedly different from the NN group, highlighting the effect of QMP on brain gene expression. Lastly, the third principal component comprises 27% of the variation and shows the effect of an interaction between the QMP and 8-Br-cGMP treatments.

Gene ontology analysis was performed using DAVID (Dennis et al. 2003). This analysis shows a significant over-representation of genes involved in carboxylic acid transmembrane activity ($p=0.00328$), GTPase regulator activity ($p=0.00612$), phototransduction ($p=0.00411$), and sensory perception ($p=0.00520$), among others (Table 1).

There was less overlap than expected by chance between significant genes in our study and previous studies examining the effects of QMP (Grozinger et al. 2003) (representation factor = 0.3) or 8-Br-cGMP treatment (Whitfield et al. 2006) (representation factor = 0.2) on brain gene expression. The representation factor is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups, where a representation number > 1 indicates more overlap than expected by chance. However, 18 genes were both significantly regulated in our study and previously found to be down-regulated by QMP treatment, and in this group there was a significant overrepresentation of genes associated with GTPase regulator activity ($p = 3.4e^{-02}$). Additionally, 5 genes were both significantly regulated in our study and previously shown to be associated with the foraging behavioral state, and in this group there was a significant overrepresentation of genes associated with kinase activity ($p = 1.0e^{-02}$).

Discussion

We found that 8-Br-cGMP treatment reduced attraction to QMP and inhibited the QMP-induced increase in *Vg* expression levels. At the brain gene expression level, 8-Br-cGMP had a larger effect on expression patterns than QMP, and, based on a principle components analysis, some of the effects of QMP differ between untreated and 8-Br-cGMP treated bees. However, there were no significant GO categories associated with the genes that were significantly different between the 8-Br-cGMP-QMP (GQ) and 8-Br-cGMP (GN) treatment groups (data not shown). Future work needs to be done in order to determine if QMP causes specific behavioral or physiological changes in 8-Br-cGMP-treated (presumably foragerlike) bees which are associated with these transcriptional effects. For example, exposure to QMP will activate pollen foraging behavior in forager bees in newly established colonies (Jaycox 1970; Higo et al 1992).

Where is the QMP response modulated?

Changes in the peripheral or central nervous system can alter perception and responses to pheromonal cues. At the level of the peripheral nervous system, changes can occur in expression or activity levels of pheromone receptors, pheromone binding proteins, or properties of the olfactory receptor neurons located in the antennae. If a single receptor system regulates all downstream behavioral and physiological response, changes in this system should result in inhibition of most or all pheromone responses. Changes in central processing could also alter the downstream behavioral and physiological responses to pheromones, though in this case responses might be modulated separately. For example, Grozinger et al. (2007) demonstrate that two components of QMP that do not elicit the retinue response still alter brain gene expression. In our studies, we did not find changes in antennal expression levels of *AmOr11*, a known receptor for the main QMP component 9-ODA (Wanner et al. 2007). These results suggest that, at least for 9-ODA, the modulation of the response to QMP is likely not occurring peripherally. However, there are many factors that could modulate peripheral responses to QMP, including pheromone binding proteins (Xu et al. 2005), neuromodulators (Linn 1984; Linn et al. 1986; Linn et al. 1992; Linn et al. 1996; Pophof 2000), or other neuronal signaling components. Further studies using electrophysiological approaches would be necessary to determine if 8-Br-cGMP has effects at the peripheral level.

What genes are regulated by QMP and 8-Br-cGMP?

Results of our brain expression analyses are consistent with the effects of 8-Br-cGMP on insects, and of QMP on honey bee workers. We found significant modulation of genes associated with phototransduction processes, which is consistent with the effects of 8-Br-cGMP on phototactic behavior in young bees (Ben-Shahar et al. 2003). Both 8-Br-cGMP (Fussnecker and Grozinger, data not shown) and QMP (Fischer and Grozinger, 2008) affect lipid levels, which is consistent with the effects we observed on sets of genes associated with lipid metabolic processes. PKG has been shown to activate at least one GTPase in an *in vitro* study (Hou et al. 2004), and it may be performing a similar role in the honey bee brain, as evidenced by the significant GO category of GTPase regulator activity. The GO category of carboxylic acid transmembrane activity may be associated with modulation of odorant reception in the olfactory glomeruli (Sachse et al. 1999), while positive regulation of antibacterial peptide biosynthetic process is interesting in that honey bee (Amdam et al. 2004) and bumble bee (König and Schmid-Hempel 1995) foragers have fewer haemocytes than nurse bees. Haemocytes are cellular components of the defense response involved in encapsulation and phagocytosis of pathogens. Thus, our results suggest that immune responses may be another forager-related physiological phenotype that is regulated by 8-Br-cGMP and/or QMP.

Comparison to previous studies

While there was less overlap than expected by chance between our study and previous analyses of the effects of QMP and 8-Br-cGMP, the genes that did overlap were in important functional categories. Transcripts significantly regulated in our study which were previously shown to be down-regulated by QMP treatment were significantly associated with GTPase regulator activity, a component of the PKG pathway (Hou et al. 2004). Those significantly regulated transcripts which also overlapped with foraging-associated transcripts from previous studies were significantly associated with kinase activity. The lack of a significant overlap between our study and these previous studies is likely due to different experimental conditions: previous studies were performed with 4-5 day old caged bees, while we used 10 day old bees. Furthermore, previous studies used double-stranded DNA arrays, rather than the oligo arrays employed in this study.

Modulation of the pheromone response in honey bees

Modulation of the pheromone response is quite prevalent in honey bees. Workers in different behavioral/physiological states have different responses to alarm, brood, and queen pheromone. (Robinson 1987; Pham-Delegue et al. 1991; Barron et al. 2002; Grozinger and Robinson 2007; Grozinger et al. 2007). In some cases, specific physiological factors associated with the transition from nursing to foraging (JH, octopamine, or, in this study, cGMP) have been shown to regulate this response (Robinson 1987; Barron et al. 2002; Grozinger and Robinson 2007; Grozinger et al. 2007). Modulating the pheromone response at a number of different levels may play an important role in maintaining and regulating the division of labor in honey bee colonies, leading to greater colony efficiency (Johnson 2010).

Since exposure to QMP slows the transition from nursing to foraging, workers must somehow become unresponsive to QMP or decrease their exposure to QMP in order to advance their behavioral development. How do these bees become unresponsive? It could be due to age-related changes in physiological factors, or it could be due to social/physical factors such as spending less time near the queen which then triggers changes in these physiological factors. For example, Johnson (2010) suggests that an increasing number of young emerging workers crowd the brood nest, resulting in older workers becoming isolated from the queen. There is clearly interplay between how the receiver's own behavior and physiology modulates the response to the pheromone, while simultaneously, the pheromone modulates the receiver's behavior and physiology.

Of the known physiological factors that regulate the transition to foraging, only 8-Br-cGMP appears to be involved in shifting behavioral, physiological, and transcriptional responsiveness to QMP. Octopamine treatment, though a strong promoter of foraging behavior (Schulz and Robinson 2001), has no effect on the retinue response (Barron et al. 2002) or QMP-mediated gene expression (Grozinger and Robinson, unpublished data). QMP reduces levels of another biogenic amine, dopamine, in the honey bee brain (Beggs et al. 2007), but dopamine treatment has no obvious effect on the retinue response (Fussnecker and Grozinger, unpublished data). JH, another strong driver of foraging behavior, inhibits the primer responses to QMP but not the retinue responses (Grozinger and Robinson 2007; Grozinger et al. 2007). However, assays with JH and OA were performed with younger bees (4-5 days old) and thus the effects of long-term treatment need to be further investigated.

Conclusion

There is increasing evidence that responses to social cues, such as pheromones, can be modified by a number of factors. In honey bees, genetic (Pankiw et al. 1994), developmental (Kocher et al., 2010), physiological (Robinson 1987; Barron et al. 2002), and environmental (Higo et al 1992) factors all play a role. Thus, the response to pheromones appears to be quite plastic and labile. This may be adaptive, particularly in animal societies, where all possible social cues may be present at any given time, but only a subset of the individuals should respond to these cues in order for the colony to function optimally.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank S. Kocher for advice on the microarray analyses, J. Flowers for expert beekeeping assistance, T. Crowgey and H. Yamamoto for assistance with the behavioral assays, and the rest of the Grozinger lab for insightful comments and helpful discussions. This research was supported by an NIH-NIDCD grant to G.E.

Robinson (subcontract to C.M.G.) and an NSF CAREER grant to C.M.G. Animal care and collection of data was in accordance with US laws.

References

- Amdam GV, Simoes ZL, Hagen A, Norberg K, Schroder K, Mikkelsen O, Kirkwood TB, Omholt SW. Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. *Exp Gerontol*. 2004; 39(5):767–773.10.1016/j.exger.2004.02.010S0531556504001019[pii] [PubMed: 15130671]
- Barron A, Schulz D, Robinson GE. Octopamine modulates responsiveness to foraging-related stimuli in honey bees (*Apis mellifera*). *J Comp Phys A*. 2002; 188(8):603–610.
- Beggs KT, Glendinning KA, Marechal NM, Vergoz V, Nakamura I, Slessor KN, Mercer AR. Queen pheromone modulates brain dopamine function in worker honey bees. *Proc Natl Acad of Sci USA*. 2007; 104(7):2460–2464.10.1073/pnas.0608224104 [PubMed: 17287354]
- Belay AT, Scheiner R, So AK, Douglas SJ, Chakabarty-Chatterjee M, Levine JD, Sokolowski MB. The foraging gene of *Drosophila melanogaster*: spatial-expression analysis and sucrose responsiveness. *J Comp Neurol*. 2007; 504(5):570–582.10.1002/cne.21466 [PubMed: 17701979]
- Ben-Shahar Y, Leung HT, Pak WL, Sokolowski MB, Robinson GE. cGMP-dependent changes in phototaxis: a possible role for the foraging gene in honey bee division of labor. *J Exp Biol*. 2003; 206(Pt 14):2507–2515. [PubMed: 12796464]
- Ben-Shahar Y, Robichon A, Sokolowski MB, Robinson GE. Influence of gene action across different time scales on behavior. *Science*. 2002; 296(5568):741–744.10.1126/science.1069911296/5568/741[pii] [PubMed: 11976457]
- Bloch G, Toma DP, Robinson GE. Behavioral rhythmicity, age, division of labor and period gene expression in the honey bee brain. *J Biol Rhythms*. 2001; 16(5):444–456. [PubMed: 11669418]
- Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol*. 2003; 4(5):P3. [PubMed: 12734009]
- Fischer P, Grozinger CM. Pheromonal regulation of starvation resistance in honey bee workers (*Apis mellifera*). *Naturwissenschaften*. 2008; 95(8):723–729.10.1007/s00114-008-0378-8 [PubMed: 18414825]
- Fluri P, Lüscher M, Wille H, Gerig L. Changes in weight of the pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker honey bees. *J Insect Physiol*. 1982; 28(1):61–68.
- Fujiwara M, Sengupta P, McIntire SL. Regulation of body size and behavioral state of *C. elegans* by sensory perception and the EGL-4 cGMP-dependent protein kinase. *Neuron*. 2002; 36(6):1091–1102. S0896627302010930 [pii]. [PubMed: 12495624]
- Fussnecker B, Grozinger C. Dissecting the role of Kr-h1 brain gene expression in foraging behavior in honey bees (*Apis mellifera*). *Insect Mol Biol*. 2008; 17(5):515–522. IMB819 [pii]10.1111/j.1365-2583.2008.00819.x. [PubMed: 18715264]
- Gadenne C, Renou M, Sreng L. Hormonal control of pheromone responsiveness in the male black cutworm, *Agrotis ipsilon*. *Cell Mol Life Sci*. 1993; 49(8):721–724.
- Gadenne C, Anton S. Central processing of sex pheromone stimuli is differentially regulated by juvenile hormone in a male moth. *J Insect Physiol*. 2000; 46(8):1195–1206. [PubMed: 10818247]
- Grozinger C, Robinson G. Endocrine modulation of a pheromone-responsive gene in the honey bee brain. *J Comp Physiol A*. 2007; 193(4):461–470.
- Grozinger CM, Fischer P, Hampton JE. Uncoupling primer and releaser responses to pheromone in honey bees. *Naturwissenschaften*. 2007; 94(5):375–379.10.1007/s00114-006-0197-8 [PubMed: 17187255]
- Grozinger CM, Sharabash NM, Whitfield CW, Robinson GE. Pheromone-mediated gene expression in the honey bee brain. *Proc Natl Acad of Sci USA*. 2003; 100(Suppl 2):14519–14525.10.1073/pnas.2335884100 [PubMed: 14573707]

- Higo HA, Colley SJ, Winston ML, Slessor KN. Effects of honey bee (*Apis mellifera* L.) queen mandibular gland pheromone on foraging and brood rearing. *Can Ent.* 1992; 124(2):409–418.
- Hou Y, Ye RD, Browning DD. Activation of the small GTPase Rac1 by cGMP-dependent protein kinase. *Cell Signal.* 2004; 16(9):1061–1069.10.1016/j.cellsig.2004.03.002S0898656804000336[pii] [PubMed: 15212766]
- Hou Y, Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 2009; 4(1):44–57. nprot.2008.211 [pii]10.1038/nprot.2008.211. [PubMed: 19131956]
- Ingram KK, Oefner P, Gordon DM. Task-specific expression of the foraging gene in harvester ants. *Mol Ecol.* 2005; 14(3):813–818. MEC2450 [pii]10.1111/j.1365-294X.2005.02450.x. [PubMed: 15723672]
- Jaycox ER. Honey bee foraging behavior: responses to queens, larvae, and extracts of larvae. *Ann Entomol Soc Am.* 1970; 63:1689–1694.
- Johnson BR. Division of labor in honeybees: form, function, and proximate mechanisms. *Behav Ecol Sociobiol.* 2010; 64(3):305–316.10.1007/s00265-009-0874-7 [PubMed: 20119486]
- Kaun KR, Hendel T, Gerber B, Sokolowski MB. Natural variation in *Drosophila* larval reward learning and memory due to a cGMP-dependent protein kinase. *Learn Mem.* 2007a; 14(5):342–349. 14/5/342 [pii]10.1101/lm.505807. [PubMed: 17522025]
- Kaun KR, Riedl CA, Chakabarty-Chatterjee M, Belay AT, Douglas SJ, Gibbs AG, Sokolowski MB. Natural variation in food acquisition mediated via a *Drosophila* cGMP-dependent protein kinase. *J Exp Biol.* 2007b; 210(Pt 20):3547–3558. 210/20/3547 [pii]10.1242/jeb.006924. [PubMed: 17921156]
- Kocher SD, Kocher SD, Ayroles JF, Stone EA, Grozinger CM. Individual variation in pheromone response correlates with reproductive traits and brain gene expression in worker honey bees. *PLoS ONE.* 2011; 5(2):e9116.10.1371/journal.pone.0009116 [PubMed: 20161742]
- König C, Schmid-Hempel P. Foraging activity and immunocompetence in workers of the bumble bee, *Bombus terrestris* L. *Proc Biol Sci.* 1995; 260(1358):225–227.
- Laidlaw H Jr. Instrumental insemination of honey bee queens: its origin and development. *Bee World.* 1987; 68:17–38.
- Linn CE Jr, Roelofs WL. Sublethal Effects of Neuroactive Compounds on Pheromone Response Thresholds in Male Oriental Fruit Moths. *Arch Insect Biochem Physiol.* 1984; 1:331–344.
- Linn CE Jr, Campbell MG, Roelofs WL. Male moth sensitivity to multicomponent pheromones: Critical role of female-released blend in determining the functional role of components and active space of the pheromone. *J Chem Ecol.* 1986; 12(3):659–668.
- Linn CE Jr, Campbell MG, Roelofs WL. Photoperiod cues and the modulatory action of octopamine and 5-hydroxytryptamine on locomotor and pheromone response in male gypsy moths, *Lymantria dispar*. *Arch Insect Biochem Physiol.* 1992; 20(4):265–284.
- Linn CE Jr, Campbell, Poole MG, Wu KR, Roelofs WL. Effects of photoperiod on the circadian timing of pheromone response in male *Trichoplusia ni*. Relationship to the modulatory action of octopamine. *J Insect Physiol.* 1996; 42(9):881–891.
- Mery F, Belay AT, So AK, Sokolowski MB, Kawecki TJ. Natural polymorphism affecting learning and memory in *Drosophila*. *Proc Natl Acad Sci U S A.* 2007; 104(32):13051–13055. 0702923104 [pii]10.1073/pnas.0702923104. [PubMed: 17640898]
- Morley JE, Alshaher MM, Farr SA, Flood JF, Kumar VB. Leptin and neuropeptide Y (NPY) modulate nitric oxide synthase: further evidence for a role of nitric oxide in feeding. *Peptides.* 1999; 20(5): 595–600. S0196-9781(99)00012-1 [pii]. [PubMed: 10465511]
- Morley JE, Kumar VB, Mattammal MB, Farr S, Morley PM, Flood JF. Inhibition of feeding by a nitric oxide synthase inhibitor: effects of aging. *Eur J Pharmacol.* 1996; 311(1):15–19. 0014299996005468 [pii]. [PubMed: 8884231]
- Pankiw T, Winston ML, Slessor KN. Variation in worker response to honey bee (*Apis mellifera* L.) queen mandibular pheromone (Hymenoptera: Apidae). *J Insect Beh.* 1994; 7(1):1–15.

- Pankiw T, Huang ZY, Winston ML, Robinson GE. Queen mandibular gland pheromone influences worker honey bee (*Apis mellifera* L.) foraging ontogeny and juvenile hormone titers. *J Insect Physiol.* 1998; 44(7-8):685–692. S0022191098000407 [pii]. [PubMed: 12769952]
- Pereira HS, Sokolowski MB. Mutations in the larval foraging gene affect adult locomotory behavior after feeding in *Drosophila melanogaster*. *Proc Natl Acad Sci USA.* 1993; 90(11):5044–5046. [PubMed: 8506349]
- Pham-Delegue MH, Trouiller E, TBakchine E, Roger B, Masson C. Age dependency of worker bee responses to queen pheromone in a four-armed olfactometer. *Ins Sociaux.* 1993; 38(3):283–292.10.1007/BF01314914
- Pophof B. Octopamine modulates the sensitivity of silkworm pheromone receptor neurons. *J Comp Physiol A.* 2000; 186(3):307–313. [PubMed: 10757246]
- Robertson HM, Wanner KW. The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res.* 2006; 16(11):1395–1403. gr.5057506 [pii]10.1101/gr.5057506. [PubMed: 17065611]
- Robinson GE. Regulation of honey bee age polyethism by juvenile hormone. *Beh Ecol and Sociobiol.* 1987; 20(5):329–338.
- Sachse S, Rappert A, Galizia CG. The spatial representation of chemical structures in the antennal lobe of honeybees: steps towards the olfactory code. *Eur J Neurosci.* 1999; 11(11):3970–3978. [PubMed: 10583486]
- Scheiner R, Sokolowski MB, Erber J. Activity of cGMP-dependent protein kinase (PKG) affects sucrose responsiveness and habituation in *Drosophila melanogaster*. *Learn Mem.* 2004; 11(3):303–311.10.1101/lm.7160411/3/303[pii] [PubMed: 15169860]
- Schulz DJ, Robinson GE. Octopamine influences division of labor in honey bee colonies. *J Comp Physiol A.* 2001; 187(1):53–61. [PubMed: 11318378]
- Slessor KN, Kaminski L-A, King GGS, Borden JH, Winston ML. Semiochemical basis of the retinue response to queen honey bees. *Nature.* 1988; 332(6162):354–356.
- Slessor KN, Winston ML, Le Conte Y. Pheromone communication in the honeybee (*Apis mellifera* L.). *J Chem Ecol.* 2005; 31(11):2731–2745.10.1007/s10886-005-7623-9 [PubMed: 16273438]
- Toth AL, Kantarovich S, Meisel AF, Robinson GE. Nutritional status influences socially regulated foraging ontogeny in honey bees. *J Exp Biol.* 2005; 208(Pt 24):4641–4649. 208/24/4641 [pii]10.1242/jeb.01956. [PubMed: 16326945]
- Touhara K, Vossell LB. Sensing odorants and pheromones with chemosensory receptors. *Annu Rev Physiol.* 2009; 71:307–332.10.1146/annurev.physiol.010908.163209 [PubMed: 19575682]
- Wang Z, Pan Y, Li W, Jiang H, Chatzimanolis L, Chang J, Gong Z, Liu L. Visual pattern memory requires foraging function in the central complex of *Drosophila*. *Learn Mem.* 2008; 15(3):133–142. 15/3/133 [pii]10.1101/lm.873008. [PubMed: 18310460]
- Wanner KW, Nichols AS, Walden KK, Brockmann A, Luetje CW, Robertson HM. A honey bee odorant receptor for the queen substance 9-oxo-2-decenoic acid. *Proc Natl Acad Sci U S A.* 2007; 104(36):14383–14388. 0705459104 [pii]10.1073/pnas.0705459104. [PubMed: 17761794]
- Whitfield CW, Ben-Shahar Y, Brillet C, Leoncini I, Crauser D, Leconte Y, Rodriguez-Zas S, Robinson GE. Genomic dissection of behavioral maturation in the honey bee. *Proc Natl Acad Sci USA.* 2006; 103(44):16068–16075. 0606909103 [pii]10.1073/pnas.0606909103. [PubMed: 17065327]
- Wyatt, TD. Pheromones and animal behaviour: communication by smell and taste. Cambridge University Press Cambridge; UK: New York: 2003.
- Xu P, Atkinson R, Jones DN, Smith DP. *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. *Neuron.* 2005; 45(2):193–200. S0896627304008451 [pii]10.1016/j.neuron.2004.12.031. [PubMed: 15664171]
- Zhukovskaya MI, Kapitsky SV. Activity modulation in cockroach sensillum: the role of octopamine. *J Insect Physiol.* 2006; 52(1):76–86. S0022-1910(05)00202-7 [pii]10.1016/j.jinsphys.2005.09.005. [PubMed: 16256134]

Abbreviations

QMP	Queen mandibular pheromone
9-ODA	(E)-9-oxodec-2-enoic acid
SDI	single drone inseminated
MDI	multi-drone inseminated
Vg	<i>vitellogenin</i> gene
Amfor	<i>foraging</i> gene
cGMP	3', 5'-cyclic guanosine monophosphate
8-Br-cGMP	8-Bromo-cGMP, an analog of cGMP
PKG	cGMP-dependent protein kinase-
qRT-PCR	quantitative real time PCR-qRT-PCR

Fig. 1.

(a) *8-Br-cGMP* treatment reduces the response to QMP in a retinue assay. One day old bees were treated for 8 days with one of four treatments: 50% sucrose and solvent slide (NN), 50% sucrose and QMP slide (NQ), 500 μ M 8-Br-cGMP and solvent slide (GN), and 500 μ M 8-Br-cGMP and QMP slide (GQ). There was a significant effect for both QMP and cGMP treatment. Asterisks denote days in which a significant difference in treatment was observed. (b) Retinue responses of bees collected for microarray analysis. These bees were treated as for 1a. Significant differences in retinue response between the NQ group and three other treatment groups were observed on days 7, 9, and 10. There was a significant effect of QMP ($p=0.0012$), but not cGMP treatment ($p=0.2629$). Asterisks denote days in which a significant difference between the NQ and GQ treatment groups was observed

Fig. 2. 8-Br-cGMP reduces effect of QMP exposure on Vg levels

One day old bees were treated during the microarray experiment for 9 days with 50% sucrose and a solvent slide (NN), 50% sucrose with a QMP slide (NQ), 500 μ M 8-Br-cGMP with a solvent slide (GN), or 500 μ M 8-Br-cGMP with a QMP slide (GQ). 8-Br-cGMP treatment significantly reduced *Vg* expression levels in the fat bodies in GQ treatment compared to the NQ treatment (ANOVA, Tukey HSD). Number of individual abdomens used is shown at the base of each bar. Different letters denote significant differences ($p < 0.05$)

Fig. 3. Hierarchical clustering analysis

Hierarchical clustering analysis using the significantly regulated genes shows that treatment with 8-Br-cGMP had a greater global effect on gene expression levels than QMP. Red and blue colors represent fold increase and decrease relative to the mean expression across all four treatment groups

Fig. 4. Principal components analysis of brain expression patterns

The first principal component accounts for 43% of the variation and shows a substantial effect of 8-Br-cGMP treatment. The second principal component explains 30% of the variation and demonstrates that the NQ group is markedly different from the NN group, demonstrating the effect of QMP on brain gene expression. Lastly, the third principal component comprises 27% of the variation and shows the effect of the interaction between the QMP and 8-Br-cGMP treatments

Table 1
Gene Ontology Analysis: Overrepresented Biological Processes in the Brain

GO Biological Process	Count	%	p-value
carboxylic acid transmembrane transporter activity	6	2.44%	0.0033
GTPase regulator activity	11	4.47%	0.0061
intracellular signaling cascade	21	8.54%	0.0366
lipid metabolic process	16	6.50%	0.0112
molecular transducer activity	29	11.79%	0.0028
phototransduction	6	2.44%	0.0041
positive regulation of antibacterial peptide biosynthetic process	4	1.63%	0.0062
response to abiotic stimulus	11	4.47%	0.0019
sensory perception	12	4.88%	0.0052
transmembrane receptor activity	17	6.91%	0.0042